Confidential Detailed Manufacturing Summary of Fat Encapsulated *Pichia kudriavzevii* ASCUSDY21

The raw materials used in the manufacture of *P. kudriavzevii* ASCUSDY21 are listed in Table 1 below. Specifications for the raw materials are provided in Appendices 009A to 009Y.

Table 1. Raw Materials and Processing Aids Used in the manufacture of *P. kudriavzevii* ASCUSDY21

Material	Function	Regulatory Status	Grade
Mannitol	Preservative	GRAS substance for use as a nutrient and/or dietary supplement (21 CFR §582.5470)	Purity ≥95%
Sucrose	Preservative	Common ingredient (*e.g.*, 21 CFR §184.1854)	Purity ≥95%
Hydrogenated glycerides	Encapsulating agent	AAFCO OP ingredient definition (hydrogenated glycerides) 33.19	Feed grade
Sodium sulfate	Encapsulating agent	AAFCO OP ingredient definition (mineral product) 57.109	Feed grade
Ammonium hydroxide	Nutrient	GRAS substance for use as a general purpose food additive (21 CFR §582.1139)	FCC
Ammonium sulfate	Nutrient	GRAS substance for use as a general purpose food additive (21 CFR §582.1143) AFFCO OP ingredient definition 57.27	FCC
Biotin	Nutrient	GRAS substance for use as a nutrient and/or dietary supplement (21 CFR §582.5159)	FCC
Calcium chloride dihydrate	Nutrient	GRAS substance for use as a general purpose food additive (21 CFR §582.1193) and sequestrant (21 CFR §582.6193) AAFCO OP ingredient definition 57.51	USP
Dextrose monohydrate	Nutrient	Common ingredient (*e.g.*, 21 CFR §168.111; 21 CFR §184.1857)	FCC
Copper sulfate pentahydrate	Nutrient	GRAS substance for use as a trace mineral (21 CFR §582.80) AAFCO OP ingredient definition (trace mineral) 57.69	USP
Dipotassium phosphate	Buffering agent	GRAS substance for use as a sequestrant (21 CFR §582.6285)	FCC
Polyglycerol polyethylene-polyoxypropylene block copolymer	Anti-foaming agent	Acceptable for use as an anti-foaming agent for the production of enzymes and DFMs in accordance with the letter issued by the FDA to the Enzyme Technical Association (ETA, Appendix 009L2)	Food-grade

Table continued on next page.

Table 1: Raw Materials and Processing Aids Used in the manufacture of *P. kudriavzevii* ASCUSDY21 (cont'd)

Material	Function	Regulatory Status	Grade
Ferric ammonium citrate	Nutrient	Anti-caking agent in salt (21 CFR §573.560) AAFCO OP ingredient definition (mineral product) 57.76	FCC
Hydrogen chloride (1 M)	pH adjustment (acid)	GRAS substance as a general purpose food additive (21 CFR §582.1057)	Feed grade
Magnesium sulfate heptahydrate	Nutrient	GRAS substance for use as a nutrient and/or dietary supplement (21 CFR §582.5443) AAFCO OP ingredient definition (mineral product) 57.88	Feed grade
Manganese sulfate monohydrate	Nutrient	GRAS substance for use as a nutrient and/or dietary supplement (21 CFR §582.5461) and trace mineral (21 CFR §582.80)	FCC
Monopotassium phosphate	Buffering agent	Permitted for use as a food additive in frozen eggs (21 CFR §160.110) – safety for use in feed assessed by ASCUS (Appendix 009Q2)	FCC
p-Aminobenzoic acid	Nutrient	Recognized vitamin ingredient – AAFCO OP ingredient definition 90.25	USP
Yeast extract	Nutrient	Yeast extract obtained by mechanical rupturing of cells is accepted for use in feed (AAFCO OP 96.11); use of autolysis in the production of the extract is not expected to introduce any different substances and should yield a product with equivalent composition – history of use in food (*e.g.*, FCC monograph established Appendix 009S2)	Food-grade
Sodium chloride	Nutrient	AAFCO OP ingredient definition (mineral product) 57.31	Food-grade
Sodium hydroxide (1 M)	pH adjustment (base)	GRAS substance for use as a general purpose food additive (21 CFR §582.1763)	Feed grade
Sodium iodide	Nutrient	GRAS substance for use as a trace mineral (21 CFR §582.80) AAFCO OP ingredient definition (mineral product) 57.108	USP
Soy peptone	Nutrient	Enzyme from soy protein; various soy protein products are accepted for use in feed, *e.g.*, hydrolyzed soy protein (AAFCO OP ingredient definition 84.63) textured soy protein product (AAFCO OP 84.64)	Feed grade
Thiamine hydrochloride	Nutrient	GRAS substance for use as a nutrient and/or dietary supplement (21 CFR §582.5875) AAFCO OP ingredient definition (recognized vitamin ingredients) 90.25	FCC
Zinc chloride	Nutrient	GRAS substance for use as a nutrient and/or dietary supplement 21 CFR §582.5985 AAFCO OP ingredient definition (mineral product) 57.117	USP

Abbreviations: OP – Official Publication; FCC – Food Chemicals Codex; USP – United States Pharmacopoeia

1 Overview

(b) (4)

2 Master Cell Bank / Working Cell Bank

(b) (4)

.

3 Fermentation

(b) (4)

(b) (4)

4 Biomass Harvest by Centrifugation

(b) (4)

(b) (4)

5 Preservation Mixture Formulation

(b) (4)

(b) (4)

6 Freeze Drying

(b) (4)

Table 2. Freeze Dryer Profile

(b) (4)

(b) (4)

7 Cryomilling

(b) (4)

8 Fat Encapsulation

(b) (4)

Table 3. Fat Encapsulated *Pichia kudriavzevii* ASCUSDY21 Recipe

Appendix A. Process Diagram of the Production of Fat Encapsulated *P. kudriavzevii* ASCUSDY21

***Pichia kudriavzevii* ASCUSDY21 Manufacturing Process** CONFIDENTIAL 8 May 2020

(b) (4)

ASCUS BIOSCIENCES

Method

Title	DY21-POE Microbe Enumeration
Version	05
Effective Date	15May2020
Author	Miranda Striluk
Approver (Signature & Date)	DocuSigned by: Martin Maylew 5/8/2020 Martin Maylew VP – Process Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable cells of Dairy-21 in Dairy-21 Palm Oil Encapsulate by counting colony forming units (CFU) on solid media.

Safety

Consult the Safety Data Sheet for all reagents prior to handling. Use caution in working with a hot water bath, hot liquids, liquid nitrogen, and extremely cold material. Liquid nitrogen can cause cold burns, frostbite, and permanent eye damage from brief exposure. Avoid skin and eye contact with liquid nitrogen and wear appropriate personal protective equipment (safety glasses and gloves) at all times. Analyst should be trained on liquid nitrogen handling before continuing this method.

Materials

Corning® 15mL Polypropylene Centrifuge Tubes (Corning 430052)
Test tubes, 13x100 mm, sterile
Test tube cap, 16 mm, polypropylene
1.5 mL polypropylene microcentrifuge tube with snap cap
1000 µL Pipette
200 µL Pipette
1000 µL pipette tips, sterile
200 µL pipette tips, sterile
Glass beads, 3 mm, sterile, new

Equipment

Laboratory Vortexer
Class I/II Biosafety Cabinet
pH meter
Mortar and Pestle
Magnetic Stir Plate

Media & Reagents

YPD Plates
Growcells 10X Phosphate Buffered Saline pH 7.4 (PBS), sterile (Growcells MRGF-6235)
Growcells 1X Phosphate Buffered Saline with 0.05% TWEEN pH 7.4, sterile (Growcells MRGF-6275)
Reagent grade 95% Ethanol
70% Ethanol
10% Bleach
Liquid Nitrogen
1N Hydrochloric Acid
1N Sodium Hydroxide

DY21-POE Microbe Enumeration

Method

1. Preparation of sterile 1X Phosphate Buffered Saline (PBS), pH 7.4

(b) (4)

2. De-encapsulation of Spray Congealed DY21-POE

(b) (4)

3. Prepare the Primary Dilution Mix

(b) (4)

4. DY21-POE Aerobic Plating

(b) (4)

(b) (4)

DY21-POE Microbe Enumeration

(b) (4)

5. Negative Control Plating

(b) (4)

6. Plate Counting

(b) (4)

DY21-POE Microbe Enumeration

Reasons for Revision

(b) (4)

ASCUS BIOSCIENCES

Method Validation Report
Version: 1

Method:
DY21-POE Microbe Enumeration Method, Version 2 Draft

Objective:
This objective of this validation was to demonstrate that changing the buffer from Phosphate Buffered Saline (PBS) to Phosphate Buffered Saline with 0.05% TWEEN does not have an impact on assay performance. (Note that TWEEN 20 is the same as Polysorbate 20.)

Results:
A summary of the CFU results from Analyst 1 and Analyst 2 were comparable and results with both buffers were similar (Table 1).

Table 1. Analyst CFU/g results for Dairy-21 Microbe Enumeration

DY21-POE Lot 18-0202-001-P48-1							
Analyst	Buffer	Avg. DY21 (CFU/g)	Standard Dev.	CV	Avg. of both Analysts DY21 (CFU/g)	STD Dev	CV
1	1X PBS	2.83E+09	(b) (4)		2.98E+09	(b) (4)	
2	1X PBS	3.12E+09					
1	1X PBST	3.01E+09			2.85E+09		
2	1X PBST	2.69E+09					

Conclusion:

PBS with Polysorbate 20 can be substituted for normal PBS for work with Dairy-21 without negative effects on the assay. This is demonstrated by obtaining comparable results with either buffer, performed by two separate analysts.

The revised method will be approved.

Deviations from the protocol:
None

Summary Report Approvals:

Name & Title	Signature
Corey Dodge Process Development	DocuSigned by: Corey Dodge 12/12/2018 FAA4AE21D1C745C...
Patricia A. Williams Quality	DocuSigned by: Patricia A Williams 12/9/2018 5B301285A10643D...

BAM: Aerobic Plate Count

January 2001

Bacteriological Analytical Manual
Chapter 3
Aerobic Plate Count

Authors: Larry Maturin (ret.) and James T. Peeler (ret)

For additional information, contact Guodong Zhang (mailto:guodong.zhang@fda.hhs.gov).

Chapter Contents

The aerobic plate count (APC) is intended to indicate the level of microorganism in a product. Detailed procedures for determining the APC of foods have been developed by the Association of Official Analytical Chemists (AOAC) (3) and the American Public Health Association (APHA) (1). The conventional plate count method for examining frozen, chilled, precooked, or prepared foods, outlined below, conforms to AOAC *Official Methods of Analysis*, sec. 966.23, with one procedural change (966.23C). The suitable colony counting range (10) is 25-250. The automated spiral plate count method for the examination of foods and cosmetics (5), outlined below, conforms to AOAC *Official Methods of Analysis*, sec. 977.27. For procedural details of the standard plate count, see ref. 2.Guidelines for calculating and reporting plate counts have been changed to conform with the anticipated changes in the 16th edition of *Standard Methods for the Examination of Dairy Products* (2) and the *International Dairy Federation* (IDF) procedures (6).

Conventional Plate Count Method

A. **Equipment and materials**

1. Work area, level table with ample surface in room that is clean, well-lighted (100 foot-candles at working surface) and well-ventilated, and reasonably free of dust and drafts. The microbial density of air in working area, measured in fallout pour plates taken during plating, should not exceed 15 colonies/plate during 15 min exposure.
2. Storage space, free of dust and insects and adequate for protection of equipment and supplies

3. Petri dishes, glass or plastic (at least 15 × 90 mm)
4. Pipets with pipet aids (no mouth pipetting) or pipettors, 1, 5, and 10 ml, graduated in 0.1 ml units
5. Dilution bottles, 6 oz (160 ml), borosilicate-resistant glass, with rubber stoppers or plastic screw caps
6. Pipet and petri dish containers, adequate for protection
7. Circulating water bath, for tempering agar, thermostatically controlled to 45 ± 1°C
8. Incubator, 35 ± 1°C; milk, 32 ± 1°C
9. Colony counter, dark-field, Quebec, or equivalent, with suitable light source and grid plate
10. Tally register
11. Dilution blanks, 90 ± 1 ml Butterfield's phosphate-buffered dilution water (R11 (/food/laboratory-methods/bam-r11-butterfields-phosphate-buffered-dilution-water)); milk, 99 ± 2 ml
12. Plate count agar (standard methods) (M124 (/food/laboratory-methods/bam-media-m124-plate-count-agar-standard-methods))
13. Refrigerator, to cool and maintain samples at 0-5°C; milk, 0-4.4°C
14. Freezer, to maintain frozen samples from -15 to -20°C
15. Thermometers (mercury) appropriate range; accuracy checked with a thermometer certified by the National Institute of Standards and Technology (NIST)

B. **Procedure for analysis of frozen, chilled, precooked, or prepared foods**

Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and others as appropriate, of food homogenate (**see** Chapter 1 (/food/laboratory-methods/bam-food-samplingpreparation-sample-homogenate) for sample preparation) by transferring 10 ml of previous dilution to 90 ml of diluent. Avoid sampling foam. Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 s. Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes. Reshake dilution bottle 25 times in 30 cm arc within 7 s if it stands more than 3 min before it is pipetted into petri dish. Add 12-15 ml plate count agar (cooled to 45 ± 1°C) to each plate within 15 min of original dilution. For milk samples, pour an agar control, pour a dilution water control and pipet water for a pipet control. Add agar to the latter two for each series of samples. Add agar immediately to petri dishes when sample diluent contains hygroscopic materials, e.g., flour and starch. Pour agar and dilution water control plates for each series of samples. Immediately mix sample dilutions and agar medium

thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify. Invert solidified petri dishes, and incubate promptly for 48 ± 2 h at 35°C. Do not stack plates when pouring agar or when agar is solidifying.

C. **Guidelines for calculating and reporting APCs in uncommon cases**

Official Methods of Analysis (3) does not provide guidelines for counting and reporting plate counts, whereas *Standard Methods for the Examination of Dairy Products*, 16th ed. (2) presents detailed guidelines; for uniformity, therefore, use APHA guidelines as modified (6,8). Report all aerobic plate counts (2) computed from duplicate plates. For milk samples, report all aerobic plate (2) counts computed from duplicate plates containing less than 25 colonies as less than 25 estimated count. Report all aerobic plate counts (2) computed from duplicate plates containing more than 250 colonies as estimated counts. Counts outside the normal 25-250 range may give erroneous indications of the actual bacterial composition of the sample. Dilution factors may exaggerate low counts (less than 25), and crowded plates (greater than 250) may be difficult to count or may inhibit the growth of some bacteria, resulting in a low count. Report counts less than 25 or more than 250 colonies as estimated aerobic plate counts (EAPC). Use the following guide:

1. Normal plates (25-250). Select spreader-free plate(s). Count all colony forming units (CFU), including those of pinpoint size, on selected plate(s). Record dilution(s) used and total number of colonies counted.
2. Plates with more than 250 colonies. When number of CFU per plate exceeds 250, for all dilutions, record the counts as too numerous to count (TNTC) for all but the plate closest to 250, and count CFU in those portions of plate that are representative of colony distribution. See ref. 2 for detailed guidelines. Mark calculated APC with EAPC to denote that it was estimated from counts outside 25-250 per plate range (*see* D-3).
3. Spreaders. Spreading colonies are usually of 3 distinct types: 1) a chain of colonies, not too distinctly separated, that appears to be caused by disintegration of a bacterial clump; 2) one that develops in film of water between agar and bottom of dish; and 3) one that forms in film of water at edge or on surface of agar. If plates prepared from sample have excessive spreader growth so that (a) area covered by spreaders, including total area of repressed growth, exceeds 50% of plate area, or (b) area of repressed growth exceeds 25% of plate area, report plates as spreaders. When it is necessary to count plates containing spreaders not eliminated by (a) or (b) above, count each of the 3 distinct spreader types as one source. For the first type, if only one chain exists, count it as a single colony. If one or more chains appear to originate from separate sources, count each source as one colony. Do not count each individual growth in such chains as a separate colony. Types 2 and 3 usually result in distinct colonies and are counted as such. Combine the spreader count and the colony count to compute the APC.

4. Plates with no CFU. When plates from all dilutions have no colonies, report APC as less than 1 times the corresponding lowest dilution used. Mark calculated APC with asterisk to denote that it was estimated from counts outside the 25-250 per plate range. When plate(s) from a sample are known to be contaminated or otherwise unsatisfactory, record the result(s) as laboratory accident (LA).

D. **Computing and recording counts (see refs 6, 8)**

To avoid creating a fictitious impression of precision and accuracy when computing APC, report only the first two significant digits. Round off to two significant figures only at the time of conversion to SPC. For milk samples, when plates for all dilutions have no colonies, report APC as less than 25 colonies estimated count. Round by raising the second digit to the next highest number when the third digit is 6, 7, 8, or 9 and use zeros for each successive digit toward the right from the second digit. Round down when the third digit is 1, 2, 3, or 4. When the third digit is 5, round up when the second digit is odd and round down when the second digit is even.

Examples

Calculated Count	APC
12,700	13,000
12,400	12,000
15,500	16,000
14,500	14,000

1. Plates with 25-250 CFU.

$$N = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2) \times (d)}$$

a. Calculate the APC as follows:

$$\frac{(31+31)\ \text{colonies}}{0.0015\ \text{ml}} = 4.1 \times 10^4$$

= 537/0.022
= 24,409
≈ 24,000

b. When counts of duplicate plates fall within and without the 25-250 colony range, use only those counts that fall within this range.

2. All plates with fewer than 25 CFU. When plates from both dilutions yield fewer than 25 CFU each, record actual plate count but record the count as less than 25 × 1/d when d is the dilution factor for the dilution from which the first counts were obtained.

Example

Colonies		
1:100	**1:1000**	**EAPC/ml (g)**
18	2	<>
0	0	<>

3. **All plates with more than 250 CFU**. When plates from both 2 dilutions yield more than 250 CFU each (but fewer than 100/cm^2), estimate the aerobic counts from the plates (EAPC) nearest 250 and multiply by the dilution.

   Example

Colonies		
1:100	**1:1000**	**EAPC/ml (g)**
TNTC	640	640,000

   TNTC, too numerous to count.
   EAPC, estimated aerobic plate count.

4. All plates with spreaders and/or laboratory accident. Report respectively as Spreader (SPR), or Laboratory Accident (LA).
5. All plates with more than an average of 100 CFU per sq cm. Estimate the APC as greater than 100 times the highest dilution plated, times the area of the plate. The examples below have an average count of 110 per sq cm.

   Example

Colonies/Dilution		
1:100	**1:1000**	**EAPC/ml (g)**
TNTC	7,150[(a)]	>6,500,000 EAPC[(b)]
TNTC	6,490	>5,900,000 EAPC

[a] Based on plate area of 65 cm^2
[b] EAPC, estimated APC
[c] Based on plate area of 59 cm^2

Spiral Plate Method

The spiral plate count (SPLC) method for microorganisms in milk, foods, and cosmetics is an official method of the APHA (2) and the AOAC (3). In this method, a mechanical plater inoculates a rotating agar plate with liquid sample. The sample volume dispensed decreases as the dispensing stylus moves from the center to the edge of the rotating plate. The microbial concentration is determined by counting the colonies on a part of the petri dish where they are easily countable and dividing this count by the appropriate volume. One inoculation determines microbial densities between 500 and 500,000 microorganisms/ml. Additional dilutions may be made for suspected high microbial concentrations.

A. **Equipment and materials**

1. Spiral plater (Spiral Systems Instruments, Inc., 7830 Old Georgetown Road, Bethesda, MD 20814)
2. Spiral colony counter (Spiral Systems) with special grid for relating deposited sample volumes to specific portions of petri dishes
3. Vacuum trap for disposal of liquids (2-4 liter vacuum bottle to act as vacuum reservoir and vacuum source of 50-60 cm Hg)
4. Disposable micro beakers, 5 ml
5. Petri dishes, plastic or glass, 150 × 15 mm or 100 × 15 mm
6. Plate count agar (standard methods) (M124 (/food/laboratory-methods/bam-media-m124-plate-count-agar-standard-methods))
7. Calculator (optional), inexpensive electronic hand calculator is recommended
8. Polyethylene bags for storing prepared plates
9. Commercial sodium hypochlorite solution, about 5% NaOCl (bleach)
10. Sterile dilution water
11. Syringe, with Luer tip for obstructions in stylus; capacity not critical
12. Work area, storage space, refrigerator, thermometers, tally, incubator, as described for Conventional Plate Count Method, above.
13. Sodium hypochlorite solution (5.25%). Available commercially.

B. **Preparation of agar plates.**

Automatic dispenser with sterile delivery system is recommended to prepare agar plates. Agar volume dispensed into plates is reproducible and contamination rate is low compared to hand-pouring of agar in open laboratory. When possible, use laminar air flow hood along with automated dispenser. Pour same quantity of agar into all plates so that same height of agar will be presented to spiral plater stylus tip to maintain contact angle. Agar plates should be level during cooling.

The following method is suggested for prepouring agar plates: Use automatic dispenser or pour constant amount (about 15 ml/100 mm plate; 50 ml/150 mm plate) of sterile agar at 60-70°C into each petri dish. Let agar solidify on level surface with poured

plates stacked no higher than 10 dishes. Place solidified agar plates in polyethylene bags, close with ties or heat-sealer, and store inverted at 0-4.4°C. Bring prepoured plates to room temperature before inoculation.

C. **Preparation of samples.**

As described in Chapter 1, select that part of sample with smallest amount of connective tissues or fat globules.

D. **Description of spiral plater.**

Spiral plater inoculates surface of prepared agar plate to permit enumeration of microorganisms in solutions containing between 500 and 500,000 microorganisms per ml. Operator with minimum training can inoculate 50 plates per h. Within range stated, dilution bottles or pipets and other auxiliary equipment are not required. Required bench space is minimal, and time to check instrument alignment is less than 2 min. Plater deposits decreasing amount of sample in Archimedean spiral on surface of prepoured agar plate. Volume of sample on any portion of plate is known. After incubation, colonies appear along line of spiral. If colonies on a portion of plate are sufficiently spaced from each other, count them on special grid which associates a calibrated volume with each area. Estimate number of microorganisms in sample by dividing number of colonies in a defined area by volume contained in same area. Studies have shown the method to be proficient not only with milk (4) but also with other foods (7,10).

E. **Plating procedure**

Check stylus tip angle daily and adjust if necessary. (Use vacuum to hold microscope cover slip against face of stylus tip; if cover slip plane is parallel at about l mm from surface of platform, tip is properly oriented). Liquids are moved through system by vacuum. Clean stylus tip by rinsing for 1 s with sodium hypochlorite solution followed by sterile dilution water for 1 s before sample introduction. This rinse procedure between processing of each sample minimizes cross-contamination. After rinsing, draw sample into tip of Teflon tubing by vacuum applied to 2-way valve. When tubing and syringe are filled with sample, close valve attached to syringe. Place agar plate on platform, place stylus tip on agar surface, and start motor. During inoculation, label petri plate lid. After agar has been inoculated, stylus lifts from agar surface and spiral plater automatically stops. Remove inoculated plate from platform and cover it. Move stylus back to starting position. Vacuum-rinse system with hypochlorite and water, and then introduce new sample. Invert plates and promptly place them in incubator for 48 ± 3 h at 35 ± 1°C.

F. **Sterility controls**

Check sterility of spiral plater for each series of samples by plating sterile dilution water. CAUTION: Prepoured plates should not be contaminated by a surface colony or be below room temperature (water can well-up from agar). They should not be excessively dry, as indicated by large wrinkles or glazed appearance. They should not

have water droplets on surface of agar or differences greater than 2 mm in agar depth, and they should not be stored at 0-4.4°C for longer than l month. Reduced flow rate through tubing indicates obstructions or material in system. To clear obstructions, remove valve from syringe, insert hand-held syringe with Luer fitting containing water, and apply pressure. Use alcohol rinse to remove residual material adhering to walls of system. Dissolve accumulated residue with chromic acid. Rinse well after cleaning.

G. **Counting grid**

1. **Description.** Use same counting grid for both 100 and 150 mm petri dishes. A mask is supplied for use with 100 mm dishes. Counting grid is divided into 8 equal wedges; each wedge is divided by 4 arcs labeled l, 2, 3, and 4 from outside grid edge. Other lines within these arcs are added for ease of counting. A segment is the area between 2 arc lines within a wedge. Number of areas counted (e.g., 3) means number of segments counted within a wedge. Spiral plater deposits sample on agar plate in the same way each time. The grid relates colonies on spiral plate to the volume in which they were contained. When colonies are counted with grid, sample volume becomes greater as counting starts at outside edge of plate and proceeds toward center of plate.

2. **Calibration.** The volume of sample represented by various parts of the counting grid is shown in operator's manual that accompanies spiral plater. Grid area constants have been checked by the manufacturer and are accurate. To verify these values, prepare 11 bacterial concentrations in range of 10^6-10^3 cells/ml by making 1:1 dilutions of bacterial suspension (use a nonspreader). Plate all Incubate both sets of plates for 48 ± 3 h at 35 ± 1°C. Calculate concentrations for each dilution. Count spiral plates over grid surface, using counting rule of 20 (described in H, below), and record number of colonies counted and grid area over which they were counted. Each spiral colony count for a particular grid area, divided by aerobic count/ml for corresponding spirally plated bacterial concentrations, indicates volume deposited on that particular grid area. Use the following formula:

$$\text{Volume (ml) for grid area} = \frac{\text{Spiral Colonies counted in area}}{\text{Baterial count/ml (APC)}}$$

$$\text{Volume (ml)} = \frac{31 + 30 \text{ colonies}}{4.1 \times 10^4 \text{ bateria/ml}} = 0.0015 \text{ ml}$$

To check total volume dispensed by spiral plater, weigh amount dispensed from stylus tip. Collect in tared 5 ml plastic beaker and weigh on analytical balance (± 0.2 mg).

 Fig. 1 10 cm plate

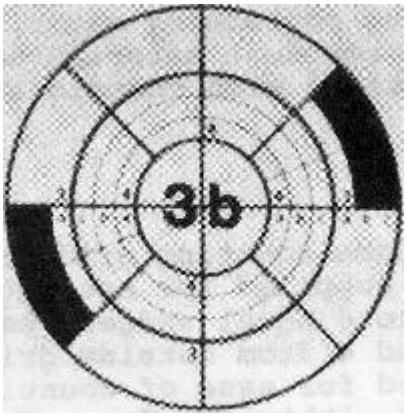

Figure 1. 10 cm plate, area (3b)

$$\frac{(31+31)\ \text{colonies}}{0.0015\,\text{ml}} = 4.1\times10^{4}$$

H. **Examination and reporting of spiral plate counts.**

Counting rule of 20. After incubation, center spiral plate over grid by adjusting holding arms on viewer. Choose any wedge and begin counting colonies from outer edge of first segment toward center until 20 colonies have been counted. Complete by counting remaining colonies in segment where 20th colony occurs. In this counting procedure, numbers such as 3b, 4c (Fig. l) refer to area segments from outer edge of wedge to designated arc line. Any count irregularities in sample composition are controlled by counting the same segments in the opposite wedge and recording results. Example of spirally inoculated plate (Fig. l) demonstrates method for determining microbial count. Two segments of each wedge were counted on opposite sides of plate with 31 and 30 colonies, respectively. The sample volume contained in the darkened segments is 0.0015 ml. To estimate number of microorganisms, divide count by volume contained in all segments counted. See example under Fig. l.

If 20 CFU are not within the 4 segments of the wedge, count CFU on entire plate. If the number of colonies exceeds 75 in second, third, or fourth segment, which also contains the 20th colony, the estimated number of microorganisms will generally be low because of coincidence error associated with crowding of colonies. In this case, count each circumferentially adjacent segment in all 8 wedges, counting at least 50 colonies, e.g., if the first 2 segments of a wedge contain 19 colonies and the third segment contains the 20th and 76th (or more), count colonies in all circumferentially adjacent first and second segments in all 8 wedges. Calculate contained volume in counted segments of wedges and divide into number of colonies.

When fewer than 20 colonies are counted on the total plate, report results as "less than 500 estimated SPLC per ml." If colony count exceeds 75 in first segment of wedge, report results as "greater than 500,000 estimated SPLC per ml." Do not count spiral plates with irregular distribution of colonies caused by dispensing errors. Report results of such plates as laboratory accident (LA). If spreader covers entire plate, discard plate. If spreader covers half of plate area, count only those colonies that are well distributed in spreader-free areas.

Compute SPLC unless restricted by detection of inhibitory substances in sample, excessive spreader growth, or laboratory accidents. Round off counts as described in I-D, above. Report counts as SPLC or estimated SPLC per ml.

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Hypertext Source: Bacteriological Analytical Manual, Edition 8, Revision A, 1998. Chapter 3.

AOAC Official Method 2013.01
Salmonella in a Variety of Foods
VIDAS® UP *Salmonella* (SPT) Method
First Action 2013
Final Action 2016

[Applicable to detection of *Salmonella* in raw ground beef (25 and 375 g), processed American cheese (25 g), deli roast beef (25 g), liquid egg (25 g), peanut butter (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), raw cod (25 g), bagged lettuce (25 and 375 g), dark chocolate (375 g), powdered eggs (25 g), instant nonfat dry milk (25 and 375 g), ground black pepper (25 g), dry dog food (375 g), raw ground turkey (375 g), almonds (375 g), chicken carcass rinsates (30 mL), and stainless steel, plastic, and ceramic environmental surfaces.]

See Tables **2013.01A** and **B** for a summary of results of the interlaboratory study. For detailed results of the interlaboratory study, *see* Tables A–F in Appendix 1 on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac).

A. Principle

The VIDAS SPT method is for use on the automated VIDAS instrument for the detection of *Salmonella* receptors using the enzyme-linked fluorescent assay. The solid-phase receptacle (SPR) serves as the solid phase, as well as the pipetting device. The interior of the SPR is coated with proteins specific for *Salmonella* receptors. Reagents for the assay are ready-to-use and predispensed in the sealed reagent strips. The instrument performs all the assay steps automatically. The reaction medium is cycled in and out of the SPR several times. An aliquot of enrichment broth is dispensed into the reagent strip. The *Salmonella* receptors present will bind to the interior of the SPR. Unbound components are eliminated during the washing steps. The proteins conjugated to the alkaline phosphatase are cycled in and out of the SPR and will bind to any *Salmonella* receptors, which are themselves bound to the SPR wall. A final wash step removes unbound conjugate. During the final detection step, the substrate (4-methylumbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methylumbelliferone), the fluorescence of which is measured at 450 nm. At the end of the assay, results are automatically analyzed by the instrument which calculates a test value for each sample. This value is then compared to internal references (thresholds) and each result is interpreted as positive or negative.

B. Apparatus and Reagents

Items (**a**)–(**h**) are available as the VIDAS SPT assay kit from bioMérieux Inc., Hazelwood, MO.

(**a**) *VIDAS or miniVIDAS automated immunoassay system.*

(**b**) *SPT reagent strips.*—60 polypropylene strips of 10 wells, each strip covered with a foil seal and label. The 10 wells contain the reagents in Table **2013.01C**.

(**c**) *SPR.*—60 SPRs coated with proteins specific for *Salmonella* receptors.

(**d**) *Standard.*—One vial (6 mL). Contains purified and inactivated *Salmonella* receptors + preservative + protein stabilizer.

(**e**) *Positive control solution.*—One vial (6 mL). Contains purified and inactivated *Salmonella* receptors + preservative + protein stabilizer.

(**f**) *Negative control solution.*—One vial (6 mL). Contains Tris-buffered saline (150 mmol/L)–Tween pH 7.6 + preservative.

(**g**) *Master lot entry (MLE) card.*—One card providing specifications for the factory master data required to calibrate the test.

(**h**) *Package insert*.

(**i**) *Disposable pipet to dispense appropriate volumes.*

(**j**) *VIDAS Heat and Go.*—Available from bioMérieux, Inc.

(**k**) *Water bath (95–100°C) or equivalent system.*

(**l**) *Stomacher®-type bag with filter.*

(**m**) *Stomacher.*—Stomacher Lab Blender 400, available from Seward Medical (London, UK); Smasher, bioMérieux, Inc., or equivalent.

(**n**) *BPW.*—Available from bioMérieux, Inc.

(**o**) *Salmonella supplement.*—Available from bioMérieux, Inc.

(**p**) *Incubators.*—Capable of maintaining 42 ± 1°C and 35 ± 1°C.

(**q**) *Diagnostic reagents.*—Necessary for culture confirmation of assays. *See* **967.27** (*see* 17.9.03).

(**r**) *IBISA chromogenic agar.*—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**s**) *ASAP chromogenic agar.*—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**t**) *Vancomycin.*—Available from bioMérieux, Inc.

C. General Instructions

(**a**) Components of the kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(**b**) Store VIDAS SPT kits at 2–8°C.

(**c**) Do not freeze reagents.

(**d**) Bring reagents to room temperature before inserting them into the VIDAS instrument.

(**e**) Mix standard, controls, and heated test portions well before using.

(**f**) Include one positive and one negative control with each group of tests.

(**g**) Return unused components to 2–8°C immediately after use.

(**h**) *See* safety precautions in the VIDAS SPT package insert (refer to the following sections in the package insert: Warnings and Precautions and Waste Disposal).

D. Preparation of Test Suspension

(**a**) *Pre-enrichment.*—Pre-enrich test portion in BPW using filter Stomacher bags to initiate growth of *Salmonella.* For 25 g test portions, add 225 mL BPW to each test portion and homogenize thoroughly for 2 min. For 375 g test portions, prewarm BPW to 42 ± 1°C, add 1125 mL to each test portion, and homogenize thoroughly for 2 min.

(**b**) After homogenization add *Salmonella* supplement to each test portion. For 25 g test portions, add 1 mL of *Salmonella* supplement, mix samples manually, and incubate for 18–24 h at 42 ± 1°C. For 375 g test portions, add 5 mL of *Salmonella* supplement, mix samples manually, and incubate for 22–26 h at 42 ± 1°C.

(**c**) After incubation, homogenize samples manually. If a water bath is used, transfer 2–3 mL enrichment broth into a tube. Seal the tube. Heat for 5 ± 1 min at 95–100°C. Cool the tube. Mix the boiled broth and transfer 0.5 mL into the sample well of the VIDAS SPT reagent strip. If the VIDAS Heat and Go is used, transfer 0.5 mL of the enrichment broth into the sample well of the VIDAS SPT reagent strip. Heat for 5 ± 1 min (*see* VIDAS Heat and Go User's

Table 2013.01A. Summary of results for the detection of *Salmonella* spp. in raw ground beef (25 g)

Method[a]	VIDAS SPT with traditional confirmation on BGSA and XLT4			VIDAS SPT with traditional confirmation on IBISA and ASAP[b]			VIDAS SPT with alternative confirmation on IBISA and ASAP[c]		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total samples analyzed	0/144	144/144	144/144	0/144	144/144	144/144	0/144	144/144	144/144
Candidate presumptive POD (CP)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)
s_r[d]	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)
s_L[e]	0.00 (0.00 +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)
s_R[f]	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)
P-value	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Candidate confirmed positive/total samples analyzed	0/144	143/144	144/144	0/144	143/144	144/144	0/144	143/144	144/144
Candidate confirmed POD (CC)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)
s_r	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)
s_R	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)
P-value	1.0000	0.4368	1.0000	1.0000	0.4368	1.0000	1.0000	0.4368	1.0000
Positive reference samples/total samples analyzed	0/144	84/144	138/144	0/144	84/144	138/144	0/144	84/144	138/144
Reference POD	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)
s_r	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)
s_R	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)
P-value	1.0000	0.6298	0.0179	1.0000	0.6298	0.0179	1.0000	0.6298	0.0179
dLPOD (candidate vs reference)	0.00 (–0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (0.01, +0.09)	0.00 (–0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (+0.01, +0.09)	0.00 (–0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (+0.01, +0.09)
dLPOD (candidate presumptive vs candidate confirmed)	0.00 (–0.03, +0.03)	0.01 (–0.02, +0.04)	0.00 (–0.03, +0.03)	0.00 (–0.03, +0.03)	0.01 (–0.02, +0.04)	0.00 (–0.03, +0.03)	0.00 (–0.03, +0.03)	0.01 (–0.02, +0.04)	0.00 (–0.03, +0.03)

[a] Results include 95% confidence intervals.

[b] Traditional confirmation on ASAP/IBISA = secondary enrichments streaked onto IBISA and ASAP.

[c] Alternative confirmation = direct streak of the primary enrichment onto IBISA and ASAP.

[d] Repeatability standard deviation.

[e] Among-laboratory standard deviation.

[f] Reproducibility standard deviation.

Table 2013.01B. Summary of results for the detection of *Salmonella* spp. in raw ground beef (375 g)

Method[a]	VIDAS SPT with traditional confirmation on BGSA and XLT4			VIDAS SPT with traditional confirmation on IBISA and ASAP[b]			VIDAS SPT with alternative confirmation on IBISA and ASAP[c]		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total samples analyzed	0/132	58/131	130/132	0/132	58/131	130/132	0/132	57/131	130/132
Candidate presumptive POD (CP)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.33, +0.54)	0.98 (+0.965, +1.00)
s_r[d]	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.44. +0.52)	0.12 (+0.11, +0.16)
s_L[e]	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.09 (0.00, +0.26)	0.00 (0.00, +0.05)
s_R[f]	0.00 (0.00, +0.23)	0.50 (+0.44, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.44, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)
P-value	1.0000	0.1551	0.5190	1.0000	0.1551	0.5190	1.0000	0.1906	0.5190
Candidate confirmed positive/total samples analyzed	0/132	58/131	130/132	0/132	59/131	130/132	0/132	58/131	130/132
Candidate confirmed POD (CC)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.45 (+0.35, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)
s_r	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.44, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)
s_L	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.09 (0.00, +0.25)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)
s_R	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)
P-value	1.0000	0.1551	0.5190	1.0000	0.2060	0.5190	1.0000	0.1551	0.5190
Positive reference samples/total samples analyzed	0/132	57/132	132/132	0/132	57/132	132/132	0/132	54/132	131/132
Reference POD	0.00 (0.00, +0.03)	0.43 (+0.35, +0.52)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.43 (+0.35, +0.52)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.41 (+0.32, +0.50)	0.99 (+0.96, +1.00)
s_r	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.49 (+0.44, +0.52)	0.09 (+0.08, +0.16)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.05 (0.00, +0.22)	0.00 (0.00, +0.04)
s_R	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.23)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.23)	0.00 (0.00, +0.23)	0.49 (+0.44, +0.52)	0.09 (+0.08, +0.10)
P-value	1.0000	0.6261	1.0000	1.0000	0.6261	1.0000	1.0000	0.3313	0.4338
dLPOD (C vs R)	0.00 (–0.03, +0.03)	0.01 (–0.12, +0.15)	–0.02 (–0.05, +0.02)	0.00 (–0.03, +0.03)	0.02 (–0.18, +0.22)	–0.02 (–0.05, +0.02)	0.00 (–0.03, +0.03)	0.03 (–0.18, +0.24)	–0.01 (–0.05, +0.03)
dLPOD (CP vs CC)	0.00 (–0.03, +0.03)	0.00 (–0.15, +0.15)	0.00 (–0.04, +0.04)	0.00 (–0.03, +0.03)	–0.01 (–0.15, +0.14)	0.00 (–0.04, +0.04)	0.00 (–0.03, +0.03)	–0.01 (–0.21, +0.23)	0.00 (–0.04, +0.04)

[a] Results include 95% confidence intervals.

[b] Traditional confirmation on ASAP/IBISA = secondary enrichments streaked onto IBISA and ASAP.

[c] Alternative confirmation = direct streak of the primary enrichment onto IBISA and ASAP.

[d] Repeatability standard deviation.

[e] Among-laboratory standard deviation.

[f] Reproducibility standard deviation.

Table 2013.01C. Reagents included in 10-well reagent strip

Wells	Reagents (SPT)
1	Sample well: 0.5 mL of enrichment broth, standard or control
2	Prewash solution (400 µL): Buffer pH 7.8 + preservative
3–5, 7–9	Wash buffer (600 µL): TRIS-buffered saline (150 mmol/L) – Tween pH 7.6 + preservative
6	Conjugate (400 µL): alkaline phosphatase-labeled proteins specific for *Salmonella* receptors + preservative
10	Reading cuvette with substrate (300 µL): 4-methyl-umbelliferyl phosphate (0.6 mmol/L) + diethanolamine[a] (DEA; 0.62 mol/L or 6.6%, pH 9.2) + preservative

[a] Irritant reagent; *see* VIDAS SPT package insert for more information.

Manual). Remove the strip and allow to cool for 10 min prior to test initiation. Perform the VIDAS test.

E. Enzyme Immunoassay

(**a**) Enter factory master calibration curve data into the instrument using the MLE card.

(**b**) Remove the kit reagents and materials from refrigerated storage and allow them to come to room temperature.

(**c**) Use one VIDAS SPT reagent strip and one VIDAS SPT SPR for each sample, control, or standard to be tested. Reseal the storage pouch after removing the required number of SPRs.

(**d**) Enter the appropriate assay information to create a work list. Enter the test code by typing or selecting "SPT," and number of tests to be run. If the standard is to be tested, identify the standard by "S1" and test in duplicate. If the positive control is to be tested, identify it by "C1." If the negative control is to be tested, identify it by "C2."

Note: The standard must be tested upon receipt of a new lot of reagents and then every 14 days. The relative fluorescence value (RFV) of the standard must fall within the set range provided with the kit.

(**e**) Load the SPT reagents strips and SPRs into the positions that correspond to the VIDAS section indicated by the work list. Verify that the color labels with the assay code on the SPRs and reagent strips match.

(**f**) Initiate the assay processing as directed in the VIDAS operator's manual.

(**g**) After the assay is completed, remove the SPRs and reagent strips from the instrument and dispose of properly.

Table 2013.01D. Interpretation of test

Test value threshold	Interpretation
<0.25	Negative
≥0.25	Positive

F. Results and Interpretation

The results are analyzed automatically by the VIDAS system. A report is printed which records the type of test performed, test sample identification, date and time, lot number, and expiration date of the reagent kit being used, each sample's RFV**,** test value, and interpreted result (positive or negative). Fluorescence is measured twice in the reagent strip's reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of the SPR. The test value is calculated by the instrument and is equal to the difference between the background reading and the final reading. The calculation appears on the result sheet. A negative result has a test value less than the threshold (0.25) and indicates that the sample does not contain *Salmonella* spp. or contains *Salmonella* spp. at a concentration below the detection limit. A positive result has a test value equal to or greater than the threshold (≥0.25) and indicates that the sample may be contaminated with *Salmonella* spp. If the background reading is above a predetermined cutoff, then the result is reported as invalid (Table **2012.01D**).

G. Confirmation

All positive VIDAS SPT results must be culturally confirmed. Confirmation should be performed using the non-heated enrichment broth stored between 2 and 8°C, and should be initiated within 72 h after the end of incubation at 42 ± 1°C. Presumptive positive results may be confirmed by isolating on selective agar plates such as IBISA or ASAP, or on the appropriate reference method selective agar plates. Typical or suspect colonies from each plate are confirmed as described in **967.27** (*see* 17.9.03). As an alternative to the conventional tube system for *Salmonella*, any AOAC-approved commercial biochemical kits may be used for presumptive generic identification of foodborne *Salmonella* as described in **978.24** (*see* 17.9.04), **989.12** (*see* 17.9.05), **991.13** (*see* 17.9.06), and **2011.17** (*see* 17.15.01).

Reference: *J. AOAC Int*. **96**, 808(2013)
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AOAC Official Method 2013.10
Listeria species in a Variety of Foods and Environmental Surfaces
VIDAS® UP *Listeria* (LPT) Method
First Action 2013
Final Action 2016

[Applicable to detection of *Listeria* in deli ham (25 and 125 g), pepperoni (25 g), beef hot dogs (25 g), chicken nuggets (25 g), chicken liver pâté (25 g), ground beef (125 g), deli turkey (125 g), cooked shrimp (25 g), smoked salmon (25 g), whole cantaloupe melon, bagged mixed salad (25 g), peanut butter (25 g), black pepper (25 g), vanilla ice cream (25 g), queso fresco (25 and 125 g), stainless steel, plastic, ceramic and concrete environmental surfaces.]

See Tables **2013.10A** and **B** for a summary of results of the collaborative study. *See* supplemental data, Tables 2A–D, for detailed results of the collaborative study on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac.

Caution: *Listeria monocytogenes* is of particular concern for pregnant women, the aged, and the infirmed. It is recommended that these concerned groups avoid handling this organism. Dispose of all reagents and other contaminated materials by acceptable procedures for potentially biohazardous materials. Some reagents in the kit contain 1 g/L concentrations of sodium azide. Check local regulations prior to disposal. Disposal of these reagents into sinks with copper or lead plumbing should be followed immediately with large quantities of water to prevent potential hazards. This kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is, therefore, recommended that these products be treated as potentially infectious and handled observing the usual safety precautions (do not ingest or inhale).

A. Principle

VIDAS® UP *Listeria* (LPT) method is for use on the automated VIDAS instrument for the detection of *Listeria* antigens using the enzyme-linked fluorescent assay (ELFA) method. The assay also incorporates phage proteins allowing an increase in sensitivity and specificity compared to traditional immunoassay. The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device. The interior of the SPR is coated with proteins specific for *Listeria* receptors. Reagents for the assay are ready-to-use and predispensed in the sealed reagent strips. All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times. An aliquot of enrichment broth is dispensed into the reagent strip. The *Listeria* receptors present will bind to the interior of the SPR. Unbound components are eliminated during the washing steps. The proteins conjugated to the alkaline phosphatase are cycled in and out of the SPR and will bind to any *Listeria* receptors, which are themselves bound to the SPR wall. A final wash step removes unbound conjugate. During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methyl-umbelliferone), the fluorescence of which is measured at 450 nm. At the end of the assay, results are automatically analyzed by the instrument, which calculates a test value for each sample. This value is then compared to internal references (thresholds) and each result is interpreted as positive or negative.

B. Apparatus and Reagents

Items (**a**)–(**h**) are available as the VIDAS UP *Listeria* (LPT) assay kit from bioMérieux (Hazelwood, MO, USA).

(**a**) *VIDAS or miniVIDAS automated immunoassay system*.

(**b**) *LPT reagent strips*.—Sixty polypropylene strips of 10 wells, each strip covered with a foil seal and label. The 10 wells contain the reagents shown in Table **2013.10C**.

(**c**) *SPR*.—Sixty SPRs coated with proteins specific for *Listeria* receptors.

(**d**) *Standard*.—One vial (1 × 6 mL). Ready-to-use. Contains purified and inactivated *Listeria* receptors + preservative + protein stabilizer.

(**e**) *Positive control solution*.—1 × 6 mL. Contains purified and inactivated *Listeria monocytogenes* antigen + preservative + protein stabilizer.

(**f**) *Negative control solution*.—1 × 6 mL. Contains Tris-buffered saline (TBS; 150 mmol/l) – Tween pH 7.6 + preservative.

(**g**) *Master Lot Entry (MLE) card*.—One card providing specifications for the factory master data required to calibrate the test: To read the MLE data, please refer to the Operator's Manual.

(**h**) *Package insert*.

(**i**) *Disposable pipet*.—To dispense appropriate volumes.

(**j**) *VIDAS Heat and Go*.—Available from bioMérieux, Inc.

(**k**) *Water bath*.—95–100°C, or equivalent.

(**l**) *Bag with filter*.

(**m**) *Smasher™ Blender/Homogenizer*.—Available from bioMérieux, Inc., or equivalent.

(**n**) *LPT broth*.—bioMérieux, Inc.

(**o**) *Incubators*.—Capable of maintaining 30 ± 1°C and 35 ± 1°C.

(**p**) *Diagnostic reagents*.—Necessary for culture confirmation of assays.

(**q**) *ALOA chromogenic agar*.—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**r**) *Tryptic Soy Agar with yeast additive*.

C. General Instructions

(**a**) Components of the kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(**b**) Store VIDAS LPT kits at 2–8°C.

(**c**) Do not freeze reagents.

(**d**) Bring reagents to room temperature before inserting them into the VIDAS instrument.

(**e**) Standard, controls, and heated test portions are mixed well before using.

(**f**) Include one positive and one negative control with each group of tests.

(**g**) Return unused components to 2–8°C immediately after use.

(**h**) *See* safety precautions in the VIDAS LPT package insert (Warnings and Precautions and Waste Disposal).

(**i**) *See* Centers for Disease Control recommendations in handling pathogens. http:/www.cdc.gov/biosafety/publications/bmb15/index.htm/

Table 2013.10A. Summary of results for the detection of *Listeria* spp. in queso fresco (25 g)[a]

	VIDAS LPT with OXA			VIDAS LPT with ALOA		
	Inoculation level					
	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/ total No. samples analyzed	1/156	80/156	156/156	1/156	80/156	156/156
Candidate presumptive POD (CP)	0.01	0.51	1.00	0.01	0.51	1.00
	(0.01, 0.04)	(0.43, 0.59)	(0.98, 1.00)	(0.01, 0.04)	(0.43, 0.59)	(0.98, 1.00)
s_r[b]	0.08	0.51	0.00	0.08	0.51	0.00
	(0.07, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.07, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.03)	(0.00, 0.13)	(0.00, 0.15)	(0.00, 0.03)	(0.00, 0.13)	(0.00, 0.15)
s_R[d]	0.08	0.51	0.00	0.08	0.51	0.00
	(0.07, 0.13)	(0.46, 0.52)	(0.00, 0.21)	(0.07, 0.13)	(0.46, 0.52)	(0.00, 0.21)
P value[e]	0.4395	0.9210	1.0000	0.4395	0.9210	1.0000
Candidate confirmed positive/ total No. samples analyzed	0/156	78/156	156/156	0/156	78/156	156/156
Candidate confirmed POD (CC)	0.00	0.50	1.00	0.00	0.50	1.00
	(0.00, 0.02)	(0.42, 0.58)	(0.98, 1.00)	(0.00, 0.02)	(0.42, 0.58)	(0.98, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)
P value	1.0000	0.9161	1.0000	1.0000	0.9161	1.0000
Positive reference samples/ total No. samples analyzed	0/156	76/156	156/156	0/156	76/156	156/156
Reference POD	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.02)	(0.41, 0.57)	(0.98, 1.00)	(0.00, 0.02)	(0.41, 0.57)	(0.98, 1.00)
s_r	0.00	0.52	0.00	0.00	0.52	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.10)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.10)	(0.00, 0.15)
s_R	0.00	0.52	0.00	0.00	0.52	0.00
	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)
P value	1.0000	0.9937	1.0000	1.0000	0.9937	1.0000
dLPOD (candidate vs reference)	0.00	0.01	0.00	0.00	0.01	0.00
	(–0.02, 0.02)	(–0.10, 0.13)	(–0.02, 0.02)	(–0.02, 0.02)	(–0.10, 0.13)	(–0.02, 0.02)
dLPOD (candidate presumptive vs candidate confirmed)	0.01	0.01	0.00	0.01	0.01	0.00
	(–0.02, 0.04)	(–0.10, 0.13)	(–0.02, 0.02)	(–0.02, 0.04)	(–0.10, 0.13)	(–0.02, 0.02)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P* value = Homogeneity test of laboratory PODs.

Table 2013.10B. Summary of results for the detection of *Listeria* spp. in queso fresco (125 g)[a]

	VIDAS LPT with OXA			VIDAS LPT with ALOA		
	Inoculation level					
	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/ total No. of samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate presumptive POD (CP)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r[b]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R[d]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value[e]	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Candidate confirmed positive/ total No. of samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate confirmed POD (CC)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Positive reference samples/ total No. of samples analyzed	0/144	69/144	144/144	0/144	69/144	144/144
Reference POD	0.00	0.48	1.00	0.00	0.48	1.00
	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9672	1.0000	1.0000	0.9672	1.0000
dLPOD (C vs R)	0.00	0.01	0.00	0.00	0.01	0.00
	(–0.03, 0.03)	(–0.10, 0.13)	(–0.03, 0.03)	(–0.03, 0.03)	(–0.10, 0.13)	(–0.03, 0.03)
dLPOD (CP vs CC)	0.00	0.00	0.00	0.00	0.00	0.00
	(–0.03, 0.03)	(–0.12, 0.12)	(–0.03, 0.03)	(–0.03, 0.03)	(–0.12, 0.12)	(–0.03, 0.03)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P* value = Homogeneity test of laboratory PODs.

Table 2013.10C. Reagents included in 10-well reagent strip

Wells	Reagents (LPT)
1	Sample well: 0.5 mL of enrichment broth, standard or control
2	Prewash solution (400 µL): TRIS-NaCl (150 mmol/L) - Tween pH 7.6 + preservative
3–5, 7–9	Wash buffer (600 µL): TRIS-NaCl (150 mmol/L) - Tween pH 7.6 + preservative
6	Conjugate (400 µL): alkaline phosphatase-labeled proteins specific for *Listeria* receptors + preservative
10	Reading cuvette with substrate (300 µL): 4-methyl-umbelliferyl phosphate (0.6 mmol/L) + diethanolamine[a] (DEA) (0.62 mol/L or 6.6%, pH 9.2) + preservative

[a] Irritant reagent: *See* VIDAS LPT package insert for more information.

D. Preparation of Test Suspension

(**a**) *Pre-enrichment.*—Pre-enrich test portion using filter Stomacher type bags to initiate growth of *Listeria.* For 25 g test portions, add 225 mL prewarmed (18–25°C) LPT broth to each test portion and homogenize thoroughly for 2 min. For cantaloupe melons, soak entire melon in approximately 1 L prewarmed (18–25°C) LPT broth. For 125 g test portions, add 375 mL prewarmed (18–25°C) LPT broth to each test portion and homogenize thoroughly for 2 min.

(**b**) *Test portions.—(1) 25 g test portions/cantaloupe melons rinses.*—After homogenization, incubate for 26–30 h at 30 ± 1°C.

(2) 125 g test portions.—After homogenization, incubate for 24–30 h at 30 ± 1°C.

From the primary enrichment broth, transfer a 1 mL aliquot into 10 mL prewarmed (18–25°C) LPT broth and incubate for 22–26 h at 30 ± 1°C.

(**c**) After incubation, homogenize samples manually. Follow appropriate instructions based on heating method.

(*1*) *Boiling.*—Transfer 2–3 mL of the enrichment broth into a tube. Seal the tube. Heat in a water bath for 5 ± 1 min at 95–100°C. Cool the tube. Mix the boiled broth and transfer 0.5 mL into the sample well of the VIDAS LPT reagent strip. Perform the VIDAS test.

(2) Heat and Go.—Transfer 0.5 mL of the enrichment broth into the sample well of the VIDAS LPT reagent strip. Heat for 5 ± 1 min (*see* VIDAS Heat and Go User's Manual). Remove the strip and allow to cool for 10 min prior to test initiation. Perform the VIDAS test.

E. Enzyme Immunoassay

(**a**) Enter factory master calibration curve data into the instrument using the MLE card.

(**b**) Remove the kit reagents and materials from refrigerated storage and let them to come to room temperature for at least 30 min.

(**c**) Use one VIDAS LPT reagent strip and one VIDAS LPT SPR for each sample, control, or standard to be tested. Reseal the storage pouch after removing the required number of SPRs.

(**d**) Enter the appropriate assay information to create a work list. Enter the test code by typing or selecting "LPT," and number of tests to be run. If the standard is to be tested, identify the standard by "S1" and test in duplicate. If the positive control is to be tested, identify it by "C1." If the negative control is to be tested, identify it by "C2."

Table 2013.10D. Interpretation of test

Test value threshold	Interpretation
<0.05	Negative
≥0.05	Positive

Note: The standard must be tested upon receipt of a new lot of reagents and then every 14 days. The relative fluorescence value (RFV) of the standard must fall within the set range provided with the kit.

(**e**) Load the LPT reagents strips and SPRs into the positions that correspond to the VIDAS section indicated by the work list. Verify that the color labels with the assay code on the SPRs and reagent strips match.

(**f**) Initiate the assay processing as directed in the VIDAS operator's manual.

(**g**) After the assay is completed, remove the SPRs and reagent strips from the instrument and dispose of properly.

F. Results and Interpretation

The results are analyzed automatically by the VIDAS system. A report is printed which records the type of test performed, the test sample identification, the date and time, the lot number and expiration date of the reagent kit being used, and each sample's RFV, test value, and interpreted result (positive or negative). Fluorescence is measured twice in the reagent strip's reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of the SPR. The test value is calculated by the instrument and is equal to the difference between the background reading and the final reading. The calculation appears on the result sheet. A "negative" result has a test value less than the threshold (0.05) and indicates that the sample does not contain *Listeria* spp. or contains *Listeria* spp. at a concentration below the detection limit. A "positive" result has a test value equal to or greater than the threshold (≥0.05) and indicates that the sample may be contaminated with *Listeria* spp. If the background reading is above a predetermined cutoff, then the result is reported as invalid (Table **2013.10D**).

G. Confirmation

All positive VIDAS LPT results must be culturally confirmed. Confirmation should be performed using the nonheated enrichment broth stored between 2–8°C and should be initiated within 72 h following the end of incubation (AFNOR Certificate No. BIO 12/33-05/12). Presumptive positive results may be confirmed by isolating on selective agar plates such as ALOA or on the appropriate reference method selective agar plates. Typical or suspect colonies from each plate are confirmed as described in appropriate reference method. As an alternative to the conventional confirmation for *Listeria*, **2012.02** VITEK 2 GP Biochemical Identification or API *Listeria* biochemical kits may be used for presumptive generic identification of foodborne *Listeria*.

Reference: *J. AOAC Int*. **97**, 431(2014)
DOI: 10.5740/jaoacint.13-372

Posted: May 2014, February 2016

Method: **Determination of Heavy Metals by ICP-MS**

Reference: **AOAC Method 2015.01**

Approved: (b) (4) Date: 4/25/19

1. Purpose

This method is to describe the steps for preparation of samples and standards to perform quantitative determination of metal impurities by microwave digestion and analysis by ICP-MS.

2. Scope

This method is applicable for the detection of metal impurities by ICP-MS. This method is suitable for a range of elements to be quantified; however, the elements of primary concern are arsenic, cadmium, lead and mercury.

3. Background

This method should be used by analysts familiar with trace element analysis and ICP-MS.

4. Responsibilities

4.1 Laboratory Co-Director authorized to assign and approve subject analysis is responsible for

- Approving Method Folder content
- Assuring the sample is fit for use
- Resolving analytical issues and deficiencies with subject analysis

4.2 Section Supervisor authorized to conduct subject analysis is responsible for

- Approving assigned analyst work
- Assuring the Method Folder is up to date including content and appendices
- Discussing any deviations with the Laboratory Co-Director

4.3 Analyst authorized to conduct this analysis is responsible for

- Reviewing Method Folder instructions prior to initiating analysis, especially for matrix applicability
- Analyzing the sample according to documented instructions
- Assessing method and instrument performance both real time and at reporting
- Addressing any deviation from instructions or specifications with the Section Supervisor
- Updating Method Folder performance data

5.0 References

5.1 Method

- AOAC INTERNATIONAL. Official Methods of Analysis, 20th ed., Method 2015.01 – Heavy Metals in Food – Inductively Coupled Plasma-Mass Spectrometry.
- FDA EAM (Elemental Analysis Manual) 4.7 Vesrion 1.1 (March 2015), P. Gray, W. Midak, J. Cheng – “Inductively Coupled Plasma-Mass Spectrometric Determination of Arsenic,

Cadmium, chromium, Lead, Mercury and Other Elements in Food Using Microwave Assisted Digestion"

- Perkin Elmer – "Determination of Elemental Impurities in Cannabis and Related Materials by Indirect Closed-Vessel Microwave Digestion and ICP-MS Analysis"

5.2 Instrumentation

- Perkin Elmer NexION 1000/2000 ICP-MS

6.0 Method Folder

6.1 Instrumentation

The analyst authorized to perform this test method must be deemed knowledgeable in the operation of the instrumentation cited in **5.2 Instrumentation**

6.2 Safety

This method does not address all safety issues associated with its use. The analyst must establish appropriate safety and health practice prior to initiating analysis. The analyst must be familiar with (b) (4) hazardous waste plan.

Reagents should be regarded as potential health hazards and exposure to these compounds should be limited.

6.3 Definitions

Analytical sample – sample, prepared by the laboratory (by homogenization, grinding, blending, etc.), from which analytical portions (aliquots) are removed for analysis.

Analytical portion – quantity of material removed from the analytical sample.

Analytical solution – solution prepared by decomposing an analytical portion and diluting to volume.

Batch – a group of analytical portions processed in a continuous sequence under relatively stable conditions. Specifically:

- Method is constant
- Instrument and its conditions (i.e. pertinent operating parameters) are constant
- Standardization is constant

Dilution Factor (DF) – factor by which concentration in a diluted solution (e.g. diluted analytical solution) is multiplied to obtain concentration in the initial solution (e.g. analytical solution).

Method Blank (MBK) – solution that is prepared using all reagents and exposed to all laboratory ware, apparatus, equipment, digestion process and analyses in the same manner as if it were an analytical portion being analyzed without the sample. The MBK is analyzed to ensure analytes have not significantly been added to the analytical portion from materials and laboratory environment.

Reagent Blank (RB) – solution that is prepared using the same labware, acids, and dilution as calibration standards, prepare a solution as if it were a calibration standard without added sample.

Reference material (RM) – food related materials developed for analytical quality control, which have reference value concentration for the element of interest.

Independent calibration verification (ICV) – solution of method analytes of known concentration obtained from a source external to the laboratory and different from the source used for instrument standardization. The ICV is used to ensure a valid standardization and to check laboratory performance.

Continuous calibration verification (CCV) – verification of one of the calibration standard points. It is used to verify the calibration accuracy during the analysis of the analytical batch.

Matrix Spike (SP) – analytical portion fortified (spiking) with the analyte before digestion. Measurement of the final concentration of the analyte is made according to the analytical method. The purpose of the spike is to determine if the preparation procedure or sample matrix contribute bias to the results.

Blank Spike (BS) – solution that is spiked with known concentration analytes and prepared using the same labware, acids, dilutions and exposed to the same digestion process as the Method Blank. The purpose is to determine the spiked analyte recoveries to determine the accuracy.

Internal Standards Solution (ISS) – non analyte solution that is added to all calibration standards, quality control and analyzed samples, which uses the isotope ratio to correct for the instrument drift and matrix interferences.

Stock standard solution – a solution containing a high concentration of the analyte purchased from a reputable commercial source. Stock standard solutions are used to prepare standard solutions and other needed analyte solutions.

Intermediate standard solution – a solution containing one or more analytes prepared in the laboratory by diluting an aliquot of stock solution.

Standard solution – a solution prepared from the dilution of stock standard or intermediate standard solutions. Standard solutions are used to standardize instrument response (absorbance) to analyte concentration.

Analytical solution detection limit (ASDL) – an estimate of the lowest concentration of the analyte element in a MBK according to the statistics of hypothesis with a 95% confidence.

Limit of detection (LOD) – an estimate of the element concentration a method can detect in an analytical portion according to the statistics of hypothesis testing with a 95% confidence.

Limit of Quantitation (LOQ) – the minimum concentration of an analyte in a specific matrix that can be reliably quantified while also meeting predefined goals for bias and imprecision.

7.0 Method Work Level Instructions

7.1 Equipment and materials

(a) Analytical Balance – capable of weighing to the nearest 0.001 gram.
(b) Digestion vials – disposable glass tubes
(c) Microwave Digestor – Milestone UltraWave
(d) ICP-MS – Perkin Elmer

7.2 Reagents and Standards

All reagents may contain impurities that may affect the integrity of the analytical results. Due to the high sensitivity of the ICP-MS, high-purity reagents, water, acids, glassware and sample tubes that are suitable for trace metal analysis must be used at all time.

(a) 100 mg/L (ppm) Gold (Au) Stock Standard
(b) 1000 mg/L (ppm) Arsenic (As) Stock Standard
(c) 1000 mg/L (ppm) Cadmium (Cd) Stock Standard
(d) 1000 mg/L (ppm) Lead (Pb) Stock Standard
(e) 1000 mg/L (ppm) Mercury (Hg) Stock Standard
(f) Nitric Acid (HNO_3) – Concentrated (sp gr 1.41), trace metal grade
(g) Hydrochloric Acid (HCl) – Concentrated, trace element grade
(h) Internal Standard Solution – 50 mg/L Germanium (Ge), 20 mg/L Gallium (Ga), 1 mg/L Indium (In), 1 mg/L Terbium (Tb)
(i) Deionized water (DI H_2O)

7.2.1 Working solutions

Please always use safety precautions when preparing solutions. Always add acid to water! Shake each solution after all the reagents are combined.

(b) (4)

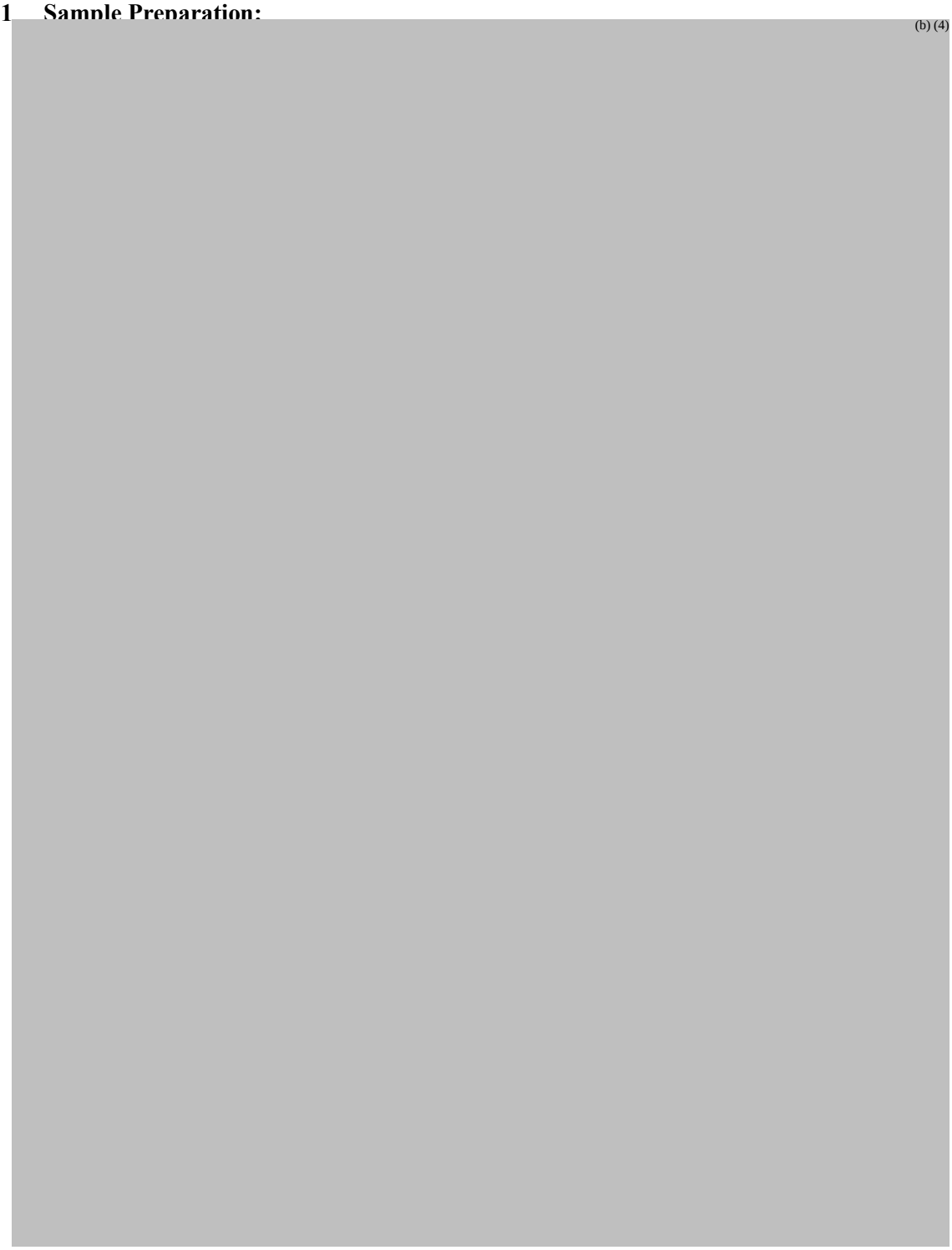
(b) (4)

7.3 Test Sample Treatment

Milestone UltraWave microwave is used to digest in order to prepare the analytical batch.

7.3.1 Sample Preparation:

(b) (4)



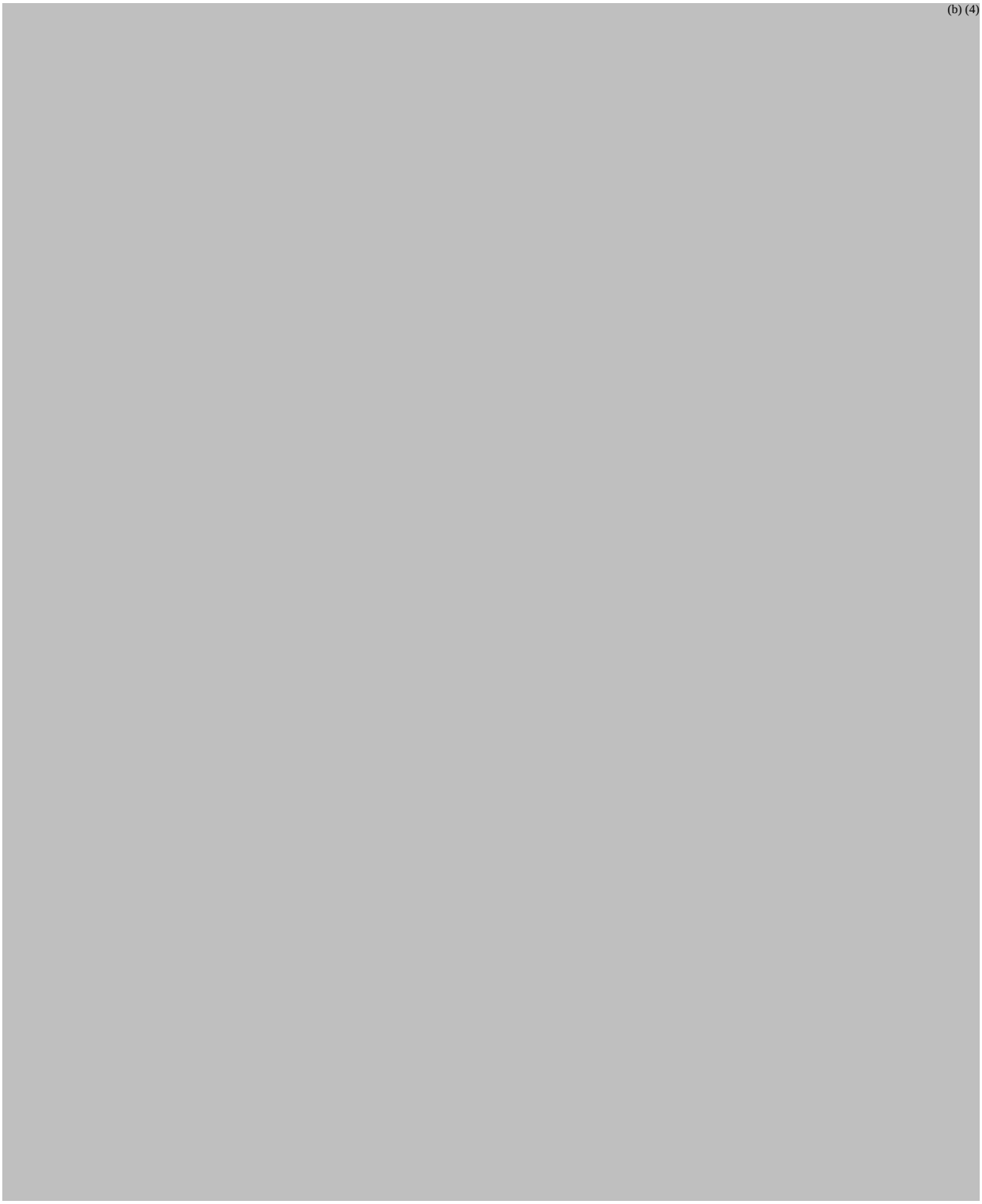
e. UltraWave Cleaning/Maintenance

(b) (4)

7.4 Instrumentation Set up

(b) (4)

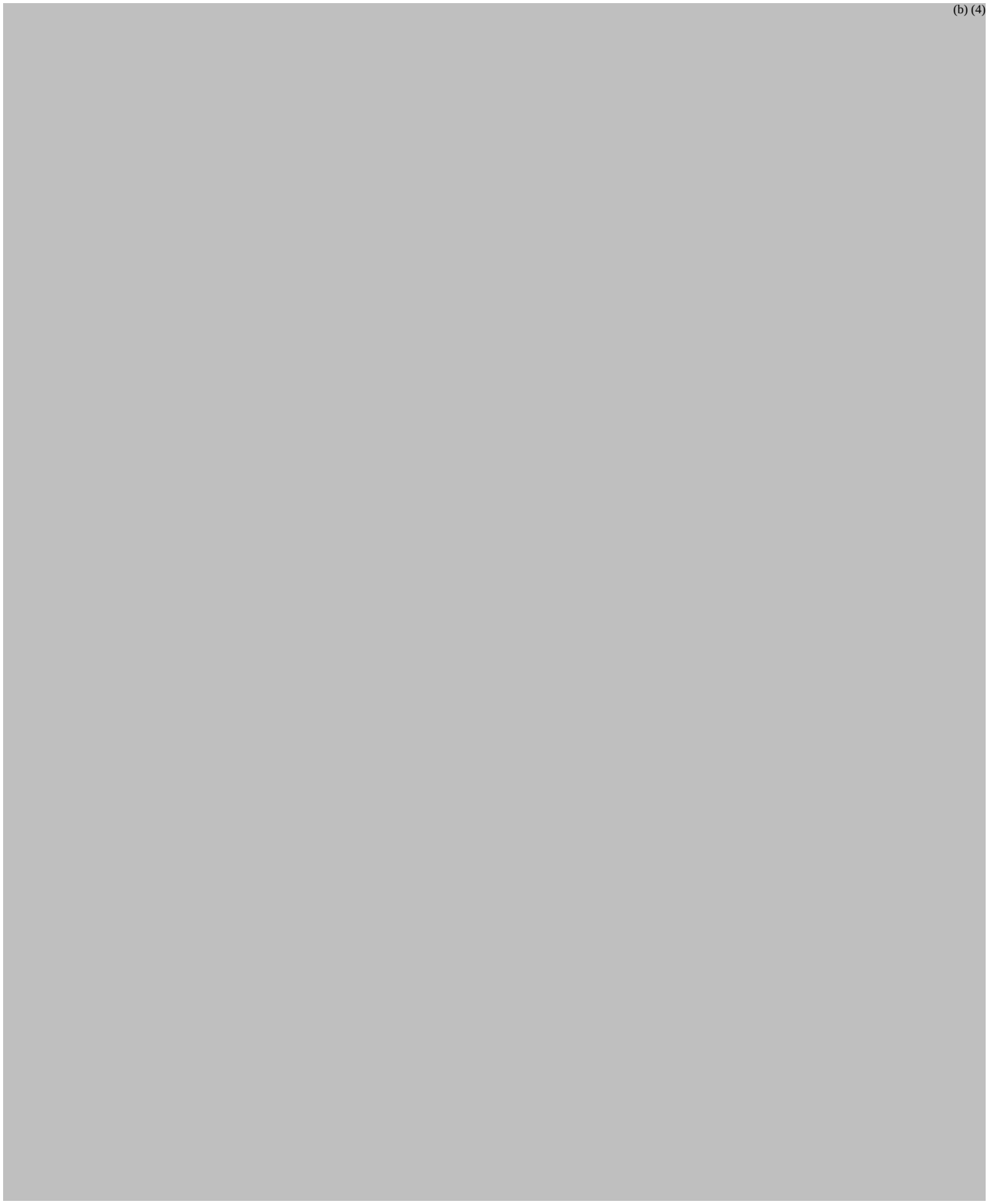
(b) (4)



Appendix A - Calibration Concentrations

(b) (4)

Appendix B - Solutions Guide



AOAC Official Method 2015.01
Heavy Metals in Food
Inductively Coupled Plasma–Mass Spectrometry
First Action 2015

Note: The following is not intended to be used as a comprehensive training manual. Analytical procedures are written based on the assumption that they will be performed by technicians who are formally trained in at least the basic principles of chemical analysis and in the use of the subject technology.

{Applicable for the determination of heavy metals [arsenic (As), CAS No. 7440-38-2; cadmium (Cd), CAS No. 7440-43-9; lead (Pb), CAS No. 7439-92-1; and mercury (Hg), CAS No. 7439-97-6] at trace levels in food and beverage samples, including solid chocolate, fruit juice, fish, infant formula, and rice, using microwave digestion and inductively coupled plasma–mass spectrometry (ICP-MS).}

Caution: Nitric acid and hydrochloric acid are corrosive. When working with these acids, wear adequate protective gear, including eye protection, gloves with the appropriate resistance, and a laboratory coat. Use an adequate fume hood for all acids.

Hydrogen peroxide is a strong oxidizer and can react violently with organic material to give off oxygen gas and heat. Adequate protective gear should be worn.

Many of the chemicals have toxicities that are not well established and must be handled with care. For all known chemicals used, consult the Material Safety Data Sheet (MSDS) in advance.

The inductively coupled plasma–mass spectrometer emits UV light when the plasma is on. UV resistant goggles should be worn if working near the plasma.

The instrument generates high levels of radio frequency (RF) energy and is very hot when the plasma is on. In the case of an instrument failure, be aware of these potential dangers.

Safely store interference reduction technology (IRT) gases, such as oxygen, in a closed, ventilated cabinet. Use adequate caution with pressurized gases. Prior training or experience is necessary to change any gas cylinders. Oxygen gas can cause many materials to ignite easily.

Following microwave digestion, samples are hot to the touch. Allow the samples to cool to room temperature before opening the digestion vessels to avoid unexpected depressurization and potential release of toxic fumes.

A. Principle

Food samples are thoroughly homogenized and then prepared by microwave digestion and the addition of dilute solutions of gold (Au) and lutetium (Lu). The Au is used to stabilize the Hg in the preparation, and the Lu is used to assess the potential loss of analyte during the microwave digestion process.

A prepared, diluted, aqueous sample digestate is pumped through a nebulizer, where the liquid forms an aerosol as it enters a spray chamber. The aerosol separates into a fine aerosol mist and larger aerosol droplets. The larger droplets exit the spray chamber while the fine mist is transported into the ICP torch.

Inside the ICP torch, the aerosol mist is transported into a high-temperature plasma, where it becomes atomized and ionized as it passes through an RF load coil. The ion stream is then focused by a single ion lens through a cylinder with a carefully controlled electrical field. For instruments equipped with dynamic reaction cell (DRC) or collision cell IRT, the focused ion stream is directed into the reaction/collision cell where, when operating with a pressurized cell, the ion beam will undergo chemical modifications and/or collisions to reduce elemental interferences. When not operating with a pressurized cell, the ion stream will remain focused as it passes through the cell with no chemical modification taking place.

The ion stream is then transported to the quadrupole mass filter, where only ions having a desired mass-to-charge ratio (m/z) are passed through at any moment in time. The ions exiting the mass filter are detected by a solid-state detector and the signal is processed by the data handling system.

B. Equipment

Perform routine preventative maintenance for the equipment used in this procedure.

An ultra-clean laboratory environment is critical for the successful production of quality data at ultra-low levels. All sample preparation must take place in a clean hood (Class 100). Metallic materials should be kept to a minimum in the laboratory and coated with an acrylic polymer gel where possible. Adhesive floor mats should be used at entrances to the laboratory and changed regularly to prevent the introduction of dust and dirt from the outside environment. Wear clean-room gloves and change whenever contact is made with anything non-ultra-clean. The laboratory floor should be wiped regularly to remove any particles without stirring up dust. *Note:* "Ultra-clean" (tested to be low in the analytes of interest) reagents, laboratory supplies, facilities, and sample handling techniques are required to minimize contamination in order to achieve the trace-level detection limits described herein.

(**a**) *Instrumentation*.—ICP-MS instrument, equipped with IRT with a free-running 40 MHz RF generator; and controllers for nebulizer, plasma, auxiliary, and reaction/collision flow control. The quadrupole mass spectrometer has a mass range of 5 to 270 atomic mass units (amu). The turbo molecular vacuum system achieves 10^{-6} torr or better. Recommended ICP-MS components include an RF coil, platinum skimmer and sampler cones, Peltier-cooled quartz cyclonic spray chamber, quartz or sapphire injector, micronebulizer, variable speed peristaltic pump, and various types of tubing (for gases, waste, and peristaltic pump). *Note*: The procedure is written specifically for use with a PerkinElmer ELAN DRC II ICP-MS (www.perkinelmer.com). Equivalent procedures may be performed on any type of ICP-MS instrument with equivalent IRT if the analyst is fully trained in the interpretation of spectral and matrix interferences and procedures for their correction, including the optimization of IRT. For example, collision cell IRT can be used for arsenic determination using helium gas.

(**b**) *Gases*.—High-purity grade liquid argon (>99.996%). Additional gases are required for IRT (such as ultra-x grade, 99.9999% minimum purity oxygen, used for determination of As in DRC mode with some PerkinElmer ICP-MS instruments).

(**c**) *Analytical balance*.—Standard laboratory balance suitable for sample preparation and capable of measuring to 0.1 mg.

(**d**) *Clean-room gloves*.—Tested and certified to be low in the metals of interest.

(**e**) *Microwave digestion system*.—Laboratory microwave digestion system with temperature control and an adequate supply of chemically inert digestion vessels. The microwave should be appropriately vented and corrosion resistant.

(*1*) The microwave digestion system must sense the temperature to within ±2.5°C and automatically adjust the microwave field output power within 2 s of sensing. Temperature sensors should be accurate to ±2°C (including the final reaction temperature of 190°C). Temperature feedback control provides the primary control performance mechanism for the method.

(*2*) The use of microwave equipment with temperature feedback control is required to control the unfamiliar reactions of unique or untested food or beverage samples. These tests may require additional vessel requirements, such as increased pressure capabilities.

(**f**) *Autosampler cups*.—15 and 50 mL; vials are precleaned by soaking in 2–5% (v/v) HNO_3 overnight, rinsed three times with reagent water/deionized water (DIW), and dried in a laminar flow clean hood. For the 50 mL vials, as these are used to prepare standards and bring sample preparations to final volume, the bias and precision of the vials must be assessed and documented prior to use. The recommended procedure for this is as follows:

(*1*) For every case of vials from the same lot, remove 10 vials.

(*2*) Tare each vial on an analytical balance, and then add reagent water up to the 20 mL mark. Repeat procedure by adding reagent water up to the 50 mL mark.

(*3*) Measure and record the mass of reagent water added, and then calculate the mean and RSD of the 10 replicates at each volume.

(*4*) To evaluate bias, the mean of the measurements must be with ±3% of the nominal volume. To evaluate precision, the RSD of the measurements must be ≤3% using the stated value (20 or 50 mL) in place of the mean.

(**g**) *Spatulas*.—To weigh out samples; should be acid-cleaned plastic (ideally Teflon) and cleaned by soaking in 2% (v/v) HNO_3 prior to use.

C. Reagents and Standards

Reagents may contain elemental impurities that could negatively affect data quality. High-purity reagents should always be used. Each reagent lot should be tested and certified to be low in the elements of interest before use.

(**a**) *DIW*.—ASTM Type I; demonstrated to be free from the metals of interest and potentially interfering substances.

(**b**) *Nitric acid (HNO_3)*.—Concentrated; tested and certified to be low in the metals of interest.

(c) *Hydrogen peroxide (H_2O_2)*.—Optima grade or equivalent, 30–32% assay.

(**d**) *Stock standard solutions*.—Obtained from a reputable and professional commercial source.

(*1*) *Single-element standards*.—Obtained for each determined metal, as well as for any metals used as internal standards and interference checks.

(*2*) *Second source standard*.—Independent from the single-element standard; obtained for each determined metal.

(*3*) *Multi-element stock standard solution*.—Elements must be compatible and stable in solutions together. Stability is determined by the vendor; concentrations are then verified before use of the standard.

(**e**) *Internal standard solution*.—For analysis of As, Cd, Pb, and Hg in food matrices, an internal standard solution of 40 µg/L rhodium (Rh), indium (In), and thulium (Tm) is recommended. Rh is analyzed in DRC mode for correction of the As signal. In addition, the presence of high levels of elements, such as carbon and chlorine, in samples can increase the effective ionization of the plasma and cause a higher response factor for arsenic in specific samples. This potential interference is addressed by the on-line addition of acetic acid (or another carbon source, such as methanol), which greatly increases the effective ionization of incompletely ionized analytes, and decreases the potential increase caused by sample characteristics. The internal standard solution should be prepared in 20% acetic acid.

Table 2015.01A. Recommended concentrations for the calibration curve

Standard	As, µg/L	Cd, µg/L	Pb, µg/L	Hg, µg/L
0	0.00	0.00	0.000	0.00
1	0.01	0.01	0.005	0.01
2	0.02	0.02	0.010	0.05
3	0.10	0.10	0.050	0.10
4	0.50	0.50	0.250	0.50
5	5.00	5.00	2.500	2.00
6	20.00	20.00	10.000	5.00

(**f**) *Calibration standards*.—Fresh calibration standards should be prepared every day, or as needed.

(*1*) Dilute the multi-element stock standard solutions into 50 mL precleaned autosampler vials with 5% HNO_3 in such a manner as to create a calibration curve. The lowest calibration standard (STD 1) should be equal to or less than the limit of quantitation (LOQ) when recalculated in units specific to the reported sample results.

(*2*) *See* Table **2015.01A** for recommended concentrations for the calibration curve.

(**g**) *Initial calibration verification (ICV) solution*.—Made up from second source standards in order to verify the validity of the calibration curve.

(**h**) *Calibration solutions*.—Daily optimization, tuning, and dual detector calibration solutions, as needed, should be prepared and analyzed per the instrument manufacturer's suggestions.

(**i**) *Certified Reference Materials (CRMs)*.—CRMs should preferably match the food matrix type being analyzed and contain the elements of interest at certified concentrations above the LOQ. Recommended reference materials include NIST SRM 1568a (Rice Flour), NIST SRM 1548a (Typical Diet), NRCC CRM DORM-3 (Dogfish Muscle), and NIST SRM 2976 (Mussel Tissue).

(**j**) *Spiking solution*.—50 mg/L Au and Lu in 5% (v/v) HNO_3. Prepared from single-element standards.

D. Contamination and Interferences

(**a**) Well-homogenized samples and small reproducible aliquots help minimize interferences.

(**b**) *Contamination*.—(*1*) Contamination of the samples during sample handling is a great risk. Extreme care should be taken to avoid this. Potential sources of contamination during sample handling include using metallic or metal-containing homogenization equipment, laboratory ware, containers, and sampling equipment.

(*2*) Contamination of samples by airborne particulate matter is a concern. Sample containers must remain closed as much as possible. Container lids should only be removed briefly and in a

clean environment during sample preservation and processing, so that exposure to an uncontrolled environment is minimized.

(**c**) *Laboratory.*—(*1*) All laboratory ware (including pipet tips, ICP-MS autosampler vials, sample containers, extraction apparatus, and reagent bottles) should be tested for the presence of the metals of interest. If necessary, the laboratory ware should be acid-cleaned, rinsed with DIW, and dried in a Class 100 laminar flow clean hood.

(*2*) All autosampler vials should be cleaned by storing them in 2% (v/v) HNO_3 overnight and then rinsed three times with DIW. Then dry vials in a clean hood before use. Glass volumetric flasks should be soaked in about 5% HNO_3 overnight prior to use.

(*3*) All reagents used for analysis and sample preparation should be tested for the presence of the metals of interest prior to use in the laboratory. Due to the ultra-low detection limits of the method, it is imperative that all the reagents and gases be as low as possible in the metals of interest. It is often required to test several different sources of reagents until an acceptable source has been found. Metals contamination can vary greatly from lot to lot, even when ordering from the same manufacturer.

(*4*) Keep the facility free from all sources of contamination for the metals of interest. Replace laminar flow clean hood HEPA filters with new filters on a regular basis, typically once a year, to reduce airborne contaminants. Metal corrosion of any part of the facility should be addressed and replaced. Every piece of apparatus that is directly or indirectly used in the processing of samples should be free from contamination for the metals of interest.

(**d**) *Elemental interferences*.—Interference sources that may inhibit the accurate collection of ICP-MS data for trace elements are addressed below.

(*1*) *Isobaric elemental interferences*.—Isotopes of different elements that form singly or doubly charged ions of the same *m/z* and cannot be resolved by the mass spectrometer. Data obtained with isobaric overlap must be corrected for that interference.

(*2*) *Abundance sensitivity*.—Occurs when part of an elemental peak overlaps an adjacent peak. This often occurs when measuring a small *m/z* peak next to a large *m/z* peak. The abundance sensitivity is affected by ion energy and quadrupole operating pressure. Proper optimization of the resolution during tuning will minimize the potential for abundance sensitivity interferences.

(*3*) *Isobaric polyatomic interferences.*—Caused by ions, composed of multiple atoms, which have the same *m/z* as the isotope of interest, and which cannot be resolved by the mass spectrometer. These ions are commonly formed in the plasma or the interface system from the support gases or sample components. The objective of IRT is to remove these interferences, making the use of correction factors unnecessary when analyzing an element in DRC mode. Elements not determined in DRC mode can be corrected by using correction equations in the ICP-MS software.

(**e**) *Physical interferences.*—(*1*) Physical interferences occur when there are differences in the response of the instrument from the calibration standards and the samples. Physical interferences are associated with the physical processes that govern the transport of sample into the plasma, sample conversion processes in the plasma, and the transmission of ions through the plasma-mass spectrometer interface.

(*2*) Physical interferences can be associated with the transfer of solution to the nebulizer at the point of nebulization, transport of aerosol to the plasma, or during excitation and ionization processes in the plasma. High levels of dissolved solids in a sample can result in physical interferences. Proper internal standardization (choosing internal standards that have analytical behavior similar to the associating elements) can compensate for many physical interferences.

Table 2015.01B. Recommended isotopes for analysis

Element	Isotope, amu	Isotopic abundance, %	Potential interferences
Cd	111	13	MoO^+
	114	29	MoO^+, Sn^+
Hg	200	23	WO^+
	202	30	WO^+
Pb[a]	Sum of 206, 207, and 208	99	OsO^+

[a] Allowance for isotopic variability of lead isotopes.

(**f**) *Resolution of interferences.*—(*1*) For elements that are subject to isobaric or polyatomic interferences (such as As), it is advantageous to use the DRC mode of the instrument. This section specifically describes a method of using IRT for interference removal for As using a PerkinElmer DRC II and oxygen as the reaction gas. Other forms of IRT may also be appropriate.

(*a*) Arsenic, which is monoisotopic, has an *m/z* of 75 and is prone to interferences from many sources, most notably from chloride (Cl), which is common in many foods (e.g., salt). Argon (Ar), used in the ICP-MS plasma, forms a polyatomic interference with Cl at *m/z* 75 [$^{35}Cl + {}^{40}Ar = {}^{75}(ArCl)$].

(*b*) When arsenic reacts with the oxygen in the DRC cell, $^{75}As^{16}O$ is formed and measured at *m/z* 91, which is free of most interferences. The potential ^{91}Zr interference is monitored for in the following ways: ^{90}Zr and ^{94}Zr are monitored for in each analytical run, and if a significant Zr presence is detected, then $^{75}As^{16}O$ measured at *m/z* 91 is evaluated against the ^{75}As result. If a significant discrepancy is present, then samples may require analysis using alternative IRT, such as collision cell technology (helium mode).

(*c*) Instrument settings used (for PerkinElmer DRC II): DRC settings for $^{91}(AsO)$ and ^{103}Rh include an RPq value of 0.7 and a cell gas flow rate of 0.6 L/min. Cell conditions, especially cell gas flow rates, may be optimized for specific analyte/matrix combinations, as needed. In such cases, the optimized methods will often have slightly different RPq and cell gas flow values.

(*2*) For multi-isotopic elements, more than one isotope should be measured to monitor for potential interferences. For reporting purposes, the most appropriate isotope should be selected based on review of data for matrix interferences and based on the sensitivity (or relative abundance) of each isotope. The table below lists the recommended isotopes to measure. Low abundance isotopes are not recommended for this method as it is specifically applicable for ultra-low level concentrations (8–10 ppb LOQs). *See* Table **2015.01B**.

(**g**) *Memory effects*.—Minimize carryover of elements in a previous sample in the sample tubing, cones, torch, spray chamber, connections, and autosampler probe by rinsing the instrument with a reagent blank after samples high in metals concentrations are analyzed. Memory effects for Hg can be minimized through the addition of Au to all standard, samples, and quality control (QC) samples.

Table 2015.01C. Digestion program for Berghof Speedwave 4 microwave

Step	Temp., °C	Ramp, min	Hold, min
1	145	1	1
2	50	1	1
3	145	1	1
4	170	1	10
5	190	1	10

E. Sample Handling and Storage

(**a**) Food and beverage samples should be stored in their typical commercial storage conditions (either frozen, refrigerated, or at room temperature) until analysis. Samples should be analyzed within 6 months of preparation.

(**b**) If food or beverage samples are subsampled from their original storage containers, ensure that containers are free from contamination for the elements of concern.

F. Sample Preparation

(**a**) Weigh out sample aliquots (typically 0.25 g of as-received or wet sample) into microwave digestion vessels.

(**b**) Add 4 mL of concentrated HNO_3 and 1 mL of 30% hydrogen peroxide (H_2O_2) to each digestion vessel.

(**c**) Add 0.1 mL of the 50 mg/L Au + Lu solution to each digestion vessel.

(**d**) Cap the vessels securely (and insert into pressure jackets, if applicable). Place the vessels into the microwave system according to the manufacturer's instructions, and connect the appropriate temperature and/or pressure sensors.

(**e**) Samples are digested at a minimum temperature of 190°C for a minimum time of 10 min. Appropriate ramp times and cool down times should be included in the microwave program, depending on the sample type and model of microwave digestion system. Microwave digestion is achieved using temperature feedback control. Microwave digestion programs will vary depending on the type of microwave digestion system used. When using this mechanism for achieving performance-based digestion targets, the number of samples that may be simultaneously digested may vary. The number will depend on the power of the unit, the number of vessels, and the heat loss characteristics of the vessels. It is essential to ensure that all vessels reach at least 190°C and be held at this temperature for at least 10 min. The monitoring of one vessel as a control for the batch/carousel may not accurately reflect the temperature in the other vessels, especially if the samples vary in composition and/or sample mass. Temperature measurement and control will depend on the particular microwave digestion system.

(*1*) *Note*: a predigestion scheme for samples that react vigorously to the addition of the acid may be required.

(*2*) The method performance data presented in this method was produced using a Berghof Speedwave 4 microwave digestion system, with the program listed in Table **2015.01C** (steps 1 and 2 are a predigestion step).

Table 2015.01D. Digestion program for CEM MARS 6 microwave

Step	Temp., °C	Ramp, min	Hold, min
1	190	20	10
2	Cool down	NA	10

(*3*) Equivalent results were achieved using the program listed in Table **2015.01D** on a CEM MARS 6 microwave digestion system using the 40-position carousel and 55 mL Xpress digestion vessels.

(*4*) For infant formula samples, the program described in Table **2015.01E** has been shown to work effectively.

Table 2015.01E. Digestion program for infant formula

Step	Temp., °C	Ramp, min	Hold, min
1	180	20	20
2	Cool down	NA	20
3	200	20	20
4	Cool down	NA	20

(**f**) Allow vessels to cool to room temperature and slowly open. Open the vessels carefully, as residual pressure may remain and digestate spray is possible. Pour the contents of each vessel into an acid-cleaned 50 mL HDPE centrifuge tube and dilute with DIW to a final volume of 20 mL.

(**g**) Digestates are diluted at least 4x prior to analysis with the 1% (v/v) HNO_3 diluent. When the metals concentration of a sample is unknown, the samples may be further diluted or analyzed using a total quantification method prior to being analyzed with a comprehensive quantitative method. This protects the instrument and the sample introduction system from potential contamination and damage.

(**h**) Food samples high in calcium carbonate ($CaCO_3$) will not fully digest. In such cases, the CRM can be used as a gauge for an appropriate digestion time.

(**i**) QC samples to be prepared with the batch (a group of samples and QC samples that are prepared together) include a minimum of three method blanks, duplicate for every 10 samples, matrix spike/matrix spike duplicate (MS/MSD) for every 10 samples, blank spike, and any matrix-relevant CRMs that are available.

G. Procedure

(**a**) *Instrument startup*.—(*1*) Instrument startup routine and initial checks should be performed per manufacturer recommendations.

(*2*) Ignite the plasma and start the peristaltic pump. Allow plasma and system to stabilize for at least 30 min.

(**b**) *Optimizations*.—(*1*) Perform an optimization of the sample introduction system (e.g., *X*-*Y* and *Z* optimizations) to ensure maximum sensitivity.

(*2*) Perform an instrument tuning or mass calibration routine whenever there is a need to modify the resolution for elements, or monthly (at a minimum), to ensure the instrument's quadrupole mass filtering performance is adequate. Measured masses should be ±0.1 amu of the actual mass value, and the resolution (measured peak width) should conform to manufacturer specifications.

(*3*) Optimize the nebulizer gas flow for best sensitivity while maintaining acceptable oxide and double-charged element formation ratios.

(*4*) Perform a daily check for instrument sensitivity, oxide formation ratios, double-charged element formation ratios, and background. If the performance check is not satisfactory, additional optimizations (a "full optimization") may be necessary.

Table 2015.01F. Summary of quality control samples

QC sample	Measure	Minimum frequency	Acceptance criteria	Corrective action
Calibration standards	Linearity of the calibration curve	Analyzed once per analytical day	Correlation coefficient ≥0.995, 1st standard ≤MRL, low standard recovery = 75–125%, all other standard recoveries = 80–120%	Reanalyze suspect calibration standard. If criteria still not met, then re-prepare standards and recalibrate the instrument.
Internal standards	Variation in sample properties between samples and standards	Each standard, blank, and sample is spiked with internal standard	60–125% recovery compared to calibration blank	If the responses of the internal standards in the following CCB are within the limit, rerun the sample at an additional 2x dilution. If not, then samples must be reanalyzed with a new calibration.
Lu digestion check spike	Assessment of potential loss during digestion	Added to every digested samples	Recovery ≥75%	Re-prepare the sample
Initial calibration verification (ICV)	Independent check of system performance	One following instrument calibration	Recovery = 90–110%	Correct problem prior to continuing analysis. Recalibrate if necessary.
Continuing calibration verification (CCV)	Accuracy	At beginning and end of analysis and one per 10 injections	Recovery = 85–115%	Halt analysis, correct problem, recalibrate, and reanalyze affected samples
Method blanks (MB)	Contamination from reagents, lab ware, etc.	Minimum of three per batch	Mean ≤ MRL; SD ≤ MDL or MBs <1/10th sample result	Determine and eliminate cause of contamination. Affected samples must be re-prepared and reanalyzed.
Method duplicates (MD)	Method precision within a given matrix	Minimum of one per 10 samples	RPD ≤ 30% or ±2x LOQ if results ≤5x LOQ	If RPD criteria not met, then sample may be re-prepared and reanalyzed, but this is not required. Sample matrix may be inhomogeneous. A post-digestion duplicate (PDD) can be analyzed to evaluate instrument precision.
Matrix spikes/matrix spike duplicates (MS/MSD)	Method accuracy and precision within a given matrix	Minimum of one per 10 samples	Recovery = 70–130% and RPD ≤ 30%	If RPD >30%, results must be qualified
Post-preparation spike (PS)	Check for matrix interference	When required (samples spiked too low/high, dilution test fails, etc.)	Recovery = 75–125%	Analyze samples using MSA or results flagged accordingly
Laboratory fortified blank (LFB) or blank spike (BS)	Method accuracy	Minimum of one per batch	Recovery = 75–125%	If LFB recovery is outside of the control limit, then batch must be re-prepared and reanalyzed
Certified Reference Material (CRM)	Method accuracy	Must be matrix-matched to samples; minimum of one per batch	Recovery = 75–125% unless limits set by CRM manufacturer are greater or element/CRM specific limits have been established	If CRM true value is ≥5x the LOQ and recovery is outside of the control limit, then batch must be re-prepared and reanalyzed

(**c**) *Internal standardization and calibration*.—(*1*) Following precalibration optimizations, prepare and analyze the calibration standards prepared as described in **C**(**e**).

(*2*) Use internal standardization in all analyses to correct for instrument drift and physical interferences. Refer to **D**(**e**)(*2*). Internal standards must be present in all samples, standards, and blanks at identical concentrations. Internal standards can be added using a second channel of the peristaltic pump to produce a responses that is clear of the pulse-to-analog detector interface.

(*3*) Multiple isotopes for some analytes may be measured, with only the most appropriate isotope (as determined by the analyst) being reported.

(*4*) Use IRT for the quantification of As using the Rh internal standard.

(**d**) *Sample analysis*.—(*1*) Create a method file for the ICP-MS.

(*2*) Enter sample and calibration curve information into the ICP-MS software.

(*3*) Calibrate the instrument and ensure the resulting standard recoveries and correlation coefficients meet specifications (**H**).

(*4*) Start the analysis of the samples.

(*5*) Immediately following the calibration, an initial calibration blank (ICB) should be analyzed. This demonstrates that there is no carryover of the analytes of interest and that the analytical system is free from contamination.

(*6*) Immediately following the ICB, an ICV should be analyzed. This standard must be prepared from a different source than the calibration standards.

(*7*) A minimum of three reagent/instrument blanks should be analyzed following the ICV. These instrument blanks can be used to assess the background and variability of the system.

(*8*) A continuing calibration verification (CCV) standard should be analyzed after every 10 injections and at the end of the run. The CCV standard should be a mid-range calibration standard.

(*9*) An instrument blank should be analyzed after each CCV (called a continuing calibration blank, or CCB) to demonstrate that there is no carryover and that the analytical system is free from contamination.

(*10*) Method of Standard Additions (MSA) calibration curves may be used any time matrix interferences are suspected.

(*11*) Post-preparation spikes (PS) should be prepared and analyzed whenever there is an issue with the MS recoveries.

(**e**) Export and process instrument data.

H. Quality Control

(**a**) The correlation coefficients of the weighted-linear calibration curves for each element must be ≥0.995 to proceed with sample analysis.

(**b**) The percent recovery of the ICV standard should be 90–110% for each element being determined.

(**c**) Perform instrument rinses after any samples suspected to be high in metals, and before any method blanks, to ensure baseline sensitivity has been achieved. Run these rinses between all samples in the batch to ensure a consistent sampling method.

(**d**) Each analytical or digestion batch must have at least three preparation (or method) blanks associated with it if method blank correction is to be performed. The blanks are treated the same as the samples and must go through all of the preparative steps. If method blank correction is being used, all of the samples in the batch should be corrected using the mean concentration of these blanks. The estimated method detection limit (EMDL) for the batch is equal to 3 times the standard deviation (SD) of these blanks.

(**e**) For every 10 samples (not including quality control samples), a matrix duplicate (MD) sample should be analyzed. This is a duplicate of a sample that is subject to all of the same preparation and analysis steps as the original sample. Generally, the relative percent difference (RPD) for the replicate should be ≤30% for all food samples if the sample concentrations are greater than 5 times the LOQ. RPD is calculated as shown below. An MSD may be substituted for the MD, with the same control limits.

$$RPD = 200 \times \frac{|S1 - S2|}{S1 + S2}$$

where S1 = concentration in the first sample and S2 = concentration in the duplicate.

(**f**) For every 10 samples (not including quality control samples), an MS and MSD should be performed. The percent recovery of the spikes should be 70–130% with an RPD ≤30% for all food samples.

(*1*) If the spike recovery is outside of the control limits, an MSA curve that has been prepared and analyzed may be used to correct for the matrix effect. Samples may be corrected by the slope of the MSA curve if the correlation coefficient of the MSA curve is ≥0.995.

(*a*) The MSA technique involves adding known amounts of standard to one or more aliquots of the processed sample solution. This technique attempts to compensate for a sample constituent that enhances or depresses the analyte signal, thus producing a different slope from that of the calibration standards. It will not correct for additive interferences which cause a baseline shift.

(*b*) The best MSA results can be obtained by using a series of standard additions. To equal volumes of the sample are added a series of standard solutions containing different known quantities of the analyte(s), and all solutions are diluted to the same final volume. For example, addition 1 should be prepared so that the resulting concentration is approximately 50% of the expected concentration of the native sample. Additions 2 and 3 should be prepared so that the concentrations are approximately 100% and 150%, respectively, of the expected native sample concentration. Determine the concentration of each solution and then plot on the vertical axis of a graph, with the concentrations of the known standards plotted on the horizontal axis. When the resulting line is extrapolated to zero absorbance, the point of interception of the abscissa is calculated MSA-corrected concentration of the analyte in the sample. A linear regression program may be used to obtain the intercept concentration.

(*c*) For results of the MSA technique to be valid, take into consideration the following limitations:

(*i*) The apparent concentrations from the calibration curve must be linear (0.995 or greater) over the concentration range of concern.

(*ii*) The effect of the interference should not vary as the ratio of analyte concentration to sample matrix changes, and the MSA curve should respond in a similar manner as the analyte.

(*2*) If the sample concentration levels are sufficiently high, the sample may be diluted to reduce the matrix effect. Samples should be diluted with the 1% (v/v) HNO_3 diluent. For example, to dilute a sample by a 10x dilution factor, pipette 1 mL of the digested sample into an autosampler vial, and add 9 mL of the 1% (v/v) HNO_3 diluent. MS/MSD sets should be performed at the same dilution factor as the native sample.

(*3*) Spike at 1–10 times the level of a historical sample of the same matrix type, or, if unknown, spike at 1–5 times a typical value for the matrix. Spiking levels should be no lower than 10 times the LOQ.

Table 2015.01G. Method blank results and LOD/LOQ, µg/kg

Method blanks	^{91}(AsO)	^{111}Cd	^{114}Cd	Pb	^{200}Hg	^{202}Hg
MB-01	2.83	0.229	0.270	1.90	1.61	0.95
MB-02	1.48	–0.088	0.270	0.14	1.48	1.13
MB-03	1.80	0.007	0.115	0.13	0.76	0.25
MB-04	1.03	0.154	0.288	0.12	1.46	0.33
MB-05	1.43	0.010	0.259	1.84	1.28	0.27
MB-06	1.07	0.105	0.096	3.02	0.87	0.76
MB-07	2.31	–0.002	0.297	2.67	0.89	0.44
MB-08	1.20	0.285	0.200	4.24	0.55	0.28
MB-09	1.05	0.002	0.182	0.09	0.96	0.25
MB-10	2.12	0.047	0.150	0.19	0.71	0.02
MB-11	2.09	–0.145	0.226	0.12	0.64	0.57
MB-12	1.44	0.037	0.165	0.18	0.45	0.50
MB-13	0.70	–0.122	0.160	0.17	0.81	0.19
MB-14	1.12	–0.001	0.074	0.14	0.85	0.21
MB-15	2.33	0.097	0.207	0.11	0.18	0.17
MB-16	1.53	–0.117	0.146	0.16	1.33	1.09
MB-17	1.79	–0.070	0.180	0.03	3.46	2.19
MB-18	1.90	0.049	0.115	0.06	3.30	2.36
MB-19	1.18	0.043	0.224	0.39	4.01	2.78
MB-20	1.24	–0.060	0.199	0.07	0.99	0.56
MB-21	0.92	0.165	0.120	0.03	0.73	0.33
MB-22	1.69	0.005	0.186	0.09	0.60	0.25
MB-23	2.13	0.171	0.152	0.08	0.41	–0.23
SD	0.54	0.113	0.063	1.18	1.01	0.77
LOD	1.6	0.50[a]	0.50[a]	3.5	3.0	2.3
LOQ	3.3	1.60[a]	1.60[a]	7.1	6.0	4.6

[a] Adjusted to conform to lowest calibration point.

(**g**) Percent recoveries of the CRMs should be 75–125% of their certified value.

(**h**) Percent recoveries of the CCV standards should be within 85–115%. Sample results may be CCV-corrected using the mean recovery of the bracketing CCVs. This should only be done after careful evaluation of the data. The instrument should show a trending drift of CCV recoveries and not just a few anomalous outliers.

(**i**) CCBs should be monitored for the effects of carryover and for possible system contamination. If carryover of the analyte at levels greater than 10 times the MDL is observed, the sample results may not be reportable.

(**j**) Absolute response of any one internal standard should not vary from the original response in the calibration blank by more than 60–125%. Some analytical samples, such as those containing concentrations of the internal standard and tissue digestates, can have a serious effect on the internal standard intensities, but this does not necessarily mean that the analytical system is out of control. In some situations, it is appropriate to reprocess the samples using a different internal standard monitored in the analysis. The data should be carefully evaluated before doing this.

Table 2015.01H. Sample-specific LOQs

Sample	LOQ, µg/kg (as received)			
	As	Cd	Pb	Hg
Infant formula	2	1	4	3
Chocolate	4	2	8	6
Rice flour	4	2	8	6
Fruit juice	1	1	2	2

(**k**) The recovery of the Lu that was spiked into the sample preparation prior to digestion should be evaluated to assess any potential loss of analyte during the process. The concentration of Lu in the sample preparation is 0.25 mg/L, and for samples diluted 4x at the instrument, this is equivalent to 62.5 µg/L at the instrument (if samples are diluted more than 4x, this must be taken into account). The Lu recovery should be no less than 75% of the original spiked concentration.

(**l**) Refer to Table **2015.01F** for a summary of all recommended quality control samples, minimum frequency at which they are to be analyzed, acceptance criteria for each, and appropriate corrective action if the acceptance criteria are not met.

I. Method Performance

(**a**) Limit of detection (LOD) and LOQ were determined through the analysis of 23 method blanks (*see* Table **2015.01G**). LOD was calculated as 3 times the SD of the results of the blanks, and LOQ was calculated as 2 times the value of the LOD, except where the resulting LOQ would be less than the lowest calibration point, in which case LOQ was elevated and set at the lowest calibration point and LOD was calculated as 1/3 of the LOQ. All LOQs achieved are ≤10 µg/kg for all food matrices and ≤8 µg/kg for liquid matrices, such as infant formula.

(**b**) Sample-specific LOQs for several matrices, based on LOQs determined by the default method, and adjusted for changes in sample mass for particular samples, are shown in Table **2015.01H**. Values have been rounded up to the nearest part-per-billion.

(**c**) Numerous relevant CRMs were analyzed to establish method accuracy. Example percent recoveries are provided in Table **2015.01I** (recoveries have been omitted for CRMs that do not provide a certified value or if the certified value is less than the LOQ).

Table 2015.01I. Recoveries for numerous relevant CRMs

Certified Reference Material	As, %	Cd, %	Pb, %	Hg, %
DOLT-4 Dogfish Liver	104	97	87	114
DORM-3 Fish Protein	105	109	94	114
DORM-4 Fish Protein	105	91	91	81
NIST 1548a Typical Diet	103	95	113	NA
NIST 1568a Rice Flour	98	99	NA	NA
NIST 1946 Lake Superior Fish Tissue	119	NA	NA	101
TORT-2 Lobster Hepatopancreas	109	104	95	116
TORT-3 Lobster Hepatopancreas	113	89	86	86

Table 2015.01J. AOAC SMPR 2012.007 (ref. 1)

Concn range, µg/kg	Repeatability, %	Reproducibility, %	Recovery, %
LOQ–100	15	32	60–115
100–1000	11	16	80–115
>1000	7.3	8	80–115

(**d**) *Standard Method Performance Requirements* (AOAC SMPR® 2012.007; 1) for repeatability, reproducibility, and recovery for the method are shown in the Table **2015.01J**. *See* Appendix A (available on the *J. AOAC Int*. website as supplemental material, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac) for detailed method performance information supporting acceptance of the method.

(**e**) *See* Appendix A for detailed method performance information supporting acceptance of the method. Method validation samples were prepared and analyzed for all applicable matrices. In general, all SMPR criteria were met for As, Cd, Hg, and Pb in the matrices apple juice, infant formula, cocoa powder, and rice flour.

References: (1) AOAC SMPR 2012.007
J. AOAC Int. **96**, 704(2013)
DOI: 10.5740/jaoac.int.2012.007

J. AOAC Int. **98**, 1113(2015)
DOI: 10.5740/jaoac.int.2015.01

Posted: September 9, 2015

ASCUS BIOSCIENCES

Method

Title	DY21 Solid Intermediate Microbe Enumeration
Version	01
Effective Date	09Dec2019
Author	Adam Taylor
Approver (Signature & Date)	DocuSigned by: Martin Mayhew 12/9/2019 D1605F1B4C3E49A... Martin Mayhew, VP – Process Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable cells of Dairy-21 in solid Dairy-21 intermediates (such as DY21 PBV and DY21 PBV Milled) by counting colony forming units (CFU) on solid media.

Safety

Consult the Safety Data Sheet for all reagents prior to handling. Use caution in working with a hot water bath, hot liquids, liquid nitrogen, and extremely cold material. Liquid nitrogen can cause cold burns, frostbite, and permanent eye damage from brief exposure. Avoid skin and eye contact with liquid nitrogen and wear appropriate personal protective equipment (safety glasses and gloves) at all times. Analyst should be trained on liquid nitrogen handling before continuing this method.

Materials

Corning® 15mL Polypropylene Centrifuge Tubes (Corning 430052) (or equivalent)
Test tubes, 13x100 mm, sterile (or equivalent)
Test tube cap, 16 mm, polypropylene (or equivalent)
1.5 mL polypropylene microcentrifuge tube with snap cap (or equivalent)
1000 µL Pipette
200 µL Pipette
1000 µL pipette tips, sterile
200 µL pipette tips, sterile
Glass beads, 3 mm, sterile

Equipment

Laboratory Vortexer
Class I/II Biosafety Cabinet
Mortar and Pestle

Media & Reagents

YPD Plates
Growcells 1X Phosphate Buffered Saline with 0.05% TWEEN pH 7.4, sterile (Growcells MRGF-6275)
70% Ethanol
10% Bleach
Liquid Nitrogen

Method

1. De-encapsulation of DY21

(b) (4)

DY21 Solid Intermediate Microbe Enumeration

(b) (4)

2. Prepare the Primary Dilution Mix

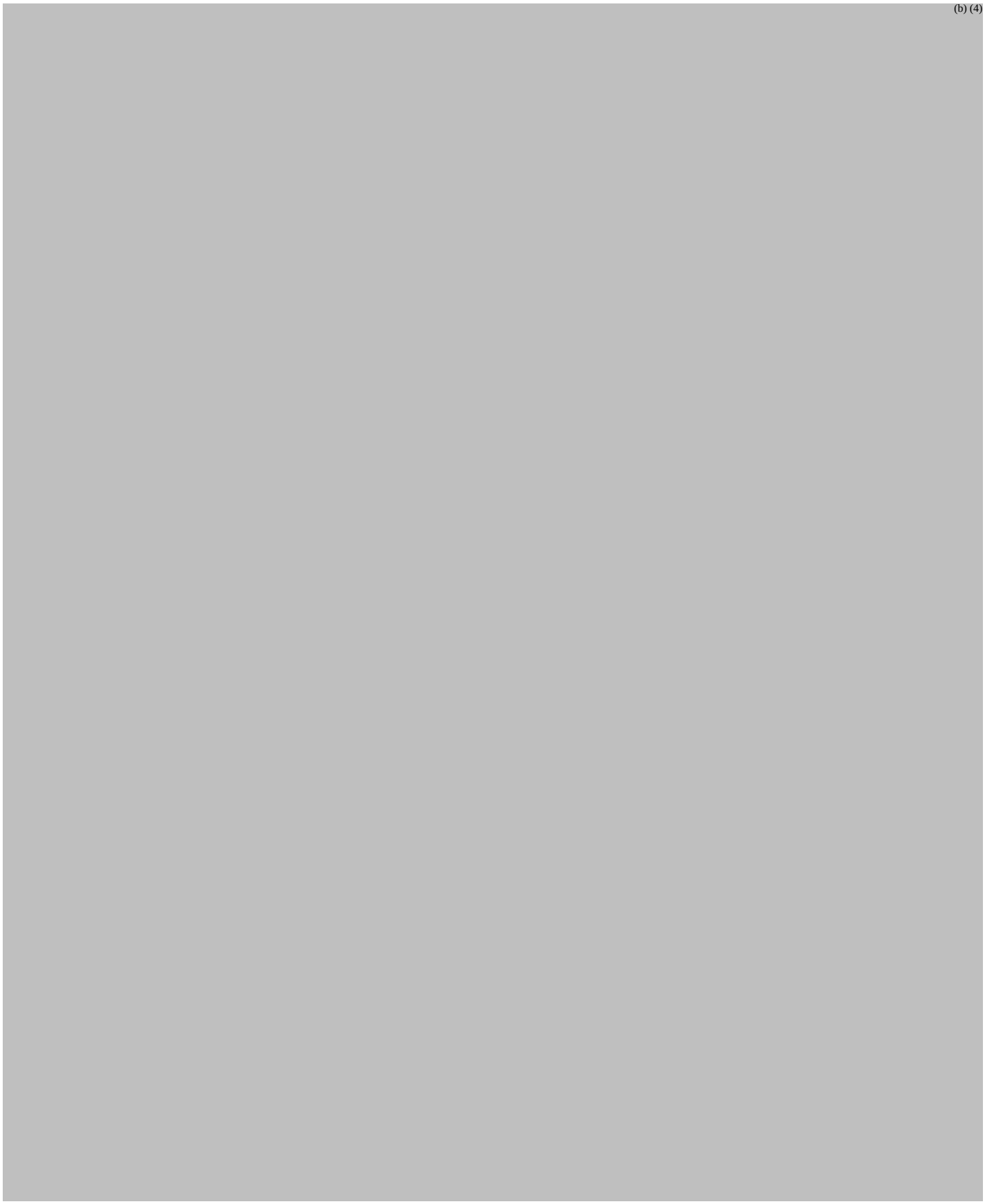
(b) (4)

3. DY21-POE Aerobic Plating

(b) (4)

DY21 Solid Intermediate Microbe Enumeration

(b) (4)



DY21 Solid Intermediate Microbe Enumeration

(b) (4)

Reasons for Revision

1. Initial version.

ASCUS BIOSCIENCES

Product Certificate of Analysis

Product Name	DY21 Milled Preservation by Vaporization
Batch Number	18-0202-001-P84-1
Date of Manufacture	22Jan2019
Storage Conditions	2-8 °C

Analytical Property	**Specification**	**Result**
DY21-POE Microbe Enumeration	> 4 x 10^8 CFU/g	(b) (4)

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

DocuSigned by:
Patricia Williams
5B301285A10643D...

12/6/2019

Patricia A. Williams
Quality

ASCUS BIOSCIENCES

Product Certificate of Analysis

Product Name	DY21 Milled Preservation by Vaporization
Batch Number	18-0202-001-P85-1
Date of Manufacture	23Jan2019
Storage Conditions	2-8 °C

Analytical Property	Specification	Result
DY21-POE Microbe Enumeration	> 4 x 10^8 CFU/g	(b) (4)

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

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Patricia A. Williams
Quality

ASCUS BIOSCIENCES

Product Certificate of Analysis

Product Name	DY21 Milled Preservation by Vaporization
Batch Number	18-0202-001-P85-2
Date of Manufacture	23Jan2019
Storage Conditions	2-8 °C

Analytical Property	**Specification**	**Result**
DY21-POE Microbe Enumeration	> 4 x 10^8 CFU/g	(b) (4)

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

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12/6/2019

Patricia A. Williams
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ASCUS BIOSCIENCES

Product Certificate of Analysis

Product Name	DY21 Palm Oil Encapsulate
Batch Number	18-0202-001-P86-1
Date of Manufacture	25Jan2019
Expiration Date	25Jan2020
Retest Date	N/A
Storage Conditions	2-8 °C

Analytical Property	**Specification**	**Result**
DY21-POE Microbe Enumeration	>4.0 X 10^7CFU/g	(b) (4)
Coliform	<10 CFU/g	
E. coli	<10 CFU/g	
Salmonella	Negative/25g	
Listeria	Negative/25g	

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

Patricia A. Williams
Quality

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6/7/2019

ASCUS BIOSCIENCES

Product Certificate of Analysis

Product Name	DY21 Palm Oil Encapsulate
Batch Number	18-0202-001-P86-2
Date of Manufacture	25Jan2019
Expiration Date	25Jan2020
Retest Date	N/A
Storage Conditions	2-8 °C

Analytical Property	Specification	Result
DY21-POE Microbe Enumeration	>4.0 X 10^7CFU/g	(b) (4)
Coliform	<10 CFU/g	
E. coli	<10 CFU/g	
Salmonella	Negative/25g	
Listeria	Negative/25g	

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

Patricia A. Williams
Quality

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Patricia Williams
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6/7/2019

Product Certificate of Analysis

Product Name	DY21 Palm Oil Encapsulate
Batch Number	18-0202-001-P87-1
Date of Manufacture	25Jan2019
Expiration Date	25Jan2020
Retest Date	N/A
Storage Conditions	2-8 °C

Analytical Property	**Specification**	**Result**
DY21-POE Microbe Enumeration	>4.0 X 10^7CFU/g	(b) (4)
Coliform	<10 CFU/g	
E. coli	<10 CFU/g	
Salmonella	Negative/25g	
Listeria	Negative/25g	

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

Patricia A. Williams
Quality

DocuSigned by:
Patricia Williams
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6/7/2019

Analysis of *Pichia kudriavzevii* ASCUSDY21 Encapsulate for Heavy Metals, Microbial & Mycotoxin Contamination

Approvers:

DocuSigned by:
Martin Mayhew
D1605F1B4C3E49A...

12/2/2019

Martin Mayhew
Vice President – Product Development & Manufacturing

Date

DocuSigned by:
Patricia A Williams
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11/21/2019

Patricia A. Williams
Quality

Date

DocuSigned by:
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11/21/2019

Howard B. Green
Regulatory

Date

Prepared by
Ascus Biosciences
San Diego, CA

November 2019

Analysis of *Pichia kudriavzevii* ASCUSDY21 Encapsulate for Heavy Metals, Microbial & Mycotoxin Contamination

Three lots of *Pichia kudriavzevii* ASCUSDY21 Encapsulate were sent for heavy metal, Mycotoxin and microbial contamination analysis at (b) (4)

The ICP-MS method (MF 24E022) was used for the heavy metal analysis of the samples and results are summarized in the following table.

Table 1. Heavy Metal Analysis of Three Lots of *Pichia kudriavzevii* ASCUSDY21 Encapsulate

Lot Number	Arsenic, ppm	Cadmium, ppm	Lead, ppm	Mercury, ppm
Detection Limit	0.002	0.002	0.002	0.002
18-0202-001-P86-1	(b) (4)			
18-0202-001-P86-2				
18-0202-001-P87-1				

ND - None Detected

P. kudriavzevii ASCUSDY21 Encapsulate was assayed for Mycotoxin (Alfatoxin M1) using the method AOAC 2000.08 and are summarized in the following table.

Table 2. Alfatoxin M1 Analysis of Three Lots of *Pichia kudriavzevii* ASCUSDY21 Encapsulate

Lot Number	Alfatoxin M1
Detection Limit	0.05 mcg/kg
18-0202-001-P86-1	(b) (4)
18-0202-001-P86-2	
18-0202-001-P87-1	

P. kudriavzevii ASCUSDY21 Encapsulate was assayed for microbial contamination using methods FDA BAM for Coliforms/E. coli, AOAC 2013.01 for Salmonella and AOAC 2013.10 for Listeria and are summarized in the following table.

Table 3. Microbial Contamination Analysis of Three Lots of *Pichia kudriavzevii* ASCUSDY21 Encapsulate

Lot Number	Coliform, CFU/g	E. coli, CFU/g	Salmonella, per 25g	Listeria, per 25g
Requirement	<10	<10	Negative	Negative
18-0202-001-P86-1	(b) (4)			
18-0202-001-P86-2				
18-0202-001-P87-1				

Given the low inclusion rate of *Pichia kudriavzevii* ASCUSDY21 Encapsulate and dilution factor in a final ration, no heavy metal or aflatoxin testing will be continued for production lots. However, all production lots of *Pichia kudriavzevii* ASCUSDY21 Encapsulate will be tested for microbial contamination for Coliform, E. coli, Salmonella and Listeria.

Attachment 1. Certificate of Analysis – Heavy Metal Analysis (Anresco No. 220190204) for *Pichia kudriavzevii* ASCUSDY21 Encapsulate

(b) (4)

Certificate of Analysis

February 22, 2019

ASCUS BIOSCIENCES
6450 Lusk Blvd, Suite E209
San Diego, CA 92121

(b) (4) No. 220190204

Sample information

Product	Three Dairy samples:1. Dairy - 21 Encapsulated Microbial Beads. Store at RT. Lot No. 18-0202-001-P86-12. Dairy - 21 Encapsulated Microbial Beads. Store at RT. Lot No. 18-0202-001-P86-23. Dairy - 21 Encapsulated Microbial Beads. Store at RT. Lot No. 18-0202-001-P87-1
Sampling	Received from Client.
Received	February 15, 2019.

Analytical Results

Analysis	Heavy MetalS
Methods	ICP-MS
Analysis Date	February 15, 2019 to February 22, 2019
Analyst	(b) (4)

Findings

Analysis	Lot No. 18-0202-001-P86-1	Lot No. 18-0202-001-P86-2	Lot No. 18-0202-001-P87-1	Detection Limit (ppm)
Arsenic, ppm	(b) (4)			0.002
Cadmium, ppm				0.002
Lead, ppm				0.002
Mercury, ppm				0.002

Reported by
(b) (4)

(b) (4)

(b) (4)

If there are any questions with this report, please contact (b) (4) 022819_1 page 1 of 1

(b) (4)

(b) (4)

Attachment 2. Certificate of Analysis – Microbial Contamination Testing (Anresco No. 220190204) *Pichia kudriavzevii* ASCUSDY21 Encapsulate

(b) (4)

Certificate of Analysis

March 9, 2019

ASCUS BIOSCIENCES
6450 Lusk Blvd, Suite E209
San Diego, CA 92121

(b) (4) No. 220190204

Sample information

Product	Three Dairy samples: 1. Dairy - 21 Encapsulated Microbial Beads. Store at RT. Lot No. 18-0202-001-P86-1 2. Dairy - 21 Encapsulated Microbial Beads. Store at RT. Lot No. 18-0202-001-P86-2 3. Dairy - 21 Encapsulated Microbial Beads. Store at RT. Lot No. 18-0202-001-P87-1
Sampling	Received from Client.
Received	February 15, 2019.

Analytical Results

Analysis Date February 15, 2019 to March 9, 2019

Findings

Sample ID	Alfatoxin M1. mcg/kg
1. Dairy - 21 Encapsulated Microbial Beads. Store at RT. Lot No. 18-0202-001-P86-1	(b) (4)
2. Dairy - 21 Encapsulated Microbial Beads. Store at RT. Lot No. 18-0202-001-P86-2	
3. Dairy - 21 Encapsulated Microbial Beads. Store at RT. Lot No. 18-0202-001-P87-1	
Detection Limit	0.05
Method	AOAC 2000.08

Reported by
(b) (4)

(b) (4)

If there are any questions with this report, please contact (b) (4) 030919_1 page 1 of 1

(b) (4) (b) (4)

Attachment 3. Certificate of Analysis – Microbial Contamination Testing (Anresco No. 220190203) *Pichia kudriavzevii* ASCUSDY21 Encapsulate

(b) (4)

Certificate of Analysis

February 20, 2019

ASCUS BIOSCIENCES
6450 Lusk Blvd, Suite E209
San Diego, CA 92121

(b) (4) No. 220190203

Sample information

Product	Three Dairy samples: 1. Dairy - 21 Encapsulated Microbial Beads. Store at RT. Lot No. 18-0202-001-P86-1 2. Dairy - 21 Encapsulated Microbial Beads. Store at RT. Lot No. 18-0202-001-P86-2 3. Dairy - 21 Encapsulated Microbial Beads. Store at RT. Lot No. 18-0202-001-P87-1
Sampling	Received from Client
Receive	February 15, 2019

Analytical Results

Methods	FDA BAM - Coliforms/E. coli AOAC 2013.01 - Salmonella AOAC 2013.10 - Listeria
Analysis Date	February 15, 2019 to February 19, 2019

Sample	Coliforms *cfu/g*	E. coli *cfu/g*	Salmonella *per 25g*	Listeria *per 25g*
1. Dairy - 21 18-0202-001-P86-1 2. Dairy - 21 18-0202-001-P86-2 3. Dairy - 21 18-0202-001-P87-1	(b) (4)			

Reported by

(b) (4)

(b) (4)

If there are any questions with this report, please contact (b) (4) 022019_1 page 1 of 1

(b) (4)

(b) (4)

Pichia kudriavzevii ASCUSDY21 Encapsulate 5°C: 12-Month Stability Summary Report

Approvers:

DocuSigned by:
Martin Mayhew
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5/12/2020

Martin Mayhew
Vice President – Product Development & Manufacturing

Date

DocuSigned by:
Patricia A Williams
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5/6/2020

Patricia A. Williams
Quality

Date

DocuSigned by:
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5/8/2020

Howard B. Green
Regulatory

Date

Prepared by
Ascus Biosciences
San Diego, Ca

May 2020

Table of Contents

Pichia kudriavzevii ASCUSDY21 Encapsulate 5°C: 12-Month Stability Summary Report

Organism: *Pichia kudriavzevii* ASCUSDY21

Testing Condition: 5°C ± 3°C

Purpose: To support the registered storage requirement of 2-8°C for 12 months.

Study Numbers: DUS1901 (Lot# 18-0202-001-P86-1)
DUS1904 (Lot# 18-0202-041-P86-2)
DUS1907 (Lot# 18-0202-001-P87-1)

Acceptance Criteria: Not Less Than 4.0 X 10^7 CFU/g

1 Results

Table 1. Results for Each Lot at Each Time Point

Results are reported in average colony forming units (CFU)/gram of *Pichia kudriavzevii* ASCUSDY21 Encapsulate.

	Avg. CFU/g			Std. Dev.		
Time (mo)	**DUS1901**	**DUS1904**	**DUS1907**	**DUS1901**	**DUS1904**	**DUS1907**
0	(b) (4)					
1						
2						
3						
6						
9						
12						

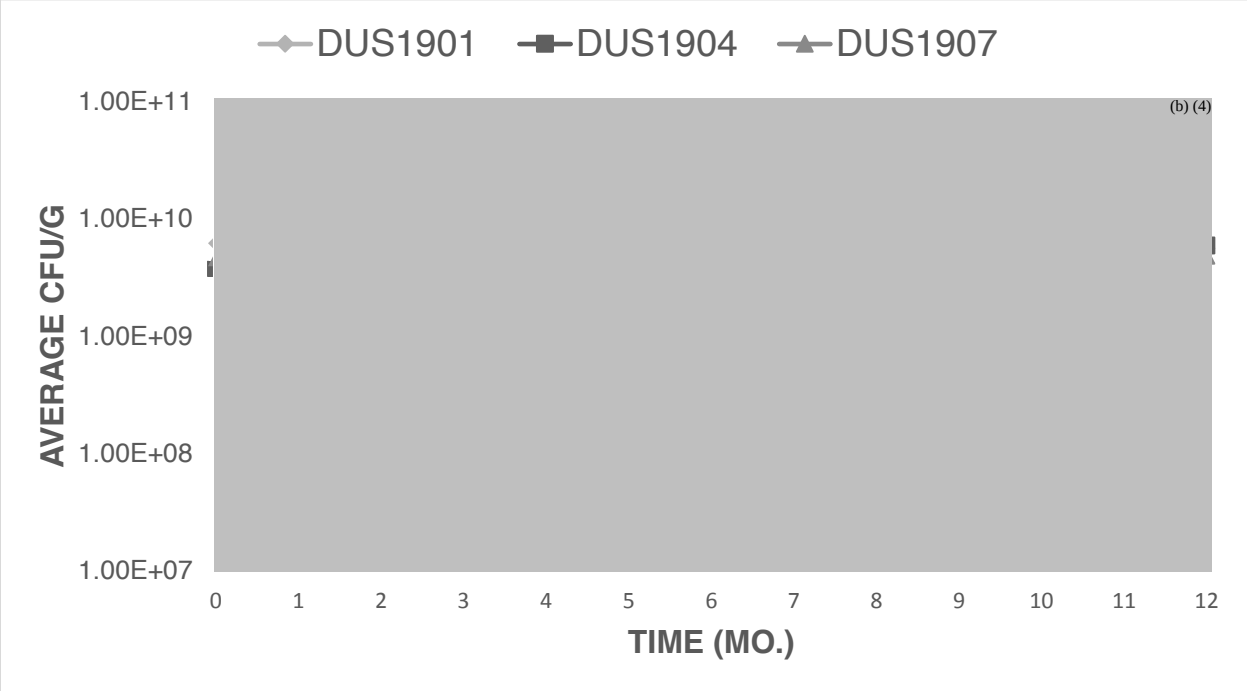

Figure 1. Graph of Results for Each Lot at Each Time Point

Results are reported in average colony forming units (CFU)/gram of *Pichia kudriavzevii* ASCUSDY21 Encapsulate.

2 Discussion

The stability study of *Pichia kudriavzevii* ASCSUSDY21 Encapsulate on three separate lots conducted at 5°C ± 3°C for 12 months resulted in no degradation of the material below the acceptance criteria of 4.00 X 10^7 CFU/g (Table 1, Figure 1).

Viability of the 3 lots remained above 1.00 X 10^9 CFU/g for the duration of this stability study, indicating *Pichia kudriavzevii* ASCSUSDY21 Encapsulate will remain above the acceptance criteria during shipping and storage excursions and refrigeration for up to 12 months at 5°C ± 3°C.

3 Deviations

Deviations in conduct with the *Pichia kudriavzevii* ASCSUSDY21 Encapsulate Microbe Enumeration Protocol V2 (Appendix 1) that were considered minor were the following:

1. Media was prepared in 1000 L volumes (Step 1.6) instead of in 500mL volumes due to high demand of media production.

2. Hot water bath temperature ranged from 65-80°C (Step 1.8) instead of 80°C to reduce condensation in the bottle while cooling the media prior to additional media additions.

4 Changes

No significant changes occurred during the stability study.

5 Appendices

Appendix 1. *Pichia kudriavzevii* ASCUSDY21 Encapsulate 5°C Stability Protocol

Appendix 2. *Pichia kudriavzevii* ASCUSDY21 Encapsulate Data Used to Summarize Table 1 and Create Figure 1

Appendix 3. Master Production Record for the three Lots

Appendix 1. *Pichia kudriavzevii* ASCUSDY21 Encapsulate 5°C Stability Protocol

Stability Protocol Title:	**DY21 POE 5ºC**
Organism:	***Pichia kudriavzevii***
Purpose:	**To support the registered storage requirement of 2-8ºC for 12 months**
Number of Samples to Place on Stability:	**9 (allows for retesting, when needed)**
Sample Storage Container:	Heat sealed 48-gauge silver metalized PET / 2.5 mil LLDPE bags made from commercial bags
Temperature Conditions:	**2-8ºC**
Acceptance Criteria:	**>4 x 10^7 cfu/g**

Tests and Timepoints:

Assay	T_0	1 month	2 months	3 months	6 months	9 months	12 months
Microbe Enumeration*	X	X	X	X	X	X	X

*DY21 POE Microbe Enumeration method

Approvals:

Howard Green Regulatory	DocuSigned by: Howard B Green 12/11/2018 0FAA38037D49453...
Corey Dodge Process Development	DocuSigned by: Corey Dodge 12/5/2018 8:16:17 AM PST FAA4AC21D1C745C...
Patricia A. Williams Quality	DocuSigned by: Patricia A Williams 12/4/2018 7:14:14 PM PST 5D301285A10643D...

Appendix 2. *Pichia kudriavzevii* ASCUSDY21 Encapsulate Data Used to Summarize Table 1 and Create Figure 1

Study Number	Scheduled Pull Date	Date Sample Was Pulled	Testing Condition	Timepoint	Batch Number	Lot Number	Analyst Initials	Sample	Mass (g)	Volume 1X PBST (mL)	DY21 Plating CFU Counts, CFU/100uL: 1E-04 A	1E-04 B	1E-04 C	1E-05 A	1E-05 B	1E-05 C	1E-06 A	1E-06 B	1E-06 C	DY 21 CFU/mL A	CFU/mL B	CFU/mL C	CFU/g A	CFU/g B	CFU/g C	Avg. CFU/g	Std. Dev.	CV
DUS1901	13-Mar-19	3/10/2019	5C	1 Mo	1	18-0202-001-P86-1	RL	76-1	0.1060	10	(b) (4)																	
								76-2	0.1041	10																		
								76-3	0.1027	10																		
	13-Apr-19	11-Apr-19	5C	2 Mo	1	18-0202-P86-1	AT	77-1	0.1005	10																		
								77-2	0.0992	10																		
								77-3	0.1009	10																		
	13-May-19	15-May-19	5C	3 Mo	1	18-0202-001-P86-1	AT/MS	78-1	0.1010	10																		
								78-2	0.1000	10																		
								78-3	0.1010	10																		
	13-Aug-19	12-Aug-19	5C	6Mo	1	18-0202-001-P86-1	RL	79-1	0.1040	10																		
								79-2	0.1010	10																		
								79-3	0.1020	10																		
	13-Nov-19	14-Nov-19	5C	9 Mo	1	18-0202-001-P86-1	AT	80-1	0.1020	10																		
								80-2	0.1047	10																		
								80-3	0.1004	10																		
	13-Feb-20	12-Feb-20	5C	12 Mo	1	18-0202-001-P86-2	AT	84-1	0.1060	10																		
								84-2	0.1000	10																		
								84-3	0.0990	10																		
DUS1904	13-Mar-19	3/10/2019	5C	1 Mo	2	18-0202-001-P86-2	RL	101-1	0.1073	10																		
								101-2	0.1020	10																		
								101-3	0.1072	10																		
	13-Apr-19	11-Apr-19	5C	2 Mo	2	18-0202-001-P86-2	AT	102-1	0.1010	10																		
								102-2	0.1009	10																		
								102-3	0.1009	10																		
	13-May-19	15-May-19	5C	3 Mo	2	18-0202-001-P86-2	AT/MS	103-1	0.1010	10																		
								103-2	0.0990	10																		
								103-3	0.1010	10																		
	13-Aug-19	12-Aug-19	5C	6 Mo	2	18-0202-001-P86-2	RL	104-1	0.1010	10																		
								104-2	0.1050	10																		
								104-3	0.1050	10																		
	13-Nov-19	14-Nov-19	5C	9 Mo	2	18-0202-001-P86-2	AT	105-1	0.0998	10																		
								105-2	0.1015	10																		
								105-3	0.1006	10																		
	13-Feb-20	12-Feb-20	5C	12 Mo	2	18-0202-001-P86-3	AT	106-1	0.0990	10																		
								106-2	0.1000	10																		
								106-3	0.1000	10																		
DUS1907	13-Mar-19	3/10/2019	5C	1 Mo	3	18-0202-001-P87-1	RL	126-1	0.1054	10																		
								126-2	0.1066	10																		
								126-3	0.1036	10																		
	11-Apr-19	4/11/2019	5C	2 Mo	3	18-0202-001-P87-1	AT	127-1	0.0999	10																		
								127-2	0.1002	10																		
								127-3	0.0993	10																		
	13-May-19	5/15/2019	5C	3 Mo	3	18-0202-001-P87-1	AT/MS	128-1	0.1010	10																		
								128-2	0.1000	10																		
								128-3	0.0990	10																		
	13-Aug-19	12-Aug-19	5C	6 Mo	3	18-0202-001-P87-1	AT	129-1	0.1000	10																		
								129-2	0.0980	10																		
								129-3	0.1010	10																		
	13-Nov-19	11/14/2019	5C	9 Mo	3	18-0202-001-P87-2	AT	130-1	0.1077	10																		
								130-2	0.1089	10																		
								130-3	0.1053	10																		
	13-Feb-20	2/12/2020	5C	12 Mo	3	18-0202-001-P87-3	AT	133-1	0.0980	10																		
								133-2	0.1000	10																		
								133-3	0.1020	10																		

Date of creation	Date of assay	Timepoint	Batch Number	Lot Number	Analyst Initials	Sample	Mass (g)	Volume 1X PBST (mL)	DY21 Plating CFU Counts, CFU/100uL: 1E-04 A	1E-04 B	1E-04 C	1E-05 A	1E-05 B	1E-05 C	1E-06 A	1E-06 B	1E-06 C	DY 21 CFU/mL A	CFU/mL B	CFU/mL C	CFU/g A	CFU/g B	CFU/g C	Avg. CFU/g	Std. Dev.	CV
25-Jan-19	29-Jan-19	T0	1	18-0202-001-P86-1	AT	86_1-1	0.1012	10	(b) (4)																	
						86_1-2	0.1018	10																		
						86_1-3	0.1015	10																		
25-Jan-19	29-Jan-19	T0	2	18-0202-001-P86-2	AT	86_2-1	0.1008	10																		
						86_2-2	0.1011	10																		
						86_2-3	0.1008	10																		
25-Jan-19	29-Jan-19	T0	3	18-0202-001-P87-1	AT	87_1-1	0.1016	10																		
						87_1-2	0.1012	10																		
						87_1-3	0.1012	10																		

Appendix 3. Master Production Record for the Three Lots

84 Project No. _____
Book No. _____ TITLE Cryoprotectant Fitz-Milled Prob

(b) (4)

[redacted]

Invented by:
Recorded by: Suzanne L. Colby Date 1/25/19

TITLE Cryogenically Fitz-Miller, Probiotic Yeast Continued Project No. 22695.01.004 Book No. 18-0202-001 85

(b) (4)

[redacted]

Recorded by: 1/23/19

Scanned by CamScanner

86 Project No. 22695
Book No. 18-0602-001 TITLE Spray Congealing DY21

(b) (4)

[redacted]

Witnessed & Understood by me,	Date	Invented by:	Date
		Recorded by:	1-25-19

Scanned by CamScanner

TITLE Spray Congeal DY21 Project No. 22695 Book No. 18-0202-001 87

(b) (4)

[redacted]

Witnessed & Understood by me, Date

Invented by: Date 1-25-19

Recorded by:

Pichia kudriavzevii ASCUSDY21 Encapsulate 25°C: 12-Month Stability Summary Report

Approvers:

DocuSigned by:
Martin Mayhew
D1605F1B4C3E49A...

5/12/2020

Martin Mayhew
Vice President – Product Development & Manufacturing

Date

DocuSigned by:
Patricia A Williams
5B301285A10643D...

5/6/2020

Patricia A. Williams
Quality

Date

DocuSigned by:
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5/8/2020

Howard B. Green
Regulatory

Date

Prepared by
Ascus Biosciences
San Diego, Ca

April 2020

Table of Contents

Pichia kudriavzevii ASCUSDY21 Encapsulate 25°C: 12-Month Stability Summary Report

Organism: *Pichia kudriavzevii* ASCUSDY21

Testing Condition: 25°C ± 3°C

Purpose: To support temperature excursions during shipping and storage.

Study Numbers: DUS1902 (Lot# 18-0202-001-P86-1)
DUS1905 (Lot# 18-0202-041-P86-2)
DUS1908 (Lot# 18-0202-001-P87-1)

Acceptance Criteria: Not Less Than 4.0 X 10^7 CFU/g

1 Results

Table 1. Results for Each Lot at Each Time Point

Results are reported in average colony forming units (CFU)/gram of *Pichia kudriavzevii* ASCUSDY21 Encapsulate.

	Avg. CFU/g			Std. Dev.		
Time (mo)	**DUS1902**	**DUS1905**	**DUS1908**	**DUS1902**	**DUS1905**	**DUS1908**
0	(b) (4)					
1						
2						
3						
6						
9						
12						

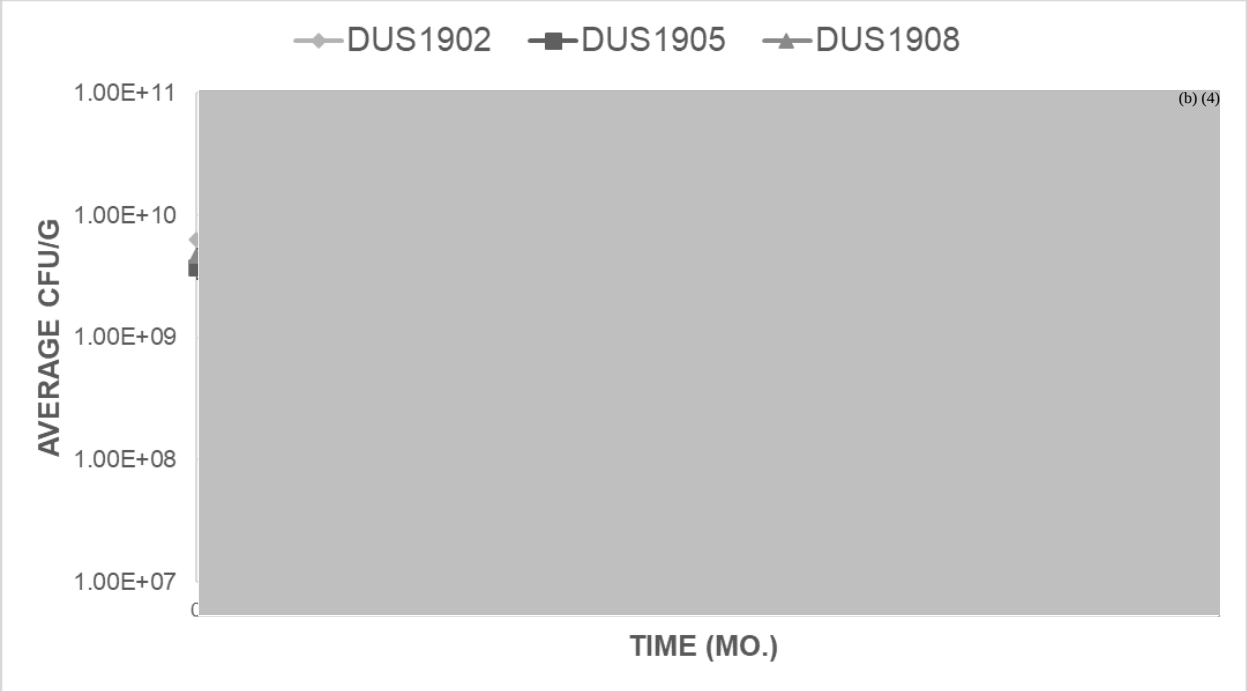


Figure 1. Graph of Results for Each Lot at Each Time Point

Results are reported in average colony forming units (CFU)/gram of *Pichia kudriavzevii* ASCUSDY21 Encapsulate.

2 Discussion

The stability study of *Pichia kudriavzevii* ASCUSDY21 Encapsulate on three separate lots conducted at 25°C ± 3°C for 12 months resulted in no degradation of the material below the acceptance criteria of 4.00 X 10^7 CFU/g (Table 1, Figure 1).

Viability of the 3 lots remained above 1.00 X 10^8 CFU/g for the duration of this stability study, indicating *P. kudriavzevii* ASCUSDY21 Encapsulate will remain above the acceptance criteria during shipping and storage excursions and refrigeration for up to 12 months at 25°C ± 3°C.

3 Deviations

Deviations in conduct with the *P. kudriavzevii* ASCUSDY21 Encapsulate Microbe Enumeration Protocol V2 (Appendix 1) that were considered minor were the following:

1. Media was prepared in 1000 L volumes (Step 1.6) instead of in 500mL volumes due to high demand of media production.

2. Hot water bath temperature ranged from 65-80°C (Step 1.8) instead of 80°C to reduce condensation in the bottle while cooling the media prior to additional media additions.

4 Changes

No significant changes occurred during the stability study.

5 Appendices

Appendix 1. *Pichia kudriavzevii* ASCUSDY21 Encapsulate 25°C Stability Protocol

Appendix 2. *Pichia kudriavzevii* ASCUSDY21 Encapsulate Data Used to Summarize Table 1 and Create Figure 1

Appendix 3. Master Production Record for the three Lots

Appendix 1. *Pichia kudriavzevii* ASCUSDY21 Encapsulate 25°C Stability Protocol

Stability Protocol Title:	DY21 POE 5°C
Organism:	*Pichia kudriavzevii*
Purpose:	To support the registered storage requirement of 2-8°C for 12 months
Number of Samples to Place on Stability:	9 (allows for retesting, when needed)
Sample Storage Container:	Heat sealed 48-gauge silver metalized PET / 2.5 mil LLDPE bags made from commercial bags
Temperature Conditions:	2-8°C
Acceptance Criteria:	>4 x 10^7 cfu/g

Tests and Timepoints:

Assay	T_0	1 month	2 months	3 months	6 months	9 months	12 months
Microbe Enumeration*	X	X	X	X	X	X	X

*DY21 POE Microbe Enumeration method

Approvals:

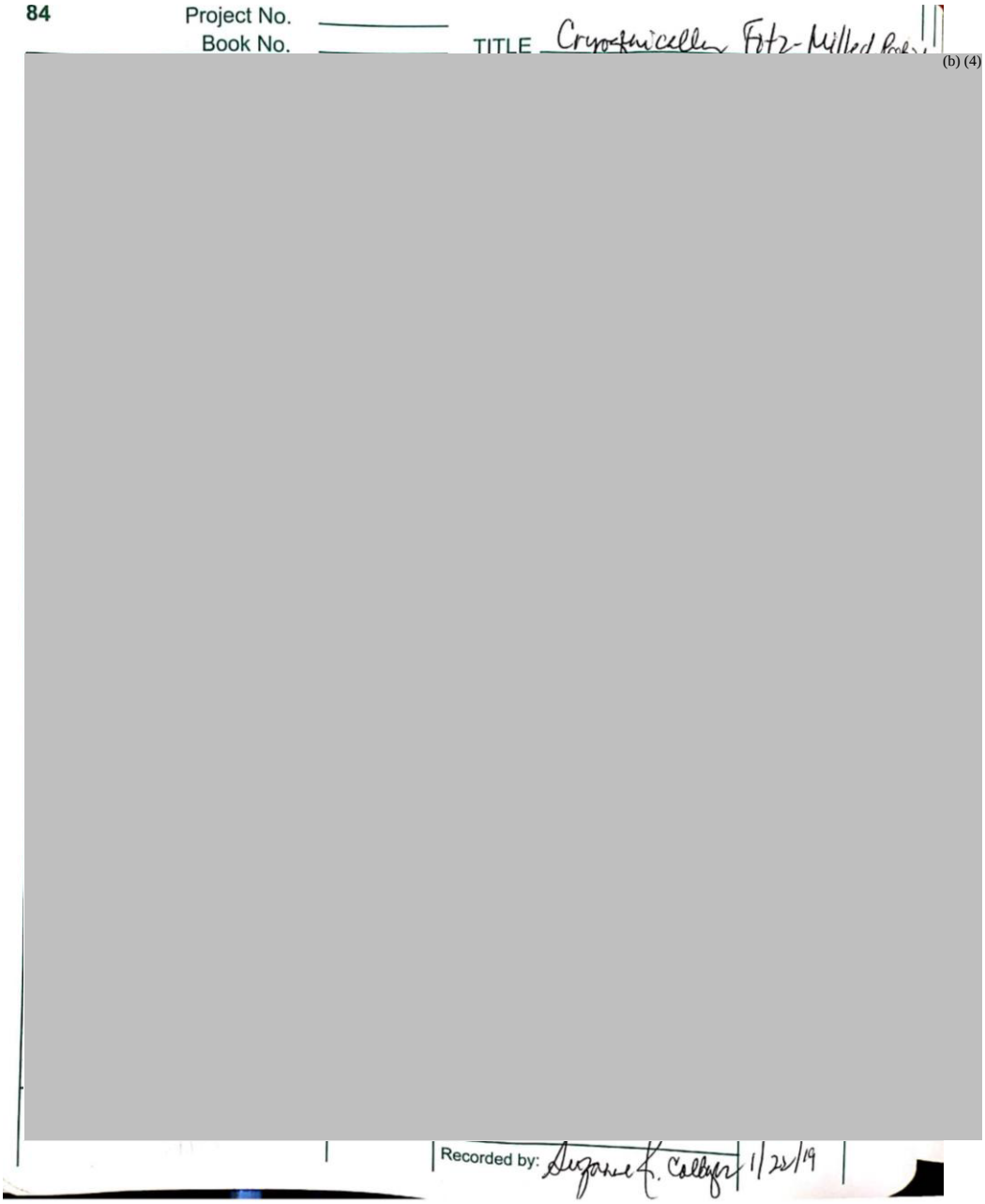
Howard Green Regulatory	DocuSigned by: Howard B Green 12/11/2018
Corey Dodge Process Development	DocuSigned by: Corey Dodge 12/5/2018 8:16:17 AM PST
Patricia A. Williams Quality	DocuSigned by: Patricia A Williams 12/4/2018 7:14:14 PM PST

Appendix 2. *Pichia kudriavzevii* ASCUSDY21 Encapsulate Data Used to Summarize Table 1 and Create Figure 1

											DY21 Plating CFU Counts, CFU/100uL									DY 21								
											1E-04			1E-05			1E-06			CFU/mL			CFU/g					
Study Number	Scheduled Pull Date	Date Sample Was Pulled	Testing Condition	Timepoint	Batch Number	Lot Number	Analyst Initials	Sample	Mass (g)	Volume 1X PBST (mL)	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	Avg. CFU/g	Std. Dev.	CV
DU51902	13-Mar-19	3/10/2019	25C	1 Mo	1	18-0202-001 P86-1	RL	85-1	0.1096	10	(b) (4)																
								85-2	0.1066	10																	
								85-3	0.1041	10																	
	13-Apr-19	4/11/2019	25C	2 Mo	1	18-0202-001 P86-1	AT	86-1	0.1001	10																	
								86-2	0.1006	10																	
								86-3	0.1004	10																	
	13-May-19	5/14/2019	25C	3 Mo	1	18-0202-P86 1	MS	87-1	0.1040	10																	
								87-2	0.1030	10																	
								87-3	0.1070	10																	
	13-Aug-19	12-Aug-19	25C	6Mo	1	18-0202-P86 1	RL	88-1	0.1080	10																	
								88-2	0.1000	10																	
								88-3	0.1060	10																	
	13-Nov-19	11/14/2019	25C	9 Mo	1	18-0202-P86 1	AT	89-1	0.1024	10																	
								89-2	0.1006	10																	
								89-3	0.1047	10																	
	13-Feb-20	2/12/2020	25C	12 Mo	1	18-0202-001 P86-1	AT	90-1	0.1030	10																	
								90-2	0.1000	10																	
								90-3	0.1020	10																	
DU51905	13-Mar-19	3/10/2019	25C	1 Mo	2	18-0202-001 P86-2	RL	110-1	0.1098	10																	
								110-2	0.1039	10																	
								110-3	0.1068	10																	
	13-Apr-19	11-Apr-19	25C	2 Mo	2	18-0202-001 P86-2	AT	112-1	0.1001	10																	
								112-2	0.1000	10																	
								112-3	0.0996	10																	
	13-May-19	14-May-19	25C	3 Mo	2	18-0202-001 P86-2	MS	113-1	0.1010	10																	
								113-2	0.1000	10																	
								113-3	0.1050	10																	
	13-Aug-19	12-Aug-19	25C	6 Mo	2	18-0202-001 P86-2	RL	114-1	0.1030	10																	
								114-2	0.1020	10																	
								114-3	0.1020	10																	
	13-Nov-19	14-Nov-19	25C	9 Mo	2	18-0202-001 P86-2	AT	115-1	0.1031	10																	
								115-2	0.1007	10																	
								115-3	0.1025	10																	
	13-Feb-20	12-Feb-20	25C	12 Mo	2	18-0202-001 P86-1	AT	116-1	0.1000	10																	
								116-2	0.1040	10																	
								116-3	0.0970	10																	
DU51908	13-Mar-19	3/10/2019	25C	1 Mo	3	18-0202-001 P87-1	RL	135-1	0.1041	10																	
								135-2	0.1030	10																	
								135-3	0.1057	10																	
	13-Apr-19	4/11/2019	25C	2 Mo	3	18-0202-001 P87-1	AT	136-1	0.1004	10																	
								136-2	0.1004	10																	
								136-3	0.0997	10																	
	13-May-19	5/14/2019	25C	3 Mo	3	18-0202-001 P87-1	MS	137-1	0.1060	10																	
								137-2	0.1020	10																	
								137-3	0.1000	10																	
	13-Aug-19	12-Aug-19	25C	6 Mo	3	18-0202-001 P87-1	AT	139-1	0.1070	10																	
								139-2	0.0980	10																	
								139-3	0.0990	10																	
	13-Nov-19	11/14/2019	25C	9 Mo	3	18-0202-001 P87-2	AT	140-1	0.1006	10																	
								140-2	0.1041	10																	
								140-3	0.1060	10																	
	13-Feb-20	2/12/2020	25C	12 Mo	3	18-0202-001 P86-1	AT	138-1	0.1100	10																	
								138-2	0.1130	10																	
								138-3	0.1050	10																	

									DY21 Plating CFU Counts, CFU/100uL									DY 21								
									1E-04			1E-05			1E-06			CFU/mL			CFU/g					
Date of creation	Date of assay	Timepoint	Batch Number	Lot Number	Analyst Initials	Sample	Mass (g)	Volume 1X PBST (mL)	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	Avg. CFU/g	Std. Dev.	CV
25-Jan-19	29-Jan-19	T0	1	18-0202-001 P86-1	AT	86_1-1	0.1012	10	(b) (4)																		
						86_1-2	0.1018	10																			
						86_1-3	0.1015	10																			
25-Jan-19	29-Jan-19	T0	2	18-0202-001 P86-2	AT	86_2-1	0.1008	10																			
						86_2-2	0.1011	10																			
						86_2-3	0.1008	10																			
25-Jan-19	29-Jan-19	T0	3	18-0202-001 P87-1	AT	87_1-1	0.1016	10																			
						87_1-2	0.1012	10																			
						87_1-3	0.1012	10																			

Appendix 3. Master Production Record for the Three Lots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Book No. _____ TITLE Cryptococcus Fatz-Millard Prob. III
(b) (4)
Recorded by: Suzanne K. Collyer 1/25/19

Scanned by CamScanner

TITLE Cryogenically Fitz-Miller, Probiotic Yeast Continued Project No. 22695.01.004 Book No. 18-0202-001 85

(b) (4)

Recorded by: 1/23/19

86 Project No. 22695
Book No. 18-0202-001 TITLE Spray Congeal DY21

(b) (4)

[redacted]

Witnessed & Understood by me,	Date	Invented by:	Date
		Recorded by:	1-25-19

TITLE Spray Congeal DY21	Project No. 22695
	Book No. 18-0202-001

87

(b) (4)

[redacted]

Witnessed & Understood by me,	Date	Invented by:	Date
		Recorded by:	1-25-19

Pichia kudriavzevii ASCUSDY21 Encapsulate 40°C – Summary Report

A

DocuSigned by:
Martin Mayhew
D1605F1B4C3E49A...

12/4/2019

Martin Mayhew
Vice President – Product Development
& Manufacturing

Date

DocuSigned by:
Patricia A Williams
5B301285A10643D...

12/4/2019

Patricia A. Williams
Quality

Date

DocuSigned by:
Howard B Green
0FAA38037D49453...

12/4/2019

Howard B. Green
Regulatory

Date

Prepared by
Ascus Biosciences
San Diego, Ca

December 2019

Revised to include the third point in Section 3 – Deviations.

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Pichia kudriavzevii ASCUSDY21 Encapsulate 40°C – Summary Report

Organism: *Pichia kudriavzevii* ASCUSDY21

Testing Condition: 40°C ± 3°C

Purpose: To support temperature excursions during shipping and storing.

Study Numbers: DUS1903 (Lot# 18-0202-001-P86-1)
DUS1906 (Lot# 18-0202-041-P86-2)
DUS1909 (Lot# 18-0202-001-P87-1)

Acceptance Criteria: Not Less Than 4.0 X 10^7 CFU/g

1 Results

Table 1. Results for Each Lot at Each Time Point

Note: Results are reported in average colony forming units (CFU)/gram for *P. kudriavzevii* ASCUSDY21 Encapsulate.

Time (wk)	Avg. CFU/g			Std. Dev.		
	DUS1903	DUS1906	DUS1909	DUS1903	DUS1906	DUS1909
0	(b) (4)					
1						
2						
3						
4						
13						
26						

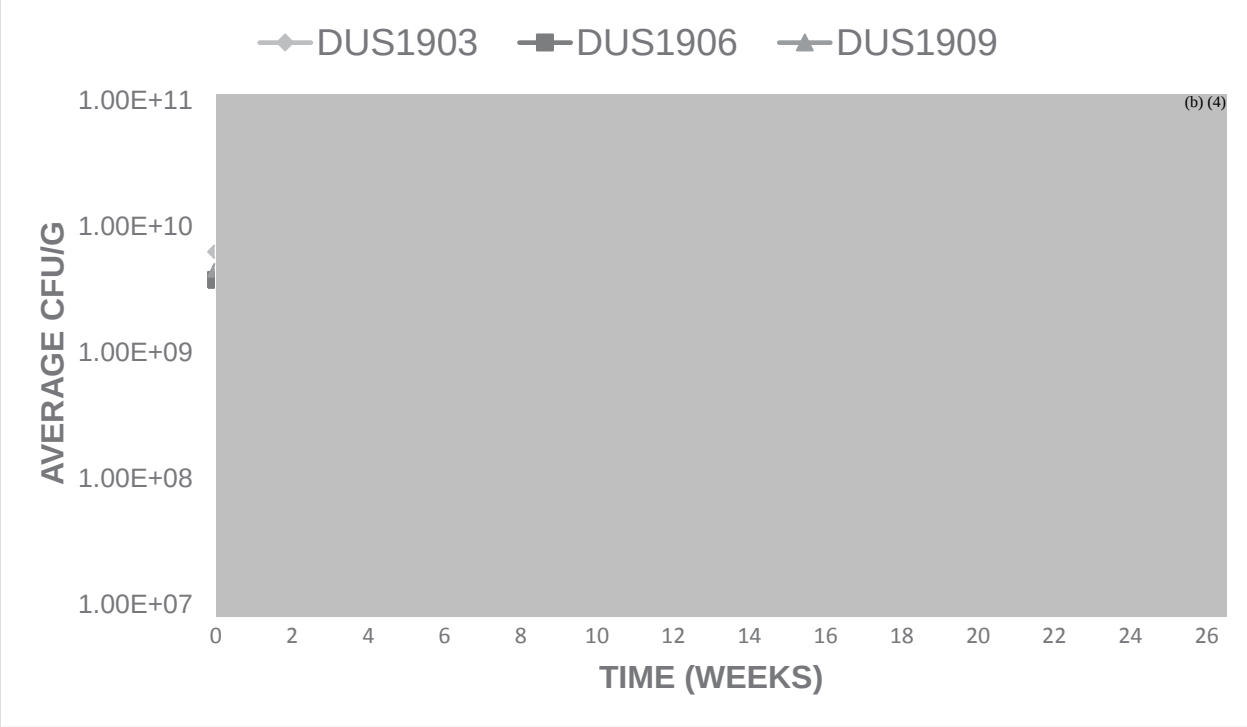


Figure 1. Graph of Results for Each Lot at Each Time Point

Note: Results are reported in average colony forming units (CFU)/gram of *P. kudriavzevii* ASCUSDY21 Encapsulate.

2 Discussion

The stability study for *P. kudriavzevii* ASCUSDY21 Encapsulate on three separate lots conducted at 40°C ± 3°C for 6 months resulted in no degradation of the material below the acceptance criteria of 4.00 X 10^7 CFU/g (Table 1, Figure 1). The study (Appendix 5) was originally designed to go 4 weeks but it was later determined to go longer as no significant degradation was seen at 4 week timepoint.

During the 26 weeks timepoint, CFU counts observed indicates that *Pichia kudriavzevii* ASCUSDY21 Encapsulate remained above the acceptance criteria during shipping and storage excursions and refrigeration for up to 6 months at 40°C ± 3°C.

3 Deviations

Deviations in conduct with the *P. kudriavzevii* ASCUSDY21 Encapsulate Microbe Enumeration Method (Appendix 3) that were considered minor were the following:

1 Media was prepared in 1000 L volumes (Step 1.6) instead of in 500 mL volumes due to high demand of media production.

2 Hot water bath temperature ranged from 65-80°C (Step 1.8) instead of 80°C to reduce condensation in the bottle while cooling the media prior to additional media additions.

3 When the 6-month timepoint was tested, some of the plates at the lowest dilution contained fewer than 10 colonies. The method indicates to use plate counts at or above 10. The low number of colonies were due to degradation, which was expected at the accelerated storage condition. The CVs for all results were acceptable, therefore, the results are valid. In addition, since the stability study was extended after the samples were placed into the chamber, there were not enough stability samples left to re-test the timepoint.

4 Changes

No significant changes occurred during the stability study.

5 Location of information

Appendix 1. *Pichia kudriavzevii* ASCUSDY21 Encapsulate 40°C Current Stability Protocol

Appendix 2. *Pichia kudriavzevii* ASCUSDY21 Encapsulate Data Used to Summarize Table 1 and Create Figure 1

Appendix 3. *Pichia kudriavzevii* ASCUSDY21 Encapsulate Microbe Enumeration Method V4

Appendix 4. Master Production Record for the 3 Lots

Appendix 5 Original Four-Week Stability Protocol for *Pichia kudriavzevii* ASCUSDY21 Encapsulate

Appendix 1. *Pichia kudriavzevii* ASCUSDY21 Encapsulate 40°C Current Stability Protocol

ASCUS BIOSCIENCES

Stability Protocol Title:	**DY21 POE 40°C**
Organism:	***Pichia kudriavzevii***
Purpose:	**To support temperature excursions during shipping and storage**
Number of Samples to Place on Stability:	**10 (allows for retesting, when needed)**
Sample Storage Container:	Heat sealed 48-gauge silver metalized PET / 2.5 mil LLDPE bags made from commercial bags
Temperature Conditions:	**37-43°C**
Acceptance Criteria:	**>4 x 10^7 cfu/g**

Tests and Timepoints:

Assay	**T_0**	**1 week**	**2 weeks**	**3 weeks**	**4 weeks**	**3 months**	**6 months**
Microbe Enumeration*	X	X	X	X	X	X	X

*DY21-POE Microbe Enumeration method

Approvals:

Howard Green Regulatory	DocuSigned by: Howard B Green 0[illegible]	5/10/2019
Corey Dodge Process Development	DocuSigned by: Corey Dodge [illegible]	5/10/2019
Patricia A. Williams Quality	DocuSigned by: Patricia Williams 5[illegible]	5/10/2019

Appendix 2. *Pichia kudriavzevii* ASCUSDY21 Encapsulate Data Used to Summarize Table 1 and Create Figure 1

											DY21 Plating CFU Counts, CFU/100uL							
											1E-04			1E-05			1E-06	
	Scheduled Pull Date	Date Sample Was Pulled	Testing Condition	Timepoint	Batch Number	Lot Number	Analyst Initials	Sample	Mass (g)	Volume 1X PBST (mL)	A	B	C	A	B	C	A	B
DUS1903	20-Feb-19	20-Feb-19	40C	1 Week	1	18-0202-001-P86-1	RL	94-1	0.1008	10								
								94-2	0.1039	10								
								94-3	0.1042	10								
	27-Feb-19	27-Feb-19	40C	2 Week	1	18-0202-001-P86-1	AT	95-1	0.1020	10								
								95-2	0.1010	10								
								95-3	0.0990	10								
	6-Mar-19	6-Mar-19	40C	3 Week	1	18-0202-001-P86-1	RL	96-1	0.1076	10								
								96-2	0.1038	10								
								96-3	0.1075	10								
	13-Mar-19	13-Mar-19	40C	4 Week	1	18-0202-001-P86-1	RL	97-1	0.1043	10								
								97-2	0.1059	10								
								97-3	0.1014	10								
	13-May-19	14-May-19	40C	3 Mo	1	18-0202-001-P86-1	AT/MS	98-1	0.1000	10								
								98-2	0.0990	10								
								98-3	0.0990	10								
	13-Aug-19	12-Aug-19	40C	6Mo	1	18-0202-001-P86-1	RL	99-1	0.1030	10								
								99-2	0.1080	10								
								99-3	0.1020	10								
DUS1906	20-Feb-19	20-Feb-19	40C	1 Week	2	18-0202-001-P86-2	RL	119-1	0.1021	10								
								119-2	0.1061	10								
								119-3	0.1070	10								
	27-Feb-19	27-Feb-19	40C	2 Week	2	18-0202-001-P86-2	AT	120-1	0.1000	10								
								120-2	0.0990	10								
								120-3	0.1000	10								
	6-Mar-19	6-Mar-19	40C	3 Week	2	18-0202-001-P86-2	RL	121-1	0.1006	10								
								121-2	0.1072	10								
								121-3	0.1044	10								
	13-Mar-19	13-Mar-19	40C	4 Week	2	18-0202-001-P86-2	RL	122-1	0.1033	10								
								122-2	0.1072	10								
								122-3	0.1032	10								
	13-May-19	14-May-19	40C	3 Mo	2	18-0202-001-P86-2	AT/MS	123-1	0.0990	10								
								123-2	0.1010	10								
								123-3	0.1010	10								
	13-Aug-19	12-Aug-19	40C	6 Mo	2	18-0202-001-P86-2	AT	124-1	0.0980	10								
								124-2	0.1030	10								
								124-3	0.0990	10								

(b) (4)

	DY 21								
	CFU/mL			CFU/g					
C	A	B	C	A	B	C	Avg. CFU/g	Std. Dev.	CV

(b) (4)

											DY21 Plating CFU Counts, CFU/100uL							
											1E-04			1E-05			1E-06	
	Scheduled Pull Date	Date Sample Was Pulled	Testing Condition	Timepoint	Batch Number	Lot Number	Analyst Initials	Sample	Mass (g)	Volume 1X PBST (mL)	A	B	C	A	B	C	A	B
DUS1909	20-Feb-19	20-Feb-19	40C	1 week	3	18-0202-001-P87-1	RL	144-1	0.1026	10								
								144-2	0.1046	10								
								144-3	0.1027	10								
	27-Feb-19	27-Feb-19	40C	2 week	3	18-0202-001-P87-1	AT	145-1	0.0990	10								
								145-2	0.0980	10								
								145-3	0.1000	10								
	6-Mar-19	6-Mar-19	40C	3 week	3	18-0202-001-P87-1	RL	146-1	0.1095	10								
								146-2	0.1039	10								
								146-3	0.1091	10								
	13-Mar-19	13-Mar-19	40C	4 week	3	18-0202-001-P87-1	RL	147-1	0.1054	10								
								147-2	0.1012	10								
								147-3	0.1064	10								
	13-May-19	14-May-19	40C	3 Mo	3	18-0202-001-P87-1	AT/MS	148-1	0.1000	10								
								148-2	0.1000	10								
								148-3	0.1010	10								
	13-Aug-19	12-Aug-19	40C	6 Mo	3	18-0202-001-P87-1	AT/RL	149-1	0.1040	10								
								149-2	0.1020	10								
								149-3	0.0980	10								

(b) (4)

							DY21 Plating CFU Counts, CFU/100uL				
							1E-04		1E-05	1E-06	
Date of assay	Timepoint	Batch Number	Lot Number	Analyst Initials	Sample	Mass (g)	Volume 1X PBST (mL)				
29-Jan-19	T0	1	18-0202-001-P86-1	AT	86_1-1	0.1012	10				
					86_1-2	0.1018	10				
					86_1-3	0.1015	10				
29-Jan-19	T0	2	18-0202-001-P86-2	AT	86_2-1	0.1008	10				
					86_2-2	0.1011	10				
					86_2-3	0.1008	10				
29-Jan-19	T0	3	18-0202-001-P87-1	AT	87_1-1	0.1016	10				
					87_1-2	0.1012	10				
					87_1-3	0.1012	10				

(b) (4)

	DY 21								
	CFU/mL			CFU/g					
C	A	B	C	A	B	C	Avg. CFU/g	Std. Dev.	CV
(b) (4)									

CFU/mL		CFU/g					
B	C	A	B	C	Avg. CFU/g	Std. Dev.	CV
(b) (4)							

Appendix 3. *Pichia kudriavzevii* ASCUSDY21 Encapsulate Microbe Enumeration Method V4

ASCUS BIOSCIENCES

Method

Title	DY21-POE Microbe Enumeration
Version	04
Effective Date	01Jul2019
Author	Miranda Striluk
Approver (Signature & Date)	Adam Taylor 6/26/2019 Adam Taylor – Ascus Technical

Scope

The purpose of this assay is to determine the number of viable cells of Dairy-21 in Dairy-21 Palm Oil Encapsulate by counting colony forming units (CFU) on solid media.

Safety

Consult the Safety Data Sheet for all reagents prior to handling. Use caution in working with a hot water bath, hot liquids, liquid nitrogen, and extremely cold material. Liquid nitrogen can cause cold burns, frostbite, and permanent eye damage from brief exposure. Avoid skin and eye contact with liquid nitrogen and wear appropriate personal protective equipment (safety glasses and gloves) at all times. Analyst should be trained on liquid nitrogen handling before continuing this method.

Materials

Corning® 15mL Polypropylene Centrifuge Tubes (Corning 430052)
Petri dishes, 100x15 mm, sterile
Test tubes, 13x100 mm, sterile
Test tube cap, 16 mm, polypropylene
1.5 mL polypropylene microcentrifuge tube with snap cap
1000 µL Pipette
200 µL Pipette
1000 µL pipette tips, sterile
200 µL pipette tips, sterile
Glass beads, 3 mm, sterile, new

Equipment

Laboratory Vortexer
Class I/II Biosafety Cabinet
pH meter
Mortar and Pestle
Magnetic Stir Plate

Media & Reagents

BD® Difco® Yeast Peptone Dextrose Broth (BD 242810)
Growcells 10X Phosphate Buffered Saline pH 7.4 (PBS), sterile (Growcells MRGF-6235)
Growcells 1X Phosphate Buffered Saline with 0.05% TWEEN pH 7.4, sterile (Growcells MRGF-6275)
Spectrum® Agar, Powder, FCC (Spectrum A1672)
Reagent grade 95% Ethanol
70% Ethanol
10% Bleach
Liquid Nitrogen
1N Hydrochloric Acid
1N Sodium Hydroxide

Confidential Page 1 of 5

DY21-POE Microbe Enumeration

Method

1. Prepare Yeast Peptone Dextrose (YPD) agar plates. This step should be performed at least 24 hours prior to commencement of testing.

(b) (4)

2. Preparation of sterile 1X Phosphate Buffered Saline (PBS), pH 7.4

(b) (4)

3. De-encapsulation of Spray Congealed DY21-POE

(b) (4)

Confidential

DY21-POE Microbe Enumeration

(b) (4)

4. Prepare the Primary Dilution Mix

(b) (4)

5. DY21-POE Aerobic Plating

(b) (4)

Confidential

Page 3 of 5

DY21-POE Microbe Enumeration

6. Negative Control Plating

(b) (4)

7. Plate Counting

(b) (4)

Confidential

DY21-POE Microbe Enumeration

(b) (4)

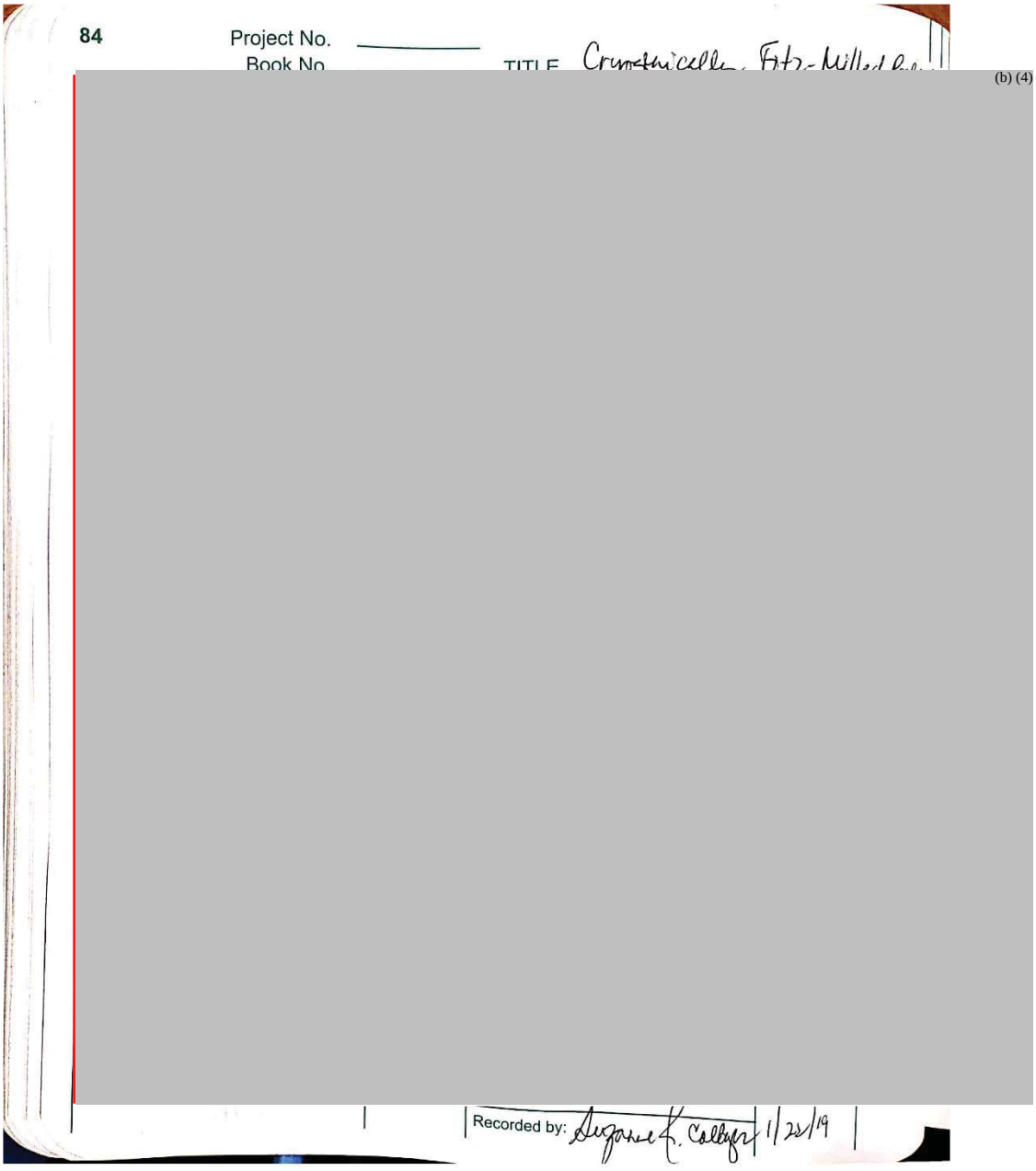
Reasons for Revision

(b) (4)

Confidential

Page 5 of 5

Appendix 4. Master Production Record for the 3 Lots



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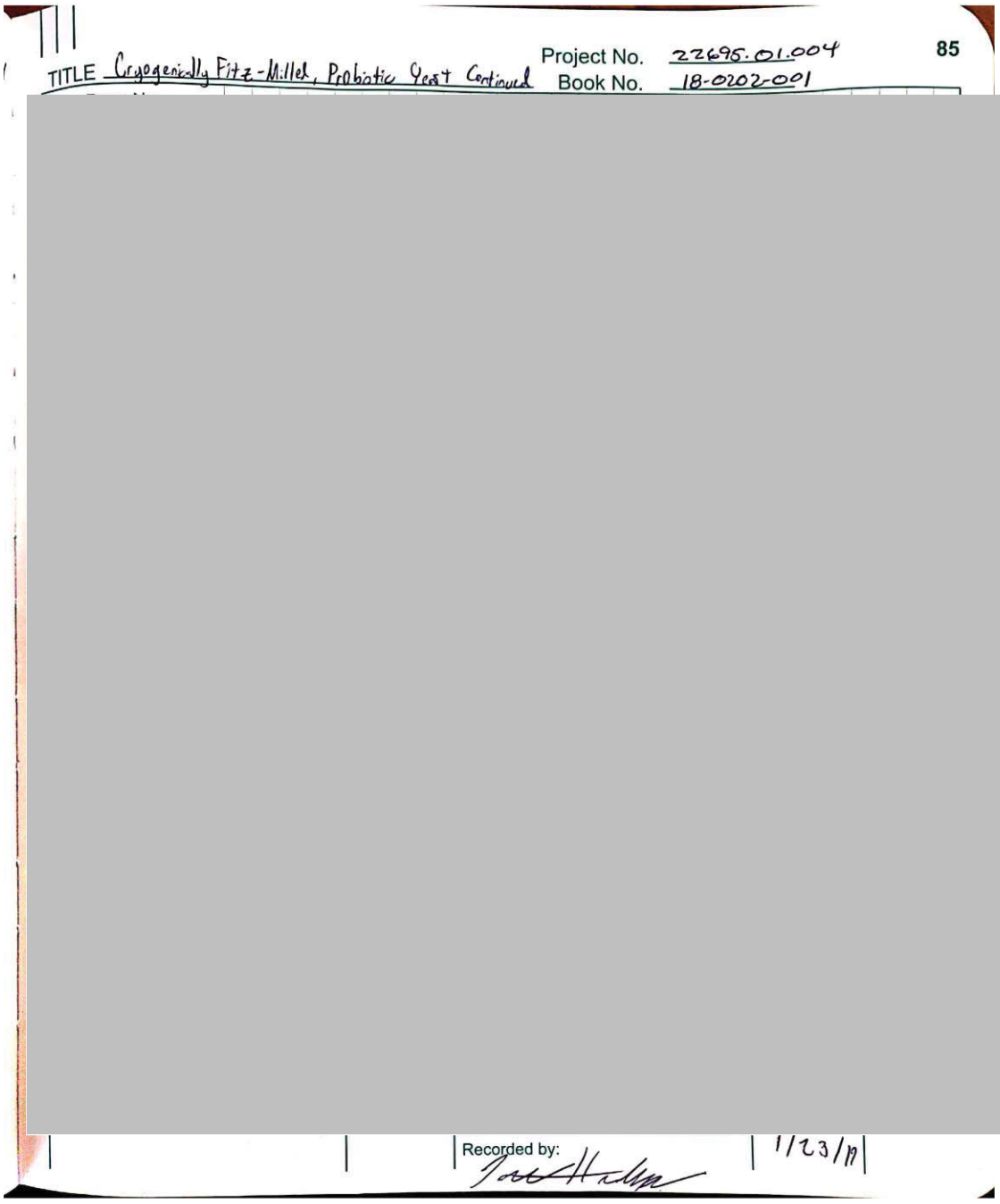
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Project No. 22695.01.004

Book No. 18-0202-001

85

(b) (4)

Recorded by:

1/23/19

86 Project No. 22695
Book No. 18-0202-001 TITLE Spray Congeals DY21

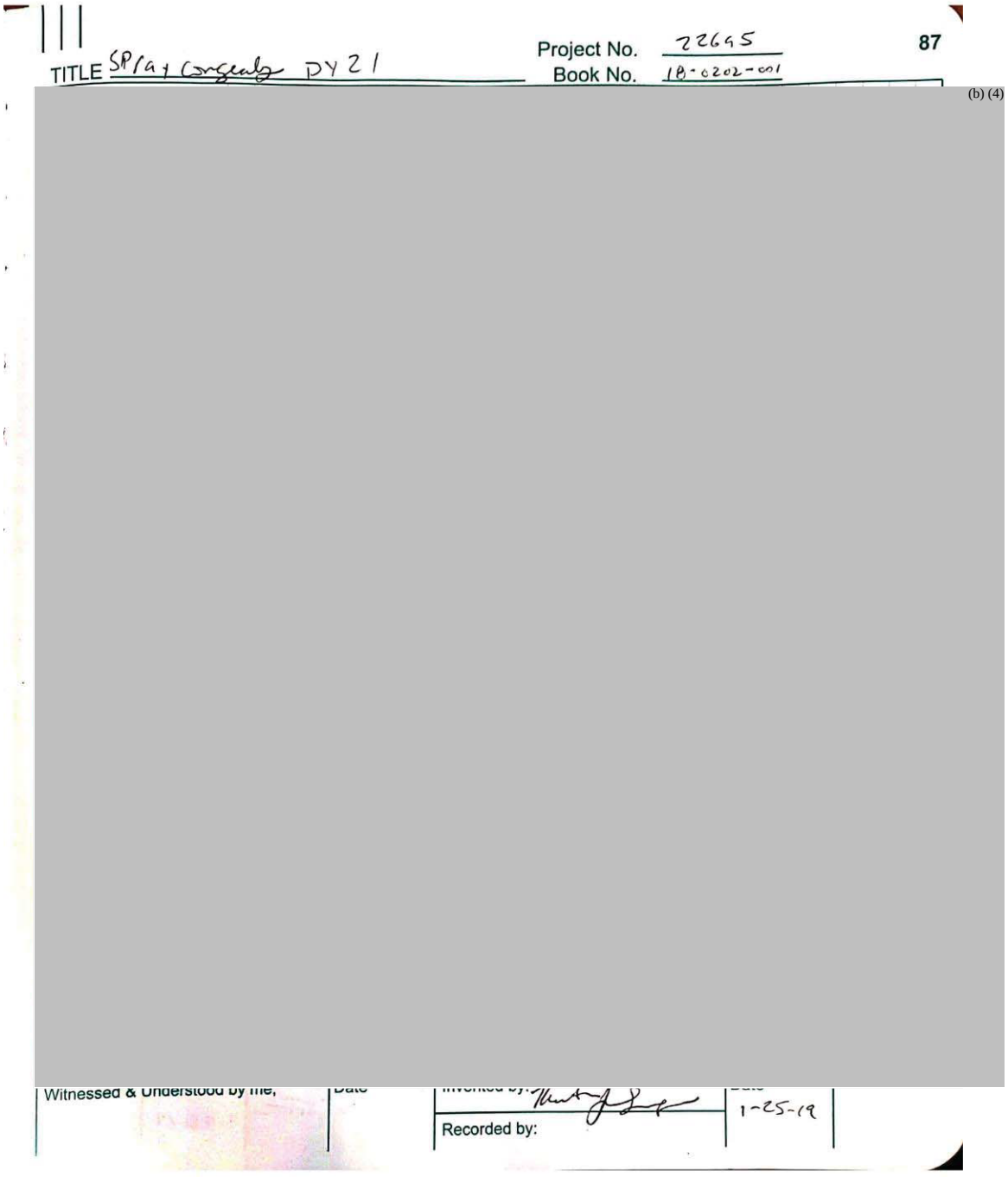
(b) (4)

[redacted]

Witnessed & Understood by me, Date

Invented by: Date 1-25-19

Recorded by:



TITLE Spray Congeal DY21 Project No. 22645 87
Book No. 18-0202-001

(b) (4)

Witnessed & Understood by me, Date

Recorded by: 1-25-19

Appendix 5. Original Four Week Stability Protocol for *Pichia kudriavzevii* ASCUSDY21 Encapsulate

Stability Protocol Title:	DY21 POE 40°C
Organism:	*Pichia kudriavzevii*
Purpose:	To support temperature excursions during shipping and storage
Number of Samples to Place on Stability:	8 (allows for retesting, when needed)
Sample Storage Container:	Heat sealed 48-gauge silver metalized PET / 2.5 mil LLDPE bags made from commercial bags
Temperature Conditions:	37-43°C
Acceptance Criteria:	>4 x 10^7 cfu/g

Tests and Timepoints:

Assay	T_0	1 week	2 weeks	3 weeks	4 weeks
Microbe Enumeration*	X	X	X	X	X

*DY21-POE Microbe Enumeration method

Approvals:

Howard Green Regulatory	DocuSigned by: Howard B Green 12/11/2018
Corey Dodge Process Development	DocuSigned by: Corey Dodge 12/5/2018 8:16:17 AM PST
Patricia A. Williams Quality	DocuSigned by: Patricia A Williams 12/4/2018 7:14:14 PM PST

unable to apply redactions

ASCUS Product Mix Uniformity Report for Protocol #1064

Approvers:

DocuSigned by:
Martin Mayhew
D1605F1B4C3E49A...

12/9/2019

Martin Mayhew
Vice President – Product Development & Manufacturing

Date

DocuSigned by:
Patricia A Williams
5B301285A10643D...

12/6/2019

Patricia A. Williams
Quality

Date

DocuSigned by:
Howard B Green
0FAA38037D49453...

12/6/2019

Howard B. Green
Regulatory

Date

Table of Contents

ASCUS Product Mix Uniformity Report for Protocol #1064

1 Objective

This homogeneity study was conducted to demonstrate that a mixture of *Clostridium beijerinckii* ASCUSDY20 and *Pichia kudriavzevii* ASCUSDY21 with diluents can be blended homogenously with premix at scale. The data will be used for regulatory agency submissions.

2 Diet Manufacturing

One dairy premix (formula number 420920M0309310 – Paulk mixer uniform) was used to determine the mixing homogeneity of an ASCUS Biosciences feed additive. A 1000 lb batch of the dairy premix was manufactured and used for the 3 replications. The basal diet did not contain the experimental test products. Feed was manufactured at the (b) (4) (b) (4). Whole grain ingredients were ground with a 3 high roller mill (Model 924). Ingredients were weighed on certified scales with lot numbers recorded, and amount was verified by the feed manufacturing investigator.

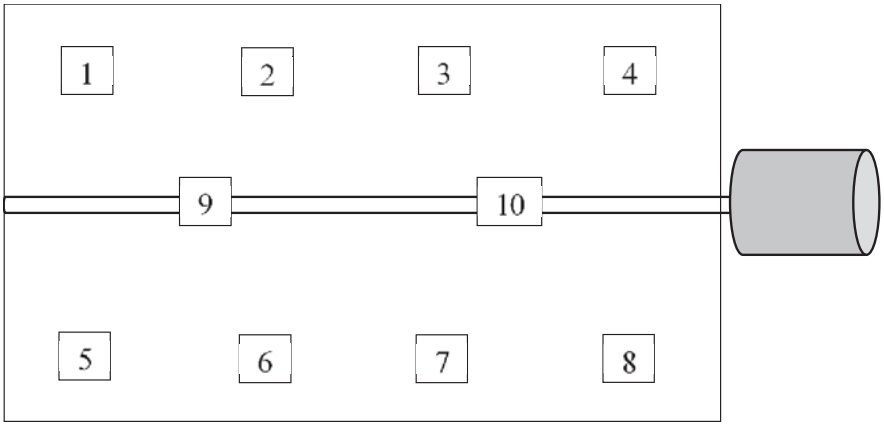
The basal mix (Appendix A) was manufactured in a 1 ton Hayes & Stolz Double Shaft Horizontal Mixer according to SOP #320. Salt and trace mineral salt were not included in the basal premix. Dry ingredients were added and mixed for 60 seconds at room temperature, followed by liquid ingredients for 120 seconds. The mixed feed was discharged and sacked off in 50 lb bags. The first and last bag of each batch were discarded, and the remaining bags were used for the mixer uniformity experiment. The batching data were recorded on the master formula sheet.

The fifty-pound bags of the basal dairy mix were used to produce the three 200 lb batches and added to a 200 lb mixer (Davis paddle mixer SS-S1; 6 cubic ft) for the study. The mixer type is representative of what is used in the dairy industry. The test article, Trace mineral (TM) salt and salt were added to the mixer and mixed for 300 seconds (SOP #857). After mixing time was complete, the mixer was turned off. A total of 10 samples were collected using a grain probe from 10 different locations in the mixer (Figure 1). Each of the 10 samples collected from the mixer was split in half using a riffle divider (Humbolt -H-3985 Sample splitter with removeable hopper, 12 chutes, 0.500” (12.70 mm). The backup individual samples will be analyzed (if necessary) using Quantab Cl titrators (Environmental Test Systems Inc., Elkhart, IN) at KSU and the remainder of the sample was shipped to ASCUS Biosciences for analysis of test article. The backup Ascus sample was held until confirmation that samples arrived at Ascus laboratory in (b) (4). The remaining feed was not fed and was destroyed.

3 Treatments

Diet Formulation	Rep/Blend
Lactation (Lact-6-1)	1
Lactation (Lact-6-1)	2
Lactation (Lact-6-1)	3

Figure 1. Sampling Locations



4 Shipping Addresses

1) Ten samples from each replication were sent to ASCUS Biosciences for analysis:

Adam Taylor
6450 Lusk Blvd
Suite E209
San Diego, Ca 92121
Phone: 707-601-2553
Fed Ex number is (b) (4)

5 Summary Report of Results from Homogeneity Study

5.1 Purpose

This homogeneity study was conducted to demonstrate that a mixture of *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21 with diluents can be blended homogenously with premix at scale. The data will be used for regulatory agency submissions.

Three separate blends of premix containing *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21 were generated at (b) (4) (b) (4) and tested at Ascus Biosciences using the method "Premix Testing of Galaxis, version 1" (Appendix D).

5.2 Assay Result Summarization

All samples were assayed in triplicate and the coefficient of variation was calculated by determining the average of triplicates for each sample point of a batch then determining the coefficient of variation of those ten samples.

5.3 Results

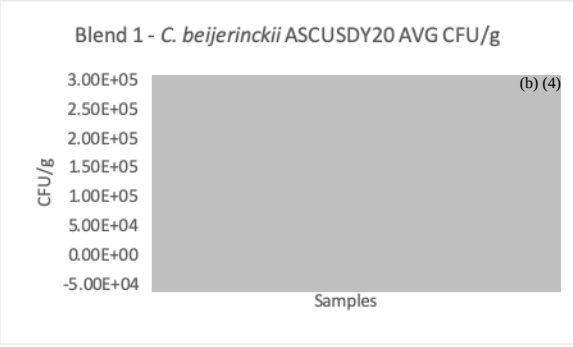
Table1. Results of 3 Blends of Galaxis 5 into Premix

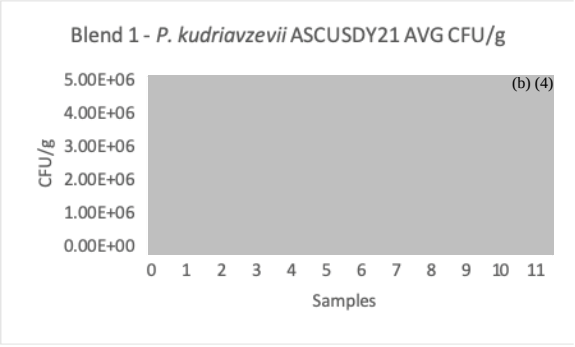
Note: Results are reported in average colony forming unites (CFU)/gram.

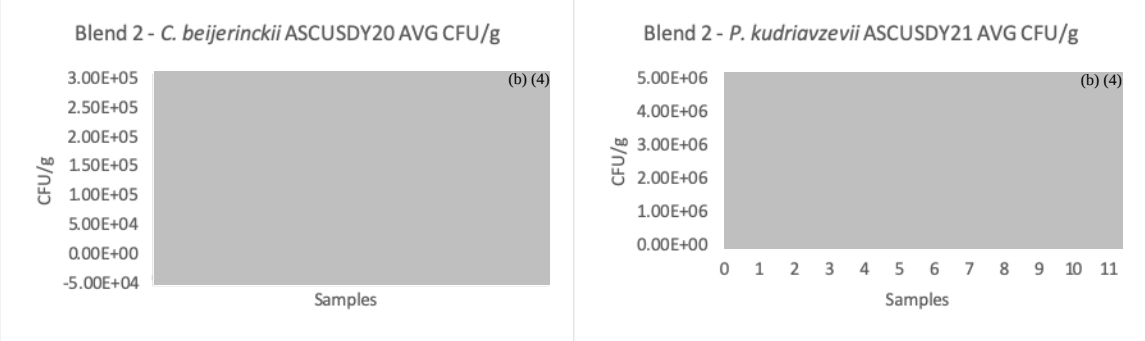
	***C. beijerinckii* ASCUSDY20**			***P. kudriavzevii* ASCUSDY21**		
	Final Result			**Final Result**		
	Avg CFU/g	**Std. Dev.**	**CV**	**Avg CFU/g**	**Std. Dev.**	**CV**
Blend 1	(b) (4)					
Blend 2						
Blend 3						

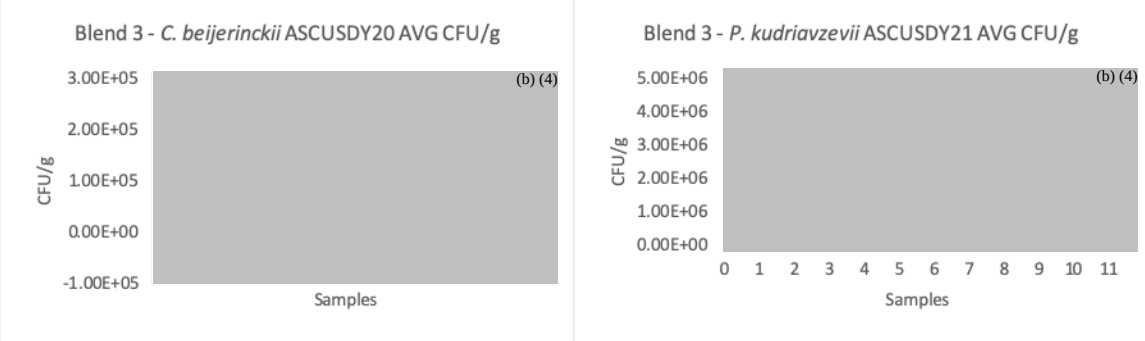
5.4 Analysis

The CV values for blend 1 are 33.38% and 24.67% respectively for *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21. The CV values for blend 2 are 61.78% and 29.69% respectively for *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21. The CV values for blend 3 are 42.69% and 28.08% respectively for *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21. Graphs of the *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21 for each blend are provided below.









5.5 Deviations and Changes

There were no deviations or changes from the protocol.

5.6 Conclusion

The protocol was executed as written and the results indicate that all 3 blends were homogenous, and the results pass per the acceptance criteria.

5.7 Appendices

Appendix A: Basal Mix

Appendix B: Basal Premix – Lot 20190729009280MM

Appendix C: Batch Sheets

Appendix D: Premix Testing of Galaxis, version 1 (Note: Galaxis is test name assigned to mixture of C. beijerinckii ASCUSDY20 and P. kudriavzevii ASCUSDY21 with calcium carbonate as carrier and hydrated sodium calcium aluminosilicate as anticaking agent)

Appendix E: Premix Testing of Galaxis 5 Method Validation Report (Note: Galaxis is test name assigned to mixture of C. beijerinckii ASCUSDY20 and P. kudriavzevii ASCUSDY21 with calcium carbonate as carrier and hydrated sodium calcium aluminosilicate as anticaking agent)

Appendix F: Excel of Data for Study

6.0 Reasons for Revision

- Addition of graphs and revision of raw data excel tables

Appendix A: Basal Mix

INGREDIENTS, %	Lactation
Premix	
Ground Corn	(b) (4)
Soy Plus	
Limestone	
Magnesium Oxide	
Vitamin E, 20,000 IU/lb	
Sodium Bicarbonate	
Megalac Essentiom	
Zinpro 120	
Zinpro 4 Plex	
Rumensin 90	
Lactation PMX	
Added with the test article	
Salt	
TM Salt	
Total	100

Activities on the day of mixing and sampling for the homogeneity study

1. (b) (4)
2. (b) (4)
3. (b) (4)

4. [redacted]

5. [redacted]

6. Once all ten samples were collected from the mixer locations, the remaining feed was collected and destroyed. The mixer was cleaned by sweeping clean then using air from compressor to blow remaining dust and feed from mixer. The mixer was inspected and bottom discharge was closed.

7. Steps 1-6 above were repeated for a total of three batches. Samples for ASCUS Biosciences were placed on ice and shipped overnight to [redacted] laboratory. Samples for salt determination (if needed) were taken by [redacted] for holding and the extra Ascus sample was put in box for storage in refrigerator. Batch sheets are presented in Appendix C.

8. Samples arrived in [redacted] and were analyzed at ASCUS Biosciences.

Appendix B: Basal Premix – Lot 20190729009280MM

(b) (4)

7/29/19 15:33

BATCH RUN SUMMARY REPORT

System: BATCHING Run ID: 29063

20190729009280MM **Formula:** 420920M030931O ~ Paulk Mixer Uniform **Work Order #:** 29955
Destination: 601 **Oper:**
Start Time: 7/29/19 10:58 **End Time:** 7/29/19 15:39 **Number of Batches:** 1 **Batch Size:** 1000 lb

Item Code	Description	Lot Code	Source Equip	Target Quantity	UOM	Actual Quantity	UOM	Dev. %	Comments
HANDADD SCALE						(b) (4)			
32007	MEGALAC R		WAREH(	25.01	lb				
52275	SODIUM BICARBONATE		WAREH(	25.01	lb				
55300	ZINPRO 4 PLEX		WAREH(	0.83	lb				
86001	Zinpro 120		WAREH(	0.43	lb				
92310	RUMENSIN 90		WAREH(	0.23	lb				
		Total for HANDADD SCALE:		**51.49**	**lb**				
MAJOR SCALE									
11102	GROUND CORN		304	661.00	lb				
41201	Soy Plus		315	237.01	lb				
52120	LIMESTONE		302	36.69	lb				
		Total for MAJOR SCALE:		**934.69**	**lb**				
MICRO SCALE									
52150	MAGNESIUM OXIDE		009	8.51	lb				
73100	VITAMIN E 20,000		006	5.32	lb				
		Total for MICRO SCALE:		**13.82**	**lb**				
		Total for 20190729009280MM:		**1,000.00**	**lb**				

Run Time: 04:40:57 **Down Time:** 00:00:00 **Total Time:** 04:40:57

(b) (4)

Page 1 of 1

FILE: 0000029063.001

RECIPE: 420920M03093 REVISION#: 1 DESC: Paulk Mixer LOT: 20190729009280MM B
RUN ID: 29063 SALES ORDER: (none) OPERATOR: F

MATERIAL	DESCRIPTION	BIN	TARGET	ACTUAL	UOM	DEV.	DEV%	OVERRIDE
11102	GROUND CORN	304	660.99	[redacted] (b) (4)				
73100	VITAMIN E 20,00	006	5.32					
41201	Soy Plus	315	237.01					
52120	LIMESTONE	302	36.69					
52150	MAGNESIUM OXIDE	009	8.51					
52275	SODIUM BICARBON	WAREHO	25.01					
32007	MEGALAC R	WAREHO	25.01					
55300	ZINPRO 4 PLEX	WAREHO	0.83					
86001	Zinpro 120	WAREHO	0.43					
92310	RUMENSIN 90	WAREHO	0.23					
TOTALS			1014.17					

BATCH START TIME 2019-07-29 15:31:06
MIXER DISCHARGE TIME 2019-07-29 15:39:23
DESTINATION: 601 BATCH SIZE: 1000 lb

	TARGET	ACTUAL		TARGET	ACTUAL
WET MIX TIME:	120	0	DRY WIX TIME:	60	180

(b) (4)

(b) (4) roduction Sheet
7/29/2019 10:59:46AM

Run ID: 29,063
System: BATCHING
Formula: 420920M030931O
Paulk Mixer Uniform
Batches: 1
Batch Size: 1,000

Item	Target Weight	Batch	Actual Weight	Lot
32007 MEGALAC R Active Lot: (None)	25.005	1	✓	
52275 SODIUM BICARBONATE Active Lot: (None)	25.005	1	✓	
55300 ZINPRO 4 PLEX Active Lot: (None)	0.830	1	✓	
86001 Zinpro 120 Active Lot: (None)	0.425	1	✓	
92310 RUMENSIN 90 Active Lot: (None)	0.225	1	✓	C93111

(b) (4)

Page 1

Appendix C: Batch Sheets

(b) (4)

Master Formula Sheet

Formula Number	420920M030931O	PI	
Formula Name/Label	Batch 1	Account	
Number of Batches		Feed Form (M/P/C)	
Batch Size	200	Packaging (Bag/Bulk)	

Ingredient		Amount	Verified
Scale	Description	(b) (4)	
HA	Paulk Mixer Uniform (Lact 6-1-18)		
HA	Salt		
HA	TM Salt		
HA	Lactation PMX		
HA	Test Ingredient Lot AD 50-14		
	Total	200	

Special Instructions:

PROJECT LEADER APPROVAL

(b) (4)

Signature _____ Date 07-30-19

(b) (4)

(b) (4)

Master Formula Sheet

Formula Number	420920M030931O	PI	
Formula Name/Label	Batch 2	Account	
Number of Batches		Feed Form (M/P/C)	
Batch Size	200	Packaging (Bag/Bulk)	

Ingredient		Amount	Verified
Scale	Description	(lbs)	Manager
HA	Paulk Mixer Uniform (Lact 6-1-18)	(b) (4)	
HA	Salt		
HA	TM Salt		
HA	Lactation PMX		
HA	Test Ingredient Lot AJ-SD-14		
	Total	200	

Special Instructions:

PROJECT LEADER APPROVAL

Signature (b) (4) Date 7-30-15

(b) (4)

(b) (4)

Master Formula Sheet

Formula Number	420920M030931O	PI	
Formula Name/Label	BATCH 3	Account	
Number of Batches		Feed Form (M/P/C)	
Batch Size	200	Packaging (Bag/Bulk)	

Ingredient		Amount	Verified
Scale	Description	(lbs)	Manager
HA	Paulk Mixer Uniform (Lact 6-1-18)	(b) (4)	
HA	Salt		
HA	TM Salt		
HA	Lactation PMX		
HA	Test Ingredient		
	Total	200	

Special Instructions:

PROJECT LEADER APPROVAL

Signature (b) (4) Date 07-30-19

(b) (4)

Appendix D: Premix Testing of Galaxis, version 1

ASCUS BIOSCIENCES

Method

Title	Premix Testing of Galaxis
Version	01
Effective Date	30Jul2019
Author	Adam Taylor
Approver (Signature & Date)	Rich La – Ascus Technical

Scope

The purpose of this assay is to determine the number of viable spores of Dairy-20 and cells of Dairy-21 in Altius and Galaxis premixes containing any or all of the following ingredients:

- Corn products
- Soy products
- Limestone

Safety

Consult the Safety Data Sheet for all reagents prior to handling. Use caution in working with a hot water bath, hot liquids, liquid nitrogen, and extremely cold material. Liquid nitrogen can cause cold burns, frostbite, and permanent eye damage from brief exposure. Avoid skin and eye contact with liquid nitrogen and wear appropriate personal protective equipment (safety glasses and gloves) at all times. Analyst should be trained on liquid nitrogen handling before continuing this method.

Materials

BD GasPak™ EZ large incubation container (BD 260672)
BD GasPak™ EZ anaerobe container system sachets (BD 260678)
Petri dishes, 100x15 mm, sterile
Test tubes, 13x100 mm, sterile
Test tube cap, 16 mm, polypropylene

Premix Testing of Galaxis

1.5 mL polypropylene microcentrifuge tube with snap cap
1000 µL Pipette
200 µL Pipette
1000 µL pipette tips, sterile
200 µL pipette tips, sterile
New glass beads, 3 mm, sterile

Equipment

Water bath set to 50°C
Laboratory Vortexer
Class I/II Biosafety Cabinet
pH meter
Mortar and Pestle

Media & Reagents

NOTE: *Comparable quality ingredients (Laboratory, NF, USP, Reagent, or ACS grade) from different suppliers may be used.*

BD® Bacto™ Tryptic Soy Broth (BD 211822)
BD® Difco® Yeast Peptone Dextrose Broth (BD 242810)
Growcells 1X Phosphate Buffered Saline pH 7.4 (PBS), sterile (Growcells MRGF-6230)
Growcells 1X Phosphate Buffered Saline with 0.05% TWEEN pH 7.4, sterile (Growcells MRGF-6275)
Growcells 10X Phosphate Buffered Saline pH 7.4 (PBS), sterile (Growcells MRGF-6235)
Research Products International Corp. GELRITE (Fisher Scientific 50-488-682)
Sigma-Aldrich® Resazurin sodium salt (Sigma-Aldrich R7017)
Spectrum® Alpha Lipoic Acid, USP (Spectrum L1506)
Spectrum® Aminobenzoic Acid, USP (Spectrum AM150)
Spectrum® Agar, Powder, FCC (Spectrum A1672)
Spectrum® Biotin, Powder, USP (Spectrum B1103)
Spectrum® L-Cysteine Hydrochloride, Monohydrate (Spectrum CY115)

Premix Testing of Galaxis

Spectrum® Ferric Ammonium Citrate, Brown, Powder, FCC (Spectrum F1000)
Spectrum® Folic Acid, Powder, USP (Spectrum FO105)
Spectrum® Niacin, Powder, USP (Spectrum NI100)
Spectrum® Phytonadione, USP (Spectrum PH195)
Spectrum® Pyridoxine Hydrochloride, USP (Spectrum PY103)
Spectrum® Riboflavin, USP (Spectrum RI103)
Spectrum® Thiamine Hydrochloride, FCC (Spectrum T1053)
Spectrum® DL-Pantothenic Acid Calcium Salt (Spectrum P2630) or Calcium Pantothenate (Spectrum CA159)
Spectrum® Vitamin B12, FCC (Spectrum C1454)
Spectrum® Polysorbate 20, FCC (Spectrum P1177)
Reagent grade 95% Ethanol
70% Ethanol
Liquid Nitrogen

Method

1. Prepare Tryptic Soy Broth and Ferric Ammonium Citrate Gelrite (TSB+FAC) Plates. This step should be performed at least 24 hours prior to commencement of testing.

(b) (4)

Ingredient	Amount/ liter
Pyridoxine Hydrochloride	(b) (4)
p- Aminobenzoic Acid	
Alpha Lipoic Acid	

Premix Testing of Galaxis

Nicotinic acid	(b) (4)
Riboflavin	
Thiamine Hydrochloride	
Calcium Pantothenate	
Biotin, Powder	
Folic Acid, Powder	
Vitamin B12	
diH2O	

(b) (4)

Premix Testing of Galaxis

(b) (4)

2. Prepare Yeast Peptone Dextrose (YPD) Agar Plates. This step should be performed at least 24 hours prior to commencement of testing.

(b) (4)

Premix Testing of Galaxis

(b) (4)

3. Prepare Sterile 1X Phosphate Buffered Saline (PBS), pH 7.4 with 0.05% Polysorbate 20

(b) (4)

Premix Testing of Galaxis

4. De-Encapsulate Spray Congealed Dairy-21

(b) (4)

5. Prepare the Primary Dilution Mix

(b) (4)

6. Heat Shock Anaerobic Plating

(b) (4)

Premix Testing of Galaxis

(b) (4)

Premix Testing of Galaxis

(b) (4)

7. Aerobic Plating

(b) (4)

Premix Testing of Galaxis

(b) (4)

8. Plate Counting and Calculations

(b) (4)

Premix Testing of Galaxis

(b) (4)

Reasons for Revision

1. Initial version (which addresses low plate counts for Dairy-20 during the method validation).

Appendix E: Premix Testing of Galaxis 5 Method Validation Report

ASCUS BIOSCIENCES

Premix Testing of Galaxis Method Validation Summary Report

Objective:
The objective of this validation protocol was to demonstrate the repeatability, robustness, and specificity of the method "Premix Testing of Galaxis".

Results:
The following parameters were tested in the validation.

Repeatability: Closeness of results obtained on the same sample of blended premix with Galaxis (premix #1) when assayed multiple times by the same person with the same reagents and equipment.

Robustness: Reliability of the method to withstand small variations such as different technicians and reagent preparations on the 3 blended premixes with Galaxis (premix #1, premix #3, premix #4).

Specificity: Accuracy of detection for DY20 and DY21 when mixed with different premixes.

A summary of the CFU results are shown in the table below. All samples fit the criteria for validation with the coefficient of variation of 75% or less.

ASCUS BIOSCIENCES

Analyst 1 Summary								
	DY20				DY21			
Sample Description	Avg. CFU/g	Avg. CFU/g	Std. Dev.	CV	Avg. CFU/g	Avg. CFU/g	Std. Dev.	CV
Sample 1A	1.50E+04							
	1.90E+04							
	1.75E+04							
Sample 1B	2.50E+04							
	2.32E+04							
	1.26E+04							
Sample 1C	2.22E+04							
	2.30E+04							
	1.50E+04							
Sample 2	1.85E+04							
	2.69E+04							
	1.54E+04							
Sample 3	2.20E+04							
	1.33E+04							
	3.91E+04							

(b) (4)

ASCUS BIOSCIENCES

Analyst 2 Summary								
	DY20				DY21			
Sample Description	Avg. CFU/g	Avg. CFU/g	Std. Dev.	CV	Avg. CFU/g	Avg. CFU/g	Std. Dev.	CV
Sample 1	2.75E+04							
	4.40E+04							
	5.96E+04							
Sample 2	5.24E+04							
	3.78E+04							
	5.89E+04							
Sample 3	5.10E+04							
	3.58E+04							
	4.74E+04							

(b) (4)

Repeatability:
From Analyst 1, the average CFU/g of samples 1A, 1B, and 1C is 1.92E4 and 3.04E6 with standard deviations of 7.72E3 and 9.97E5 for DY20 and DY21 respectively. The coefficient of variance for these samples was 9% and 33% for DY20 and DY21 respectively. These results indicate that the assay is repeatable.

Robustness:
All samples tested by both analysts had CVs less than 75%, indicating low variation of results when slight variations of the method occur.

Specificity:
DY20 and DY21 were able to be detected by both analysts when mixed into these different types of premixes.

ASCUS BIOSCIENCES

Deviations:
There was a dose change from 1g Galaxis 5 per pound of premix to 5g Galaxis 5 per pound of premix to more accurately reflect dosing on farms. This only impacted the sample preparation and no deviation is required.

Conclusion:
Repeatability, robustness, and specificity were tested for the Premix Testing of Galaxis method, and in all parameters this method validation has passed. The method is validated for the testing of premix (containing corn, soy, and /or limestone materials) and Galaxis at a dose of approximately 5g/pound.

The method will be approved.

Raw data is included in the executed protocol and can be found on the Ascus Biosciences Google Drive.

Approvers:

Rich La Ascus Technical	DocuSigned by: Rich La C4F920E8CAE2489...	7/29/2019
Patricia A. Williams Quality	DocuSigned by: Patricia Williams 5B301285A10643D...	7/29/2019

Appendix F: Excel Data for Study

Blend 1								DY20 Anaerobic Plating, CFU/100uL								
								1E+00			1E-01			1E-02		
Sample Description	Sample	Test Tube + Cap (g)	Sample + Tube & Cap (g)	Actual Mass (g)	Volume PBST (mL)	Heat Shock Start	Heat Shock End	A	B	C	A	B	C	A	B	C
1	PDM-1	6.287	7.290	1.003	10	11:16AM	11:26AM	(b) (4)								
	PDM-2	6.325	7.356	1.031	10	11:16AM	11:26AM									
	PDM-3	6.278	7.339	1.061	10	11:16AM	11:26AM									
2	PDM-1	6.248	7.259	1.011	10	11:16AM	11:26AM									
	PDM-2	6.254	7.261	1.007	10	11:16AM	11:26AM									
	PDM-3	6.386	7.427	1.041	10	11:16AM	11:26AM									
3	PDM-1	6.339	7.354	1.015	10	11:16AM	11:26AM									
	PDM-2	6.213	7.241	1.028	10	11:16AM	11:26AM									
	PDM-3	6.316	7.338	1.022	10	11:16AM	11:26AM									
4	PDM-1	6.242	7.277	1.035	10	11:16AM	11:26AM									
	PDM-2	6.210	7.263	1.053	10	11:16AM	11:26AM									
	PDM-3	6.312	7.354	1.042	10	11:16AM	11:26AM									
5	PDM-1	6.572	7.618	1.046	10	12:19PM	12:29PM									
	PDM-2	6.620	7.628	1.008	10	12:19PM	12:29PM									
	PDM-3	6.534	7.584	1.050	10	12:19PM	12:29PM									
6	PDM-1	6.628	7.669	1.041	10	12:19PM	12:29PM									
	PDM-2	6.594	7.595	1.001	10	12:19PM	12:29PM									
	PDM-3	6.551	7.594	1.043	10	12:19PM	12:29PM									
7	PDM-1	6.628	7.647	1.019	10	12:19PM	12:29PM									
	PDM-2	6.638	7.644	1.006	10	12:19PM	12:29PM									
	PDM-3	6.559	7.626	1.067	10	12:19PM	12:29PM									
8	PDM-1	6.481	7.497	1.016	10	1:14PM	1:24PM									
	PDM-2	6.596	7.601	1.005	10	1:14PM	1:24PM									
	PDM-3	6.518	7.544	1.026	10	1:14PM	1:24PM									
9	PDM-1	6.551	7.548	0.997	10	1:14PM	1:24PM									
	PDM-2	6.499	7.511	1.012	10	1:14PM	1:24PM									
	PDM-3	6.515	7.544	1.029	10	1:14PM	1:24PM									
10	PDM-1	6.710	7.719	1.009	10	1:14PM	1:24PM									
	PDM-2	6.597	7.615	1.018	10	1:14PM	1:24PM									
	PDM-3	6.667	7.667	1.000	10	1:14PM	1:24PM									

Table 1. *C. beijerinckii* ASCUSDY20 - Blend 1 Data

DY20 Analysis												
CFU/mL			CFU/mL			CFU/mL						
A	B	C	A	B	C	A	B	C	CFU/g	Avg. CFU/g	Std. Dev.	CV
(b) (4)												

Table 2. *C. beijerinckii* ASCUSDY20 - Blend 1 Analysis

Blend 1								DY21 Aerobic Plating, CFU/100uL								
								1E-01			1E-02			1E-03		
Sample Description	Sample	Test Tube + Cap (g)	Sample + Tube & Cap (g)	Actual Mass (g)	Volume PBST (mL)	Heat Shock Start	Heat Shock End	A	B	C	A	B	C	A	B	C
1	PDM-4	6.620	7.643	1.023	10	N/A	N/A	(b) (4)								
	PDM-5	6.652	7.671	1.019	10	N/A	N/A									
	PDM-6	6.695	7.736	1.041	10	N/A	N/A									
2	PDM-4	6.577	7.608	1.031	10	N/A	N/A									
	PDM-5	6.549	7.570	1.021	10	N/A	N/A									
	PDM-6	6.529	7.621	1.092	10	N/A	N/A									
3	PDM-4	6.734	7.745	1.011	10	N/A	N/A									
	PDM-5	6.890	7.914	1.024	10	N/A	N/A									
	PDM-6	6.793	7.817	1.024	10	N/A	N/A									
4	PDM-4	6.802	7.809	1.007	10	N/A	N/A									
	PDM-5	6.797	7.824	1.027	10	N/A	N/A									
	PDM-6	6.732	7.780	1.048	10	N/A	N/A									
5	PDM-4	6.715	7.808	1.093	10	N/A	N/A									
	PDM-5	6.706	7.744	1.038	10	N/A	N/A									
	PDM-6	6.735	7.744	1.009	10	N/A	N/A									
6	PDM-4	6.651	7.734	1.083	10	N/A	N/A									
	PDM-5	6.779	7.795	1.016	10	N/A	N/A									
	PDM-6	6.734	7.777	1.043	10	N/A	N/A									
7	PDM-4	6.762	7.792	1.030	10	N/A	N/A									
	PDM-5	6.774	7.833	1.059	10	N/A	N/A									
	PDM-6	6.772	7.854	1.082	10	N/A	N/A									
8	PDM-4	6.491	7.499	1.008	10	N/A	N/A									
	PDM-5	6.611	7.606	0.995	10	N/A	N/A									
	PDM-6	6.618	7.630	1.012	10	N/A	N/A									
9	PDM-4	6.660	7.669	1.009	10	N/A	N/A									
	PDM-5	6.520	7.528	1.008	10	N/A	N/A									
	PDM-6	6.533	7.533	1.000	10	N/A	N/A									
10	PDM-4	6.451	7.452	1.001	10	N/A	N/A									
	PDM-5	6.778	7.786	1.008	10	N/A	N/A									
	PDM-6	6.669	7.676	1.007	10	N/A	N/A									

Table 3. *P. kudriavzevii* ASCUSDY21- Blend 1 Data

DY 21 Analysis												
CFU/mL			CFU/mL			CFU/mL						
A	B	C	A	B	C	A	B	C	CFU/g	Avg. CFU/g	Std. Dev.	CV
(b) (4)												

Table 4. *P. kudriavzevii* ASCUSDY21- Blend 1 Analysis

Blend 2								DY20 Anaerobic Plating, CFU/100uL								
								1E+00			1E-01			1E-02		
Sample Description	Sample	Test Tube + Cap (g)	Sample + Tube & Cap (g)	Actual Mass (g)	Volume PBST (mL)	Heat Shock Start	Heat Shock End	A	B	C	A	B	C	A	B	C
1	PDM-1	6.635	7.689	1.054	10	3:15PM	3:25PM	(b) (4)								
	PDM-2	6.561	7.650	1.089	10	3:15PM	3:25PM									
	PDM-3	6.582	7.610	1.028	10	3:15PM	3:25PM									
2	PDM-1	6.632	7.648	1.016	10	3:15PM	3:25PM									
	PDM-2	6.572	7.616	1.044	10	3:15PM	3:25PM									
	PDM-3	6.576	7.607	1.031	10	3:15PM	3:25PM									
3	PDM-1	6.635	7.679	1.044	10	3:15PM	3:25PM									
	PDM-2	6.589	7.592	1.003	10	3:15PM	3:25PM									
	PDM-3	6.575	7.673	1.098	10	3:15PM	3:25PM									
4	PDM-1	6.588	7.616	1.028	10	3:15PM	3:25PM									
	PDM-2	6.559	7.634	1.075	10	3:15PM	3:25PM									
	PDM-3	6.573	7.602	1.029	10	3:15PM	3:25PM									
5	PDM-1	6.577	7.629	1.052	10	3:15PM	3:25PM									
	PDM-2	6.628	7.675	1.047	10	3:15PM	3:25PM									
	PDM-3	6.556	7.559	1.003	10	3:15PM	3:25PM									
6	PDM-1	6.641	7.736	1.095	10	3:15PM	3:25PM									
	PDM-2	6.631	7.729	1.098	10	3:15PM	3:25PM									
	PDM-3	6.563	7.653	1.090	10	3:15PM	3:25PM									
7	PDM-1	6.580	7.662	1.082	10	3:04PM	3:14PM									
	PDM-2	6.584	7.620	1.036	10	3:04PM	3:14PM									
	PDM-3	6.569	7.646	1.077	10	3:04PM	3:14PM									
8	PDM-1	6.587	7.596	1.009	10	3:04PM	3:14PM									
	PDM-2	6.592	7.627	1.035	10	3:04PM	3:14PM									
	PDM-3	6.612	7.673	1.061	10	3:04PM	3:14PM									
9	PDM-1	6.613	7.668	1.055	10	3:04PM	3:14PM									
	PDM-2	6.626	7.619	0.993	10	3:04PM	3:14PM									
	PDM-3	6.613	7.632	1.019	10	3:04PM	3:14PM									
10	PDM-1	6.726	7.742	1.016	10	3:04PM	3:14PM									
	PDM-2	6.719	7.756	1.037	10	3:04PM	3:14PM									
	PDM-3	6.627	7.704	1.077	10	3:04PM	3:14PM									

Table 5. *C. beijerinckii* ASCUSDY20 – Blend 2 Data

DY20 Analysis												
CFU/mL			CFU/mL			CFU/mL						
A	B	C	A	B	C	A	B	C	CFU/g	Avg. CFU/g	Std. Dev.	CV
(b) (4)												

Table 6. *C. beijerinckii* ASCUSDY20 - Blend 2 Analysis

Blend 2								DY21 Aerobic Plating, CFU/100uL								
								1E-01			1E-02			1E-03		
Sample Description	Sample	Test Tube + Cap (g)	Sample + Tube & Cap (g)	Actual Mass (g)	Volume PBST (mL)	Heat Shock Start	Heat Shock End	A	B	C	A	B	C	A	B	C
1	PDM-4	6.731	7.815	1.084	10	N/A	N/A	(b) (4)								
	PDM-5	6.775	7.873	1.098	10	N/A	N/A									
	PDM-6	6.812	7.821	1.009	10	N/A	N/A									
2	PDM-4	6.690	7.707	1.017	10	N/A	N/A									
	PDM-5	-0.122	0.956	1.078	10	N/A	N/A									
	PDM-6	6.898	7.911	1.013	10	N/A	N/A									
3	PDM-4	6.734	7.733	0.999	10	N/A	N/A									
	PDM-5	6.903	7.916	1.013	10	N/A	N/A									
	PDM-6	6.830	7.879	1.049	10	N/A	N/A									
4	PDM-4	6.790	7.785	0.995	10	N/A	N/A									
	PDM-5	6.715	7.768	1.053	10	N/A	N/A									
	PDM-6	6.899	7.937	1.038	10	N/A	N/A									
5	PDM-4	6.718	7.754	1.036	10	N/A	N/A									
	PDM-5	6.974	8.034	1.060	10	N/A	N/A									
	PDM-6	6.779	7.881	1.102	10	N/A	N/A									
6	PDM-4	6.831	7.841	1.010	10	N/A	N/A									
	PDM-5	6.698	7.783	1.085	10	N/A	N/A									
	PDM-6	6.898	7.920	1.022	10	N/A	N/A									
7	PDM-4	6.722	7.724	1.002	10	N/A	N/A									
	PDM-5	6.715	7.728	1.013	10	N/A	N/A									
	PDM-6	6.656	7.652	0.996	10	N/A	N/A									
8	PDM-4	6.639	7.654	1.015	10	N/A	N/A									
	PDM-5	6.797	7.875	1.078	10	N/A	N/A									
	PDM-6	6.775	7.782	1.007	10	N/A	N/A									
9	PDM-4	6.524	7.555	1.031	10	N/A	N/A									
	PDM-5	6.872	7.877	1.005	10	N/A	N/A									
	PDM-6	6.745	7.783	1.038	10	N/A	N/A									
10	PDM-4	6.713	7.712	0.999	10	N/A	N/A									
	PDM-5	6.804	7.885	1.081	10	N/A	N/A									
	PDM-6	6.713	7.773	1.060	10	N/A	N/A									

Table 7. *P. kudriavzevii* ASCUSDY21- Blend 2 Data

DY21 Analysis												
CFU/mL			CFU/mL			CFU/mL						
A	B	C	A	B	C	A	B	C	CFU/g	Avg. CFU/g	Std. Dev.	CV
[redacted]												

(b) (4)

Blend 3								DY20 Anaerobic Plating, CFU/100uL								
								1E+00			1E-01			1E-02		
Sample Description	Sample	Test Tube + Cap (g)	Sample + Tube & Cap (g)	Actual Mass (g)	Volume PBST (mL)	Heat Shock Start	Heat Shock End	A	B	C	A	B	C	A	B	C
1	PDM-1	6.607	7.690	1.083	10	5:06PM	5:16PM	(b) (4)								
	PDM-2	6.630	7.640	1.010	10	5:06PM	5:16PM									
	PDM-3	6.604	7.665	1.061	10	5:06PM	5:16PM									
2	PDM-1	6.600	7.694	1.094	10	5:06PM	5:16PM									
	PDM-2	6.579	7.595	1.016	10	5:06PM	5:16PM									
	PDM-3	6.595	7.627	1.032	10	5:06PM	5:16PM									
3	PDM-1	6.623	7.692	1.069	10	5:06PM	5:16PM									
	PDM-2	6.633	7.685	1.052	10	5:06PM	5:16PM									
	PDM-3	6.630	7.667	1.037	10	5:06PM	5:16PM									
4	PDM-1	6.582	7.622	1.040	10	5:06PM	5:16PM									
	PDM-2	6.573	7.643	1.070	10	5:06PM	5:16PM									
	PDM-3	6.586	7.604	1.018	10	5:06PM	5:16PM									
5	PDM-1	6.020	7.700	1.680	10	3:43PM	3:53PM									
	PDM-2	6.563	7.600	1.037	10	3:43PM	3:53PM									
	PDM-3	6.570	7.612	1.042	10	3:43PM	3:53PM									
6	PDM-1	6.655	7.742	1.087	10	3:43PM	3:53PM									
	PDM-2	6.592	7.680	1.088	10	3:43PM	3:53PM									
	PDM-3	6.565	7.565	1.000	10	3:43PM	3:53PM									
7	PDM-1	6.589	7.661	1.072	10	3:43PM	3:53PM									
	PDM-2	6.614	7.650	1.036	10	3:43PM	3:53PM									
	PDM-3	6.578	7.638	1.060	10	3:43PM	3:53PM									
8	PDM-1	6.518	7.532	1.014	10	4:12PM	4:22PM									
	PDM-2	6.617	7.628	1.011	10	4:12PM	4:22PM									
	PDM-3	6.772	7.772	1.000	10	4:12PM	4:22PM									
9	PDM-1	6.526	7.554	1.028	10	4:12PM	4:22PM									
	PDM-2	6.522	7.523	1.001	10	4:12PM	4:22PM									
	PDM-3	6.627	7.633	1.006	10	4:12PM	4:22PM									
10	PDM-1	6.499	7.501	1.002	10	4:12PM	4:22PM									
	PDM-2	6.644	7.642	0.998	10	4:12PM	4:22PM									
	PDM-3	6.748	7.762	1.014	10	4:12PM	4:22PM									

Table 9. *C. beijerinckii* ASCUSDY20 - Blend 2 Data

DY20 Analysis												
CFU/mL			CFU/mL			CFU/mL						
A	B	C	A	B	C	A	B	C	CFU/g	Avg. CFU/g	Std. Dev.	CV
(b) (4)												

Table 10. *C. beijerinckii* ASCUSDY20 - Blend 2 Analysis

Blend 3								DY21 Aerobic Plating, CFU/100uL								
								1E-01			1E-02			1E-03		
Sample Description	Sample	Test Tube + Cap (g)	Sample + Tube & Cap (g)	Actual Mass (g)	Volume PBST (mL)	Heat Shock Start	Heat Shock End	A	B	C	A	B	C	A	B	C
1	PDM-4	6.709	7.720	1.011	10	N/A	N/A	(b) (4)								
	PDM-5	6.811	7.887	1.076	10	N/A	N/A									
	PDM-6	6.719	7.731	1.012	10	N/A	N/A									
2	PDM-4	6.780	7.821	1.041	10	N/A	N/A									
	PDM-5	6.684	7.770	1.086	10	N/A	N/A									
	PDM-6	6.636	7.638	1.002	10	N/A	N/A									
3	PDM-4	6.770	7.782	1.012	10	N/A	N/A									
	PDM-5	6.600	7.600	1.000	10	N/A	N/A									
	PDM-6	6.675	7.721	1.046	10	N/A	N/A									
4	PDM-4	6.599	7.678	1.079	10	N/A	N/A									
	PDM-5	6.606	7.686	1.080	10	N/A	N/A									
	PDM-6	6.745	7.804	1.059	10	N/A	N/A									
5	PDM-4	6.771	7.790	1.019	10	N/A	N/A									
	PDM-5	6.683	7.732	1.049	10	N/A	N/A									
	PDM-6	6.599	7.634	1.035	10	N/A	N/A									
6	PDM-4	6.807	7.833	1.026	10	N/A	N/A									
	PDM-5	6.747	7.811	1.064	10	N/A	N/A									
	PDM-6	6.734	7.757	1.023	10	N/A	N/A									
7	PDM-4	6.768	7.847	1.079	10	N/A	N/A									
	PDM-5	6.724	7.745	1.021	10	N/A	N/A									
	PDM-6	6.676	7.753	1.077	10	N/A	N/A									
8	PDM-4	6.596	7.600	1.004	10	N/A	N/A									
	PDM-5	6.661	7.677	1.016	10	N/A	N/A									
	PDM-6	6.611	7.620	1.009	10	N/A	N/A									
9	PDM-4	6.801	7.799	0.998	10	N/A	N/A									
	PDM-5	6.754	7.780	1.026	10	N/A	N/A									
	PDM-6	6.555	7.565	1.010	10	N/A	N/A									
10	PDM-4	6.677	7.697	1.020	10	N/A	N/A									
	PDM-5	6.812	7.815	1.003	10	N/A	N/A									
	PDM-6	6.798	7.806	1.008	10	N/A	N/A									

Table 11. *P. kudriavzevii* ASCUSDY21- Blend 3 Data

DY21 Analysis												
CFU/mL			CFU/mL			CFU/mL						
A	B	C	A	B	C	A	B	C	CFU/g	Avg. CFU/g	Std. Dev.	CV
(b) (4)												

ASCUS BIOSCIENCES

Method

Title	DY21-POE Microbe Enumeration
Version	05
Effective Date	15May2020
Author	Miranda Striluk
Approver (Signature & Date)	DocuSigned by: Martin Maylew 5/8/2020 Martin Maylew VP – Process Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable cells of Dairy-21 in Dairy-21 Palm Oil Encapsulate by counting colony forming units (CFU) on solid media.

Safety

Consult the Safety Data Sheet for all reagents prior to handling. Use caution in working with a hot water bath, hot liquids, liquid nitrogen, and extremely cold material. Liquid nitrogen can cause cold burns, frostbite, and permanent eye damage from brief exposure. Avoid skin and eye contact with liquid nitrogen and wear appropriate personal protective equipment (safety glasses and gloves) at all times. Analyst should be trained on liquid nitrogen handling before continuing this method.

Materials

Corning® 15mL Polypropylene Centrifuge Tubes (Corning 430052)
Test tubes, 13x100 mm, sterile
Test tube cap, 16 mm, polypropylene
1.5 mL polypropylene microcentrifuge tube with snap cap
1000 µL Pipette
200 µL Pipette
1000 µL pipette tips, sterile
200 µL pipette tips, sterile
Glass beads, 3 mm, sterile, new

Equipment

Laboratory Vortexer
Class I/II Biosafety Cabinet
pH meter
Mortar and Pestle
Magnetic Stir Plate

Media & Reagents

YPD Plates
Growcells 10X Phosphate Buffered Saline pH 7.4 (PBS), sterile (Growcells MRGF-6235)
Growcells 1X Phosphate Buffered Saline with 0.05% TWEEN pH 7.4, sterile (Growcells MRGF-6275)
Reagent grade 95% Ethanol
70% Ethanol
10% Bleach
Liquid Nitrogen
1N Hydrochloric Acid
1N Sodium Hydroxide

DY21-POE Microbe Enumeration

Method

1. (b) (4)

2. De-encapsulation of Spray Congealed DY21-POE

(b) (4)

3. Prepare the Primary Dilution Mix

(b) (4)

4. DY21-POE Aerobic Plating

(b) (4)

DY21-POE Microbe Enumeration

(b) (4)

(b) (4)

5. Negative Control Plating

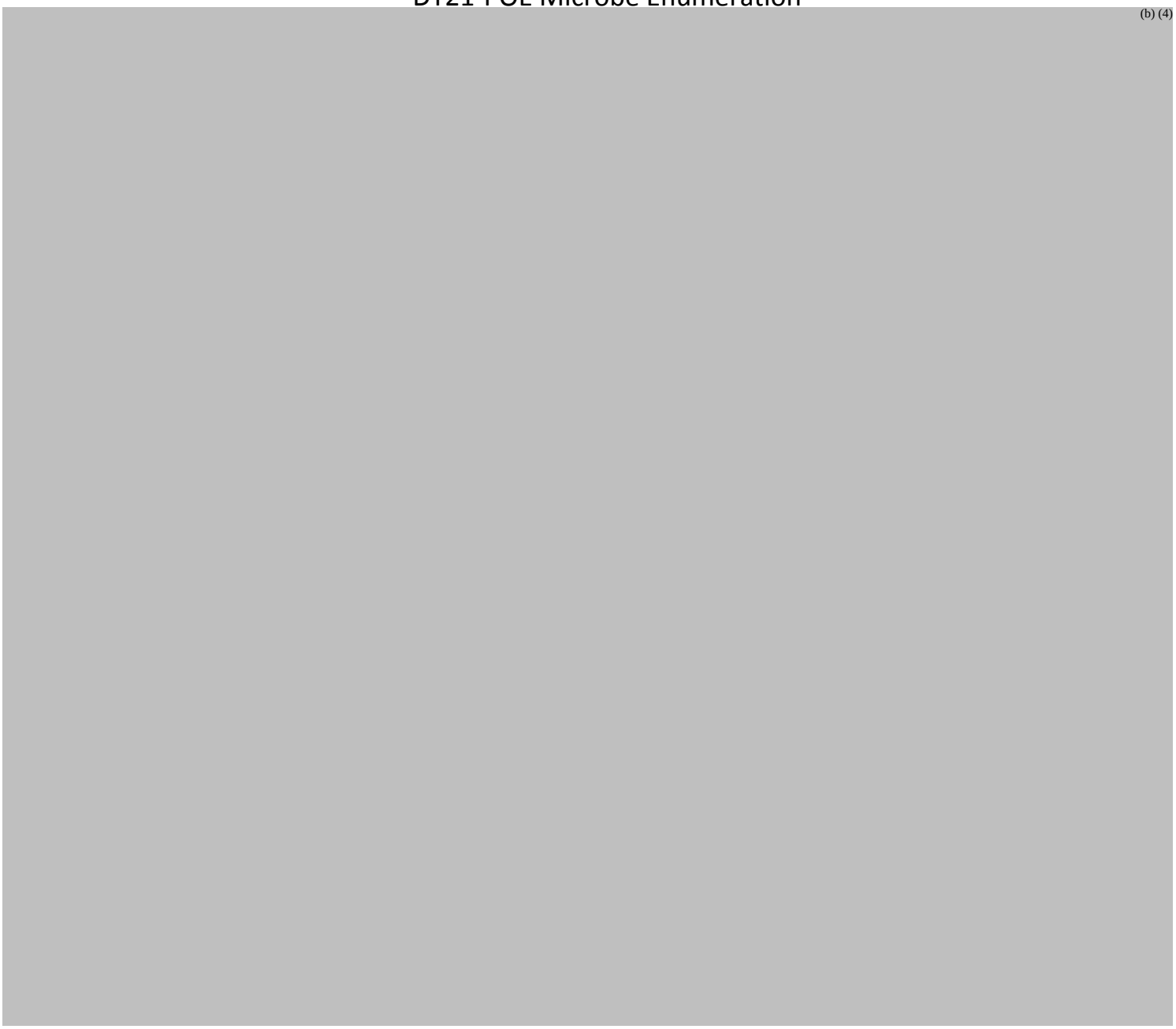
(b) (4)

6. Plate Counting

(b) (4)

DY21-POE Microbe Enumeration

(b) (4)



Pariza Decision Tree as applied to *Pichia kudriavzevii* ASCUSDY21

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology?
→ YES, go to 2.

2. Has the strain genome been sequenced?
→ YES, go to 3.

3. Is the strain genome free of genetic elements, encoding virulence factors, and/or toxins associated with pathogenicity?
→ YES, go to 4.

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA?
→ YES, go to 5.

5. Does the strain produce antimicrobial substances?
→ NO, go to 6.

6. Has the strain been genetically modified using rDNA techniques?
→ NO, go to 8b.

8b For strains to be used in animal feeds: Was the strain isolated from a feed (for example, silage) that has a history of safe consumption by target animals, for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an 'incidental isolate')?
→ NO, go to 13b.

13b For strains to be used in animal feeds: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies?
→ None anticipated from a review of the published literature. Safety is based on (a) natural occurrence and prevalence of *Pichia kudrivzevii* in the rumen of ruminants and in fermented foods; and (b) characterization of the strain to indicate absence of any anticipated virulence factors for pathogenicity or anti-fungal resistance of concern. Go to 14b.

14b The strain is deemed by ASCUS Biosciences, Inc. to be safe for use in the manufacture of feeds, probiotics, and dietary supplements for animal consumption.

Search Strategy for Literature Review: *Pichia kudriavzevii*

A literature search was conducted up to November 6, 2019 in order to identify potential information related to the safety of *Pichia kudriavzevii* as a source of viable microorganisms for ruminants.

Taxonomy

The following species names were used to identify all pertinent safety data: *Pichia kudriavzevii*, *Candida krusei*, *Issatchenkia orientalis*, *Candida glycerogenes*, *Candida acidothermophilum* (Douglass *et al*., 2018; Subramanya *et al*., 2017).

Search Strategy

The overall search strategy is described in Table 1. The relevant database was searched using the keyword/search terms listed in Tables 2 to 6. Initially, a search was conducted using Web of Science which was considered sufficiently representative of the body of available information. From these identified publications, the pertinent studies were reviewed for citations to other relevant information. A further search was performed using Google Scholar using the cited by functionality for pertinent publications. Finally, reviews and previous scientific opinions by authoritative bodies were reviewed in order to ensure the completeness of the literatures search. A summary of the search output is provided below.

Table 1: Literature Search and Selection Strategy		
Step 1	Records identified using selected literature databases	Web of Science
	Record total records (titles/abstracts) identified through electronic search	
Step 2	Merge search results and exclude duplicates	
Step 3	Screen titles/abstracts and exclude obviously irrelevant records	
Step 4	Review full texts and assess for relevance and eligibility for inclusion	
Step 5	Review full texts for citations and use Google Scholar to identify 'cited by' records of relevance	
Step 6	Review authoritative body opinions and reviews for any additional references not identified in the above search	

Table 2:	**Topic Specific Search Terms – *Pichia kudriavzevii***			
Search strategy for safety of species (*P. kudriavzevii*)	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Pichia kudriavzevii*	Merge, exclude duplicates n=32 Screen for relevance n=16
		Term 2	Toxi* (n=13) Pathogen* (n=11) Safe* (n=8) Disease (n=7) Infection (n=12) Virulence (n=2)	
Search strategy for safety of *P. kudriavzevii* for cattle	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Pichia kudriavzevii*	Merge, exclude duplicates n=5 Screen for relevance n=0
		Term 2	Ruminant (n=1) Calves (n=1) Cow* (n=3) Cattle (n=2)	
Search strategy for history of use of *P. kudriavzevii* for use in food and feed	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Pichia kudriavzevii*	Merge, exclude duplicates n=31 Screen for relevance n=16
		Term 2	Food* (n=39) Feed* (n=17)	

Search: Term 1 in combination with one or more of Term 2; Boolean search techniques were applied.

Table 3:	**Topic Specific Search Terms – *Candida krusei***			
Search strategy for safety of species (*P. kudriavzevii*)	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Candida krusei*	Merge, exclude duplicates n=1377 [Representative reviews and EFSA citations used only]
		Term 2	Toxi* (n=127) Pathogen* (n=619) Safe* (n=64) Disease (n=305) Infection (n=1344) Virulence (n=113)	
Search strategy for safety of *P. kudriavzevii* for cattle	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Candida krusei*	Merge, exclude duplicates n=19 Screen for relevance n=9 [bovine mastitis only]
		Term 2	Ruminant (n=0) Calves (n=14) Cow* (n=29) Cattle (n=14)	
Search strategy for history of use of *P. kudriavzevii* for use in food and feed	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Candida krusei*	Merge, exclude duplicates n=73 Screen for relevance n=16
		Term 2	Food* (n=80) Feed* (n=31)	

Search: Term 1 in combination with one or more of Term 2; Boolean search techniques were applied.

Table 4:	**Topic Specific Search Terms – *Issatchenkia orientalis***			
Search strategy for safety of species (*P. kudriavzevii*)	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Issatchenkia orientalis*	Merge, exclude duplicates n=26 Screen for relevance n=6
		Term 2	Toxi* (n=9) Pathogen* (n=18) Safe* (n=6) Disease (n=5) Infection (n=8) Virulence (n=2)	
Search strategy for safety of *P. kudriavzevii* for cattle	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Issatchenkia orientalis*	Merge, exclude duplicates n=4 Screen for relevance n=4
		Term 2	Ruminant (n=1) Calves (n=0) Cow* (n=3) Cattle (n=0)	
Search strategy for history of use of *P. kudriavzevii* for use in food and feed	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Issatchenkia orientalis*	Merge, exclude duplicates n=25 Screen for relevance n=22
		Term 2	Food* (n=34) Feed* (n=13)	

Search: Term 1 in combination with one or more of Term 2; Boolean search techniques were applied.

Table 5:	**Topic Specific Search Terms – *Candida glycerinogenes***			
Search strategy for safety of species (*P. kudriavzevii*)	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Candida glycerinogenes*	Merge, exclude duplicates n=1 Screen for relevance n=1
		Term 2	Toxi* (n=0) Pathogen* (n=1) Safe* (n=0) Disease (n=0) Infection (n=1) Virulence (n=0)	
Search strategy for safety of *P. kudriavzevii* for cattle	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Candida glycerinogenes*	Merge, exclude duplicates n=0
		Term 2	Ruminant (n=0) Calves (n=0) Cow* (n=0) Cattle (n=0)	
Search strategy for history of use of *P. kudriavzevii* for use in food and feed	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Candida glycerinogenes*	Merge, exclude duplicates n=2 Screen for relevance n=2
		Term 2	Food* (n=1) Feed* (n=1)	

Search: Term 1 in combination with one or more of Term 2; Boolean search techniques were applied.

Table 6: Topic Specific Search Terms – *Candida acidothermophilum*				
Search strategy for safety of species (*P. kudriavzevii*)	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Candida acidothermophilum*	Merge, exclude duplicates n=1 Screen for relevance n=1
		Term 2	Toxi* (n=0) Pathogen* (n=1) Safe* (n=0) Disease (n=1) Infection (n=1) Virulence (n=0)	
Search strategy for safety of *P. kudriavzevii* for cattle	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Candida acidothermophilum*	Merge, exclude duplicates n=0
		Term 2	Ruminant (n=0) Calves (n=0) Cow* (n=0) Cattle (n=0)	
Search strategy for history of use of *P. kudriavzevii* for use in food and feed	Keywords/search terms [Database: Web of Science; search by topic]	Term 1	*Candida acidothermophilum*	Merge, exclude duplicates n=1 Screen for relevance n=0
		Term 2	Food* (n=0) Feed* (n=1)	

Search: Term 1 in combination with one or more of Term 2; Boolean search techniques were applied

Microbiome Safety for *Pichia kudriavzevii* ASCUSDY21

1 Objectives

The objective of this work is to:

1. Elucidate the roles of rumen microbiome in rumen digestive health via literature review.
2. Identify the typical microbial composition of the rumen microbial community of dairy cows using external datasets and peer reviewed manuscripts.
3. Identify examples and methods of rumen microbiome manipulation in peer reviewed manuscripts.
4. Corroborate if daily administration of *Pichia kudriavzevii* DAIRY21 increases its abundance beyond abundances typically observed in the rumen using in-house data.

2 Literature Review

The rumen microbiome is crucial for the digestion of feed and supplies necessary nutrients to ruminants (Faichney, 1996; Huws *et al*., 2018). The rumen hosts a diverse group of microorganisms that work closely to degrade plant materials. The fermentation process converts nearly all dietary carbohydrates to volatile fatty acids (VFA), predominantly butyrate, acetate, and propionate. These three major VFAs play key roles in host metabolism. The butyrate pool in rumen is the smallest of the three (Sutton *et al*., 2003). It is predominantly metabolized by rumen mucosa and almost all of the absorbed butyrate was converted to ketone bodies (Weigand *et al*., 1975; Cook *et al*., 1969). Studies have also linked butyrate to the development of rumen papillary and calf gastrointestinal tracts (Weigand *et al*., 1975; Górka *et al*., 2018). Further, direct infusion of butyrate into the rumen has shown increases in milk fat production without changing milk yield (Huhtanen *et al*., 1993). Unlike butyrate, acetate and propionate are both absorbed by rumen and passed to extra-ruminal tissues for metabolism (Cook and Miller, 1965). Propionate, in particular, can be converted into glucose via gluconeogenesis in the liver. Studies show that gluconeogenesis provides up to 90% of the glucose required by ruminants, and over half of the glucose produced is derived from propionate (Leng *et al*., 1967; Young, 1977). Thus, a large rumen propionate pool is needed to support the basic ruminant metabolism. Yost *et al*., (1977) reported that rumen propionate pool size is directly related to the amount of feed intake and significant differences between individuals were observed, highlighting the rumen fermentation differences among animals. In addition, direct infusion of propionate into the rumen

has been shown to increase milk protein production, but decrease milk yield (Rook and Balch, 1961). Acetate absorbed through rumen epithelium was predominantly metabolized by extra-ruminal tissues other than liver (Cook and Miller, 1965). Direct infusion of acetate into the rumen has been shown to improve the yield of milk, as well as the amount of milk fat produced (Rook and Balch, 1961). Interestingly, Sabine and Johnson (1964) found only 40-50% of the infused acetate was used by the host, suggesting acetate may play an equally important role if not more in the development of rumen microbiome. The study also reported a large variability of acetate usage among animals, again highlighting the individual host differences which the rumen microbiomes are likely contributing to.

Besides its importance in fulfilling ruminant carbon needs, rumen microorganisms are also pivotal in providing nitrogen. Published studies estimate that approximately 60-90% of protein absorbed by ruminant duodenum arises from a microbial source (Wallace *et al*., 1997; Broudiscou and Jouany, 1995). The association between rumen nitrogen use efficiency and microbiome has also been widely reported (Huws *et al*., 2018; Bach *et al*., 2005; Edwards *et al*., 2008). To further elucidate the roles of rumen microbiome, Lin *et al*. (2019) identified microbial activities and their corresponding host genetic responses, emphasizing the symbiotic relationship between host nutrient needs and rumen microorganisms. Therefore, changes in rumen microbiome could directly influence ruminant nutrient balance.

The importance of rumen microbiome, especially its unique ability in cellulose degradation, has long been discussed (Woodman and Stewart, 1928; Woodman, 1930). Hungate (1957) attempted to characterize the rumen microbiome by anaerobic cultivation. These studies provided a glimpse into rumen bacterial diversity as well as the metabolic potential of select bacterial species. However, the development of molecular biology and Next-Generation Sequencing (NGS) techniques have revealed that many of the cultivation techniques leveraged by Hungate only characterized a small proportion of the rumen microbial community. A large proportion of the rumen microbiome is considered “unculturable”, and hence dismissed in early rumen microbiology experiments (Jannasch and Jones, 1959; Staley, 1985; Pace, 1997; Steen *et al*., 2019). Since then, the use of molecular techniques (Pace, 1997; Zuckerkandl and Pauling, 1965; Schwartz and Dayhoff, 1978; Woese, Kandler and Wheelis, 1990) leveraging NGS have greatly advanced our ability to characterize rumen microbiome and its associations with animal health and nutrition, as well as environmental factors (Wallace *et al*., 1997; Rodriguez-R and Konstantinidis, 2014; Jami and Mizrahi, 2012; Kumar *et al*., 2015; Wallace *et al*., 2019; Petri *et al*., 2013; Huws *et al*., 2018; Henderson *et al*., 2015; Deusch *et al*., 2017; Mizrahi and Jami, 2018; Sasson *et al*., 2017; Weimer, 2015; Furman *et al*., 2020).

Marker gene amplicon sequencing is one of the most commonly used methods of rumen microbiome characterization (Sirohi *et al*., 2012). Typically, the small subunit ribosomal RNA (16S rRNA) gene is used to evaluate bacterial and archaeal community composition, while the internal transcribed spacer (ITS) between the 18S and 28S rRNA is used to characterize fungal community composition (Mizrahi and Jami, 2018). Several studies have linked the rumen microbiome profile to animal performance and milk production and is now considered an indicator of rumen digestive health (Jami and Mizrahi, 2012; Kumar *et al*., 2015; Lima *et al*., 2015). Rumen microbiome is highly variable depending on several factors, including age, breed, diet, location, farm management practices, and lactation stage (Wallace *et al*., 2019; Henderson

et al., 2015; Furman *et al*., 2020; Pitta *et al*., 2016). To better study the microbiome in context of the observed individuality, many studies have focused on identifying and characterizing the core rumen microbiomes (Jami and Mizrahi, 2012; Kumar *et al*., 2015; Wallace *et al*., 2019; Petri *et al*., 2013; Henderson *et al*., 2015; Furman *et al*., 2020; Lima *et al*., 2015; Xue *et al*., 2018; Kittelmann *et al*., 2013; Fouts *et al*., 2012). The concept of core microbiome, a common assemblage of microorganisms that exists in or is associated with a specific habitat, was first introduced and applied to differentiate human microbiomes associated with healthy and diseased conditions (Turnbaugh *et al*., 2007; Turnbaugh and Gordon 2009; Turnbaugh *et al*., 2009). Since then, core microbiomes have been identified in a broad spectrum of environments including agroecosystems, monogastric animals, and ruminants (Shade and Handelsman, 2012; Yeoh *et al*., 2017; Toju *et al*., 2018; Lowe *et al*., 2012; Dougal *et al*., 2013).

The rumen microbial community composition is constantly in flux. The microbial population has been shown to change over time in response to a variety of factors, including diet composition, time after feeding, season, and stage of lactation. Additionally, there are groups of microorganisms that are unique to particular breeds of cow (i.e. Jersey or Holstein), regions, and individual animals that further increase the inherent complexity of the microbial community native to the rumen. Despite this variability, there is a core microbiome that appears in the majority of animals. This core has been investigated at Ascus Biosciences, as well as in independent academic studies. Although the results are variable at times and defining a “normal healthy" rumen is challenging, there are several phyla that tend to appear across all ruminants. Henderson *et al*. (2015) reported 32 different species of ruminants globally shared a core assembly of rumen bacteria. Consistent with other studies (Jami and Mizrahi, 2012; Deusch *et al*., 2017; Lima *et al*., 2015; Xue *et al*., 2018; Jami *et al*., 2014; Schären *et al*., 2018), members of Bacteroidetes, Firmicutes, Proteobacteria, and Fibrobacteres were among the topmost abundant bacteria identified regardless of animal origin and diet. The fungal rumen community, although much less abundant than the bacterial rumen community, tends to fall into the following phyla: Ascomycota, Basidiomycota, Neocallimastigomycota, and Zygomycota (Kumar *et al*., 2015; Lima *et al*., 2015; Kittelmann *et al*., 2013; Fouts *et al*., 2012; Tapio *et al*., 2017; Langda *et al*., 2020; Dias *et al*., 2017; Paul *et al*., 2018; Belanche *et al*., 2019; Mendes de Almeida *et al*., 2012; Vargas-Bello-Pérez, Cancino-Padilla and Romero, 2016; Ishaq *et al*., 2017). Neocallimastigales used to be an order within Chyrtridiomycota, however in 2012, these anaerobic fungi were placed into a separate phylum called Neocallimastigomycota (Adl *et al*., 2012). Although this change was proposed 7 years ago, some species of Neocallimastigomycota are still listed as members of Chyrtridiomycota in public databases. For the sake of clarity, instances of ‘Chytridiomycota’ have been replaced with ‘Neocallimastigomycota’ in this report.

Many published manuscripts described the rumen bacterial dynamics. Studies reporting the core bacterial communities from dairy rumen (Jami and Mizrahi, 2012; Wallace *et al*., 2019; Petri *et al*., 2013; Furman *et al*., 2020; Lima *et al*., 2015; Xue *et al*., 2018; Dias *et al*., 2017) and a wide range of ruminants (Henderson *et al*., 2015) are summarized in Table 1. Ascus has also conducted surveys and the results corroborate published numbers (Table 2).

Table 1. The Average Abundance of Major Rumen Bacterial Phyla from Published Studies.

	Percent Relative Abundance								
	Bacterial Core Microbiome								
	Adult Dairy Cows						Pre-weaning Dairy Calves		Ruminants (32 species)
Major Rumen Bacterial Phylum	Xue et al., 2018	Petri et al., 2013	Jami et al., 2012	Lima et al., 2014[a]	Wallace et al., 2019[b]	Furman et al., 2020[c]	Dias et al., 2017[d]	Furman et al., 2020[e]	Henderson et al., 2015[f]
Bacteroidetes	20.68±0.18	32.8	51	33.6-40.7	56	1-75	15-30	1-75	38.7±1.4
Fibrobacteres	0.86±0.02	0.1-15	0.02-0.48	< 1	6	< 1	NA	NA	3.1±0.1
Firmicutes	21.67±0.18	43.2	41.6	42.5-49.65	16	10-80	30-90	10-80	44.2±1.8
Proteobacteria	0.52±0.01	14.3	5.46	1-12	8	1-70	1-10	1-70	2.8±0.1
Tenericutes	0.44±0.01	NA	0.69	1-3	< 1	<1	NA	NA	1.4±0
Spirochaetes	1.35±0.04	0.5-15	< 1	< 1	5	1-5	NA	NA	1±0

[a] values were estimated from Fig 1

[b] values estimated from Fig 1B

[c] values estimated from Fig 2A (60 - 700 days of life)

[d] pre-weaning calf (7-63 days old) rumen microbiome. Values estimated from Figure 2B

[e] values estimated from Fig 2A (1 - 59 days of life)

[f] approximation from supplementary Table 1 using the most abundant groups

Table 2. The Average Abundance of Major Rumen Bacterial Phyla from Ascus Surveys.

Major Rumen Bacterial Phylum	Percent Relative Abundance Ascus Conducted Surveys Adult Diary Cows Survey 1	Survey 2	Survey 3	Survey 4
Bacteroidetes	36.67	24.75	36.339	44.35
Fibrobacteres	1.53	3.71	0.49	1.15
Firmicutes	46.82	61.85	48.41	46.98
Proteobacteria	5.49	3.63	11.2	3.36
Tenericutes	1.26	1.2	0.43	0.7
Spirochaetes	2.72	1.7	0.66	0.55

Despite the recognition of their significant roles in rumen, the diversity characterization of rumen fungal communities is lagging far behind rumen bacteria (Mizrahi and Jami, 2018; Comtet-Marre *et al*., 2017). This is due to: 1) the understanding of fungi is generally limited to date and frequently, the fungal community profiles were not reported; 2) fungal marker genes varied largely among fungal phylogeny and researches frequently target different regions that apply to their specific research questions. For example, published manuscripts, Kittleman, *et al*., (2013), Dias, *et al*. (2017), Paul *et al*. (2018), and Tapio *et al*. (2017), describing the dairy rumen fungal community using an ITS primer set (MN100 and MNGM2) bias towards members of Neocallimastigomycota. This led to the primary identification of Neocallimastigomycota in dairy rumen and neglecting other fungal groups. Below, from the available and applicable literature, we summarized the average abundance of major fungal groups in dairy rumen (Kumar *et al*., 2015; Fouts *et al*., 2012; Mendes de Almeida *et al*., 2012; Ishaq *et al*., 2017) and other ruminants (Langda *et al*., 2020; Belanche *et al*., 2019) (Table 3). Ascus conducted survey results are reported in Table 4. The average abundance of major rumen fungal phyla from Ascus surveys are also consistent with the published studies.

Table 3. The Average Abundance of Major Rumen Fungal Phyla from Published Studies.

Major Rumen Fungal Phylum	Percent Relative Abundance					
	Dairy Cow				Other Ruminants	
	Kumar et al., 2015	Mendes de Almeida et al., 2012[a]	Ishaq et al., 2017[b]	Fouts et al., 2012[c]	Belanche et al., 2019[d]	Langda et al., 2019[e]
Ascomycota	27	85	5-68	47-68	1-9	18-30
Basidiomycota	3		1-3	2-10	8-20	< 1
Neocallimastigomycota	1	Cannot be cultivated aerobically	26-92	30-50	71-92	52-78
Zygomycota	< 1	15	< 1		NA	< 1
unidentified	68	NA	1-5		NA	0.1-0.5

[a] aerobic cultivation based

[b] values estimated from Fig 2

[c] values estimated from Fig 2C

[d] values estimated from Fig 4B

[e] values estimated from Fig 2D

Table 4. The Average Abundance of Major Rumen Fungal Phyla from Ascus Conducted Surveys.

Major Rumen Fungal Phylum	Percent Relative Abundance	
	Ascus Surveys (Dairy Cows)	
	Survey 1	Survey 2
Ascomycota	36.57	58.09
Basidiomycota	12.54	0.042
Neocallimastigomycota	50.86	41.86
Zygomycota	0.0047	0.0003
unidentified	0.03	0

As more rumen microbiomes were studied, it became clear that diet was the major determinant of observed microbiome differences (Kumar *et al*., 2015; Deusch *et al*., 2017; Mizrahi and Jami, 2018; Belanche *et al*., 2019; Johnson and Johnson, 1995; Brulc *et al*., 2009; Carberry *et al*., 2014). This indicates the direct impact of diet on rumen microbial populations. Indeed, few strong co-occurrence patterns were observed among rumen microbes, suggesting that shifts within core microbiome were based on the pool of available metabolites produced during ingesta fermentation. Hence, modifying either diet or microbiome could influence the rumen fermentation process (Wallace *et al*., 2019; Furman *et al*., 2020; Moraïs and Mizrahi, 2019; Belanche *et al*., 2012).

Numerous studies suggested that microbiome shifts improved digestibility (Wallace *et al*., 2019; Weimer, 2015; Comtet-Marre *et al*., 2017; Moraïs and Mizrahi, 2019; Yáñez-Ruiz *et al*., 2015). Based on the current literature, Moraïs and Mizrahi (2019) summarized that multiple microbial community states exist within the rumen depending on the rumen metabolic needs. The flow of metabolites and energy were passed on from one functional group to the next rather than from one group to another. While individual microbial species may be able to carry out similar functions, Moraïs and Mizrahi (2019) hypothesize that microbial interactions drive larger changes in overall fermentation patterns. Hence, identifying the optimal microbial interactions could improve digestibility (Weimer, 2015). Sasson *et al*. (2017) reported that the differences in cows' ability to harvest energy was correlated with a group of heritable rumen microorganisms. Wallace *et al*. (2019) extended the study with a bigger cohort of animals. Similar results were reported, where specifically that rumen digestibility differences were associated with heritable core rumen microbiomes. This is also consistent with other studies showing that early colonization of microbes through vaginal birth could improve rumen digestibility significantly (Furman *et al*., 2020; Yáñez-Ruiz *et al*., 2015). While a microbiome-led breeding program could be used to preserve the optimal microbial interactions and improve rumen digestibility, it is not the most efficient and the outcome may be difficult to predict. Many other methods have been reported to promote efficient microbial interactions by shifting rumen microbiome (Weimer, 2015).

3 Altering the Microbiome

Throughout the history of agriculture, humans have long been manipulating rumen microbiomes to enhance rumen digestibility and fermentation profiles. For centuries, Swedish farmers have fed cud from healthy cattle to another with ruminal indigestion (Brag and Hansen, 1994). This method was later scientifically evaluated and became a common practice called rumen transfaunation (Brag and Hansen, 1994; DePeters and George, 2014). Ribeiro *et al*. (2017) recently conducted a study where 70% of the barley fed domestic cattle's rumen content was replaced by foraging bison rumen content repeatedly. The study found the procedure significantly improved cattle N digestibility. In another study, mixed rumen contents from two healthy cows were fed to 45 cattle with primary and secondary digestive issues (Steiner *et al*., 2020). After the transfaunation, it was observed that the sick animals had increased appetite and improved rumen digestibility. However, the exotic microbiome may not consistently establish due to significant host physiological differences. While the introduced microbiome did not interfere with normal rumen function, inconsistent establishment of a new microbiome was observed, and some were reverted back to a state similar to the original microbiome (Zhou *et al*., 2018; Weimer *et al*., 2010).

Alternative to transfaunation, in-feed supplementation of native and non-native microorganisms have also been used to treat rumen indigestion (McAllister *et al*., 2011; Nagpal *et al*., 2015). Unlike transfaunation, the process promotes the shifts of the native rumen microbiome instead of introducing exotic microbial communities. In-feed supplementation is non-invasive and eliminates the danger of accidental pathogen feeding. Many different microorganisms have been isolated and used as direct fed microbial products (DFM) in treating rumen digestion issues (McAllister *et al*., 2011; Nagpal *et al*., 2015; Meissner *et al*., 2010; Stein *et al*., 2006). The DFMs in use today include members of bacteria and fungi. Studies have shown that they are capable of out-competing rumen pathogens, moderating rumen pH (by utilizing overproduced lactic acid or increasing the production of volatile fatty acids propionate) and improving fiber digestion by excreting cellulolytic/hydrolytic enzymes. Thus, introducing microorganisms to promote microbiome changes and to optimize microbial interactions is a valid method of improving rumen digestibility.

To compare the impact of DFM and diet on rumen microbiome, Ishaq *et al*. (2017) conducted a study where yeast was administered to animals fed either a high-fiber diet or a high-grain diet and the changes in rumen fungal and protozoal microbiomes were evaluated.

This experiment showed that diet had far greater influence on the composition of the microbiome than the supplementation of yeast. In Table 2 from the manuscript (see below), the AMOVA analysis shows that feeding of yeast created no significant difference in fungal microbiome composition between control and treatment cows on the same diet type (e.g. high-fiber yeast vs. high-fiber control). Similar results were observed for ANOSIM analysis. Diet, however, did create statistically significant differences in microbiome composition. Thus, although DFM supplementation may impact the rumen microbiome and fermentation, the amount of change isn't as dramatic and significant as diet formulation.

TABLE 2 | Comparison of treatments by AMOVA, ANOSIM, and UniFrac, for rumen fungi and protozoa for cows receiving two dietary treatments with or without yeast supplementation under SARA conditions.

	Fungal ITS					Protozoal 18S				
	AMOVA	ANOSIM		Weighted UniFrac		AMOVA	ANOSIM		Weighted UniFrac	
	P	R	P	W	P	P	R	P	W	P
Location	**	0.13	*	0.65	**	*	0.08	**	0.87	**
Epimural x Fluid	**	0.05	ns	0.65	**	**	0.10	*	0.99	**
Epimural x Solid	T[a]	0.06	ns	0.55	**	*	0.08	*	1	**
Fluid x Solid	**	0.28	**	0.77	**	*	0.07	*	0.61	**
HF x HG	**	0.93	**	1	**	**	0.10	**	0.65	**
C x Y	ns	0.01	ns	0.48	**	ns	0.00	ns	0.61	**
Treatment	**	0.51	**	0.83	*	**	0.15	**	0.87	**
HFC x HGC										
Epimural	**	0.91	**	1	**	ns	0.40	*	1	**
Fluid	n/a	n/a	n/a	n/a	n/a	ns	0.00	ns	0.65	**
Solid	**	0.95	**	1	**	ns	0.11	ns	0.74	**
HFY × HGY										
Epimural	**	0.82	**	1	**	ns	0.31	*	1	ns
Fluid	n/a	n/a	n/a	n/a	n/a	ns	0.19	*	0.5	**
Solid	T[a]	0.85	T1	1	**	ns	0.00	ns	0.85	**
HFC × HFY										
Epimural	ns	0.03	ns	0.61	*	ns	−1.8	ns	0.96	**
Fluid	ns	0.01	ns	0.55	*	ns	0.03	ns	0.66	**
Solid	ns	0.00	ns	0.79	*	ns	0.00	ns	0.65	**
HGC × HGY										
Epimural	ns	0.02	ns	0.74	**	ns	0.31	*	0.95	*
Fluid	n/a	n/a	n/a	n/a	n/a	ns	0.00	ns	0.72	**
Solid	ns	0.00	ns	0.63	**	ns	0.02	ns	0.67	**
HFC x HGY										
Epimural	**	0.84	**	1	**	*	0.32	T[a]	0.95	*
Fluid	n/a	n/a	n/a	n/a	n/a	ns	0.00	ns	0.53	**
Solid	**	0.84	**	1	**	ns	0.00	ns	0.74	**

[a]Values were significant only before Bonferroni correction.

Diets include high fiber (HF) or high grain (HG), locations include Epimural (E), fluid (F), or solid (S), and treatments include yeast (Y) or Control (C). Significance is determined as $P < 0.05$, $*P < 0.001$, $**P > 0.05$ (ns), or not enough comparisons to make (n/a). Significance was adjusted by Bonferroni where appropriate.

4 Typical microbiome composition of dairy cows receiving *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21

Ascus conducted an experiment to assess the effects of the administration of native rumen microbes on the rumen microbiome community. The experiment was conducted on 24 dairy cows (8 animals per group): one group of animals received *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21 ("Microbes 1"), a second group received *C. beijerinckii* ASCUSDY20, *P. kudriavzevii* ASCUSDY21, and another native rumen bacterium ("Microbes 2"), and the third group served as control ("No microbes"). The average abundance of major fungal phyla and major bacterial phyla were reported in Table 5 and Table 6, respectively. For the ease of comparison, the abundance of major rumen fungal and bacteria phyla from published literature were also included. In this administration experiment, it can be seen that the addition of *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21 to dairy cows did not significantly alter the rumen fungal or bacterial composition when compared to the control group. Abundances of all fungal and bacterial phyla are within the standard ranges observed in animals not fed native rumen microbes. The average abundance of each phylum tended to be similar across experimental groups. The abundance of all fungal and bacterial phyla is also within the ranges reported in literature (Table 5 and Table 6). Therefore, directly feeding C. beijerinckii ASCUSDY20 and P. kudriavzevii ASCUSDY21 did not alter dairy rumen fungal communities beyond their natural states. This corroborates with Ascus' assessment that administering *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21 to dairy cows do not shift their rumen microbiomes beyond the natural ranges.

Table 5. Abundance of Major Rumen Fungal Phyla from the Ascus Experiments as Compared to Published Data.

Major Rumen Fungal Phylum	Percent Relative Abundance						
	Ascus Experiment			Published Dairy Rumen Data			
	Microbes 1	Microbes 2	No microbes	Kumar et al., 2015	Mendes de Almeida et al., 2012[a]	Ishaq et al., 2017[b]	Fouts et al., 2012[c]
Ascomycota	31.89	31.33	31.5	27	85	5-68	47-68
Basidiomycota	7.33	7.99	9.63	3		1-3	2-10
Neocallimastigomycota	60.42	60.16	58.06	1	Cannot be cultivated aerobically	26-92	30-50
Zygomycota	0.00091	0.0003	0.0016	< 1	15	< 1	
unidentified	0.46	0.52	0.8	68	NA	1-5	

[a] aerobic cultivation based

[b] values estimated from Fig 2

[c] values estimated from Fig 2C

Table 6. Abundance of Major Rumen Bacterial Phyla from the Ascus Experiment as Compared to the Published Data.

Major Rumen Bacterial Phylum	Percent Relative Abundance								
	Ascus Experiment			Published Dairy Rumen Data					
	Microbes 1	Microbes 2	No microbes	Xue et al., 2018	Petri et al., 2013	Jami et al., 2012	Lima et al., 2014[a]	Wallace et al., 2019[b]	Furman et al., 2020[c]
Bacteroidetes	35.53	36.02	36.3	20.68±0.18	32.8	51	33.6-40.7	56	1-75
Fibrobacteres	0.43	0.42	0.54	0.86±0.02	0.1-15	0.02-0.48	< 1	6	< 1
Firmicutes	55.73	54.87	54.56	21.67±0.18	43.2	41.6	42.5-49.65	16	10-80
Proteobacteria	4.45	4.47	4.66	0.52±0.01	14.3	5.46	1-12	8	1-70
Spirochaetes	0.97	0.72	0.57	0.44±0.01	NA	0.69	1-3	< 1	<1
Tenericutes	0.53	0.69	0.65	1.35±0.04	0.5-15	< 1	< 1	5	1-5

[a] values were estimated from Fig 1

[b] values estimated from Fig 1B

[c] values estimated from Fig 2A (60 - 700 days of life)

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FINAL REPORT

PREPARED BY / DATE: (b) (4) / May 25, 2016

PROJECT NUMBER: DE1601

STUDY TITLE: Rumen microbial inoculation efficacy trial.

INVESTIGATOR: (b) (4)

BIOSTATISTICIAN: (b) (4)

MONITORS: (b) (4)

SPONSOR: Ascus Biosciences, Inc.

OBJECTIVE: Evaluate the effect of inoculating an Ascus Biosciences selection of rumen microbes on milk composition and yield

DATE OF INITIATION: January 18th, 2016

DATE ENDED: March 9th, 2016

(b) (4)

Signature: (b) (4)

Date: May 25th, 2016

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EXECUTIVE SUMMARY

A total of 16 multiparous Holsteins cows were brought into (b) (4) facilities and individually housed for a total of 52 days. All cows underwent a 10-day period for surgery recovery and adaptation to new facilities and diet. Cows were randomly allocated to two study groups; a) Inoculated: A selection of microbes suspended in buffer solution were inoculated via ruminal cannula once a day during the intervention period; and, b) Control: Cows were inoculated only with buffer control. The intervention period lasted a total of 32 days. Also, outcomes of interest were measured for an additional 10 days after the last inoculation day. A treatment by week interaction was observed for milk yield, fat corrected milk (FCM), energy corrected milk (ECM), and protein yield. A tendency for a treatment by week interaction was also observed for fat yield, feed efficiency (FE), and rumen pH. The interaction for yields was mainly the result of milk yield diverging between the two treatments within the first 2-3 weeks of the study and coming back together toward the end of the Intervention period. A tendency for a higher milk fat percentage for Inoculated vs. the Control was observed. Although the treatment by week interaction was not significant, it can be observed that milk fat percentages was numerically similar within the first two weeks due probably to adaptation and numerically higher for Intervention during weeks three to five. The difference on milk fat percentage was not observed during the follow-up period when cows were not inoculated with microbes. The results obtained in this study are very promising and encourage to further research efficacy of these or additional microbes on milk yield and composition with a larger number of animals.

JUSTIFICATION AND HYPOTHESIS

Ascus Biosciences identified rumen microbial populations which are affected by diet-induced changes in milk fat composition. Therefore, the hypothesis was that inoculating these microbes directly into the rumen would increase milk fat content.

OBJECTIVE

The study objective was to evaluate the effect of inoculating an Ascus Biosciences selection of microbes on milk composition and yield.

MATERIALS AND METHODS

Animals and Facilities

A total of 16 cows were enrolled into the study. The cows were multiparous Holsteins (second and third lactation) that were brought on January 18, 2016 from a local dairy farm into (b) (4) facilities (b) (4) (b) (4) The animal selection criteria included cows between 60 and 120 days in milk (DIM), daily milk production of 36 kg or more, and somatic cell count (SCC) below 200,000 cells/mL in accordance with the previous DHIA monthly test.

Upon arrival, cows were housed individually in box stalls bedded with almond shells where they were fed twice a day total mixed ration (TMR) diet offered at libitum and had free access to water except for short periods during milking. Cows were milked twice a day (4:30 am and 4:00 pm) at a conventional milking parlor. In the two days after arrival, all cows were surgically fitted with a ruminal cannula on the left flank fossa (Bar Diamond 10 cm 1 C Cannula, Parma, ID).

Experimental Design

Treatment Groups

The cows were randomly allocated to two study groups of 8 cows each:

Inoculated: A selection of microbes suspended in buffer solution personnel were inoculated via ruminal cannula once a day during the intervention period. Cows assigned to I received study IDs 1, 3, 5, 7, 9, 11, 13 and 15.

Control: Cows were inoculated only with buffer control once a day during the intervention period. Cows assigned to C received study IDs 2, 4, 6, 8, 10, 12, 14 and 16.

Study Periods

Pre-Intervention Period

All cows underwent a 10-day period for surgery recovery and adaptation to new facilities and diet. During this period, (b) (4) personnel conducted daily health assessments.

Intervention Period

Immediately after the morning milking cows were inoculated via the rumen cannula by Ascus Biosciences personnel for 32 days.

Post-Intervention Period

Outcomes of interest were measured for an additional 10 days after the last inoculation day.

Rumen Inoculation

Each animal was either inoculated with microbes or with a buffer control via the ruminal cavity in accordance to Ascus Biosciences protocol.

Sampling and Measurements

Feed Intake

Animals were fed twice a day individually in separate feed containers after the morning and afternoon milkings. Feed weights were recorded twice a day at each feeding during Pre-Intervention days 5 to 10, Intervention and Post-Intervention periods. Prior day refusals were weighted and discarded daily before the morning feeding.

Cow Weight

All cows were weighted individually after the morning milking before new feed was administered using a PS-2000 scale (Salter Brecknell, Fairmont, MN) on the last day of Pre-Intervention period, and then on Intervention days 7, 14, 21, and 28; and Post-Intervention days 1, 6 and 10.

Milk Yield

Milk weighs were collected at each milking from ICAR approved Waikato MKV milk meters (Waikato, Hamilton, New Zealand) installed on each milking unit long milk hose.

Milk Sampling

Two composite milk samples per cow were collected at each milking on the last day of Pre-Intervention period, during the Intervention and Post-Intervention period. The Waikato Milk Meter retains a small percentage of the yield in a calibrated flask from which two milk samples were collected into 2 oz vials. One sample was analyzed using near-infrared spectroscopy (NIR) for crude protein, fat, and milk urea nitrogen (MUN) at the (b) (4)). The second sample was stored frozen at -20°C at (b) (4) laboratory and shipped to Ascus Biosciences Laboratory at the end of the experiment.

Rumen Digesta Sampling

Rumen samples were collected once a day prior to inoculation after the morning milking on Intervention days 1, 2, 3, 5, 8, 11, 14, 17, 20, 23, 26, 29, and 32; and Post Intervention days 1, 4, 7 and 10. Two composite rumen samples were collected into 15 mL conicals from the dorsal, central, anterior and caudal parts of the rumen, consisting of both fluid and particulate. Rumen samples required the fixing of cells with 10% stock solution of 5% phenol and 95% ethanol. Conicals were sealed with parafilm and shipped frozen to Ascus Biosciences facility for microbial analysis.

Rumen pH

Rumen pH was measured on the last day of the Pre-Intervention period, and daily during the Intervention before inoculation and Post-Intervention periods. The rumen digesta was hand stirred and then scooped with a 13 mL vial. The pH was recorded immediately after ruminal fluid collection using a pH meter (Hanna Instruments, Woonsocket, RI).

TMR Sampling

One sample of TMR was collected on Pre-Intervention day 9, Intervention days 6, 13, 20, and 27; and Post-Intervention days 1, 5, and 9. TMR ingredients are reported in Table 2 and nutrient composition on Table 3. TMR samples were always collected one day before fecal sampling. TMR samples were collected using the quartering method at the different sampling times, stored frozen in vacuum-sealed bags and shipped to (b) (4) at the end of the study to be analyzed using the NIR1 Plus Package. The NIR 1 Analysis includes tests for Dry Matter, Moisture, Crude Protein, ADF Protein, NDF Protein, Soluble Protein, ADF, NDF, NDFom, Lignin, Starch, Sugar, Fat, Ash, Calcium (Ca), Phosphorus (P), Magnesium (Mg), and Potassium (K). The NIR 1 Plus package in addition to what is evaluated in the NIR1 Package provides 30 hr NDF Digestibility with Kd Rate, NDF Digestibility at 120 and 240 hrs, uNDF120, and uNDF240.

Fecal Sampling

Feces were collected from the rectum using a palpation sleeve immediately after weighing the cows. Fecal samples were collected on the last day of the Pre-Intervention period, and then on Intervention days 7, 14, 21 and 28; and Post-Intervention days 2, 6 and 10. Approximately 55 g of feces was placed into 2 oz. vials, stored frozen and shipped at the end of the trial to (b) (4), (b) (4) to be analyzed using the NIR1 Plus Package.

Outcomes Evaluated

Dry Matter Intake (DMI)

It is the feed consumed (Kg) in an as fed basis times the dry matter percentage of the feed obtained from the laboratory analysis The feed consumed was calculated by subtracting the amount of feed refused (not eaten) from the feed weight administered to cows on a daily basis.

Milk Yield

Daily milk yield was calculated as the sum of both morning and afternoon milk weights (Kg).

3.5% Fat Corrected Milk (FCM)

Milk yield value corrected for 3.5% fat using formula from NRC (2001): [(0.4324 × kg of milk) + (16.216 × kg of fat)].

Energy Corrected Milk (ECM)

Milk yield value corrected for 3.5% fat and 3.2% true protein using formula from NRC (2001): [(0.3246 × kg of milk) + (12.86 × kg of fat) + (7.04 × kg of true protein)].

Milk Components Percentage

Daily milk crude protein (%), fat (%), lactose (%), and MUN concentration (mg/dL) were calculated as the average of both morning and afternoon milk samples analysis results.

Milk Components Yield

Obtained multiplying daily milk crude protein (%), fat (%), lactose (%) and MUN (mg/dL) by the daily milk yield (Kg).

Feed Efficiency

Defined as Kg of 3.5% FCM produced per Kg of DM consumed.

Daily Body Weight Gain

Calculated as the difference in body weight between two measures divided by the number of days in between.

Rumen pH

pH reading from the days which was measured.

Fecal Matter

It was evaluated dry matter (DM), starch, NDF, protein, and lignin.

Apparent Nutrient Digestibility

Includes a NIR Plus evaluation of feed and associated fecal matter to generate an evaluation of apparent nutrient digestibility. In order to calculate nutrient digestibility 240-hr in vitro digestion is was performed and undigested NDF at 240 hr (uNDFom240) is used as a marker. It assumes the amount of uNDFom240 is constant in both the feces and the feed so the relative differences between the feed and feces will give the estimate of digestibility. It allows to determine the amount of CP, NDF and starch in the manure without having to measure the quantity of manure cows are producing.

Study Incidences

During the Pre-Intervention period, Cow 10 which was assigned to Control had a displaced abomasum, which negatively led to a loss of appetite, drop in milk yield and mild diarrhea. The sick animal was removed from the study and data from this cow was not used in the analysis. This cow was replaced by another cow on January 30th, 2016 (Intervention day 3) and data from this cow was used in the analysis.

In addition, cows with study IDs 8, 14, 16 had health problems (fever, displaced abomasum, etc) with episodes of anorexia and low milk production. Finally, cows 3 and 7 although healthy produced less milk than expected due to a large daily variation in milk production.

Statistical Analysis and Results Layout

Milk production, milk composition, body weight gain and rumen pH were measured daily on 16 cows for 32 days during treatment application and another 10 days after inoculation. Fecal nutrients concentration and nutrients apparent digestibility were measured by pooling two cows within the same treatment group such that 8 experimental units were available for analysis. Therefore, the present report is structured in three sections: 1) The first section (SECTION I) presents the results of the statistical analysis of dry matter intake (DMI), milk production, milk composition, body weight gain and rumen pH during the Intervention period; 2) The second section (SECTION II) includes graphical representation of dry matter intake, milk production, milk composition, body weight gain and rumen pH during the Intervention and Post-Intervention periods; and, 3) The third section (SECTION III) presents the results of the statistical analysis of digestibility.

SECTION I: Dry Matter Intake, Milk Production and Composition, Body Weight Gain and Rumen pH During the Intervention Period

Statistical Analysis

Data was analyzed using the SAS/STAT software, Version 9.3 of the SAS System for PC. Copyright © 2014 SAS Institute Inc., Cary, NC, USA. Daily values were originally analyzed implementing random coefficients models with linear and quadratic terms. However, due to the small sample size and the model complexity, for several of the outcomes the model convergence was not obtained. Therefore, daily values were averaged to produce weekly means. Week 5 averages included only 4 days while the remaining weeks included 7 daily values. Weekly DMI, milk yield, milk composition, body weight gain and rumen pH were analyzed as repeated measures using the MIXED procedure available within SAS/STAT software. The model included the fixed effect of treatment (Control vs. Inoculated), time (week 1, 2, 3, 4 and 5) and their interaction. Milk yield and DMI measured the three days prior to treatment application, were averaged and used as covariate for the corresponding outcome variable. Cow within treatment was the subject of the repeated statement. The covariance structure that provided the best fit according to the Bayes Information Criterion (BIC) was chosen. The covariance structure employed consisted of unstructured for DMI, milk protein and lactose percentages and fat yield, compound symmetry for milk urea nitrogen, and first order autoregressive for the remaining outcomes. Furthermore, where appropriate separate residual variances for each treatment were estimated as they provided a better fit according to BIC. When a significant treatment by time interaction was observed, treatment means within week were compared using the SLICE option. Significance was declared at p-value <0.05 and tendency was declared at 0.05≤ p-value <0.10.

A total of two analyses were conducted on the collected data: 1. The first analysis (n=16) included all collected observation on all cows; and, 2. The second analysis (n=11) excluded three cows (study IDs 8, 14 and 16) from Control that had health events and two cows from Intervention (study IDs 3 and 7) because of large daily milk production variability. All the analyses were executed using the previously described models, except that for analyses two the covariance structure for the repeated measures was reassessed. The covariance structure employed consisted of unstructured for feed efficiency, compound symmetry for fat percentage and milk urea nitrogen, and first order autoregressive for the remaining outcomes. Analysis 1 is reported in the Results section while analyses 2 is reported as Appendix B.

Results

Treatment least square means, fixed effects and covariance parameters estimates of the analysis including all cows (analysis 1) are reported in Table I-1 and Figures I-1 to I-13. A treatment by week interaction was observed for milk yield (P = 0.0025, Figure I-2), FCM (P = 0.0026, Figure I-3), ECM (P = 0.0019, Figure I-4), and protein yield (P = 0.0012, Figure I-8). A tendency for a treatment by week interaction was also observed for fat yield (P = 0.0880, Figure I-9), feed efficiency (FE, P = 0.0671, Figure I-11) and rumen pH (P = 0. 0741, Figure I-13). The interaction for yields was mainly the result of milk yield diverging between the two treatments within the first 2-3 weeks of the study, but not toward the end of the Intervention period.

A tendency for a higher milk fat percentage for Inoculated vs. the Control was observed (P = 0.0991). Although the treatment by week interaction was not significant (P = 0.2677, Figure I-6), it can be observed that milk fat percentages were numerically similar within the first two weeks and numerically higher for Intervention during weeks three to five. No other main effect was either significant or tended to be significant without also having a significant treatment by week effect.

Comment: The statistical analysis performed included all the weekly means when the treatment was applied; as such treatment by time interactions should be the main focus. Treatment main effects and least square means included the weekly values at the beginning of the Intervention period when cows still not responded to treatment due to adaptation. Furthermore, as the number of cows was not very large the main focus should be effect size and not the lack or presence of statistical significance.

Table I-1: Dry matter intake, milk production and composition, BW gain and rumen pH least square means (± SEM) of cows assigned to Control and Inoculated.

	Treatment		Fixed Effects[1]			
Outcome	Control	Inoculated	Cov	Tx	Week	Tx*Week
				----------Pr > F----------		
DMI, kg	26.2 ± 2.8	30.2 ± 1.2	0.0030	0.2201	0.0001	0.1910
Milk yield, kg	25.7 ± 1.9	30.6 ± 1.9	0.0020	0.0791	0.3996	0.0025
FCM, kg	27.7 ± 2.5	32.5 ± 2.5	--	0.1883	0.2221	0.0026
ECM, kg	27.2 ± 2.4	32.1 ± 2.4	--	0.1669	0.1968	0.0019
Milk components, %						
Crude Protein	3.08 ± 0.06	3.27 ± 0.11	--	0.1553	0.1119	0.3125
Fat	3.87 ± 0.08	4.06 ± 0.08	--	0.0991	0.0876	0.2677
Lactose	4.64 ± 0.10	4.73 ± 0.03	--	0.3787	0.6162	0.5016
Milk components yield, kg						
Crude Protein	0.80 ± 0.07	0.97 ± 0.07	--	0.1183	0.0545	0.0012
Fat	1.01 ± 0.10	1.20 ± 0.10	--	0.1818	0.1304	0.0880
MUN, mg/dL	6.17 ± 0.60	7.41 ± 0.45	--	0.1222	<0.0001	0.3440
FCM/DMI	1.22 ± 0.07	1.10 ± 0.07	--	0.2835	<0.0001	0.0671
BW gain, kg/day	0.78 ± 0.44	1.46 ± 0.43		0.2838	0.4960	0.3335
Rumen pH	6.24 ± 0.09	6.05 ± 0.09	--	0.1600	0.0044	0.0741

[1]Cov= covariate effect, Tx = treatment effect, Day = day effect; Tx*Day = treatment by day interaction.

Figure I-1: Dry matter intake (kg) daily means (no fill) and covariate adjusted weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 2201) and treatment by time interaction (P = 0.1910). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

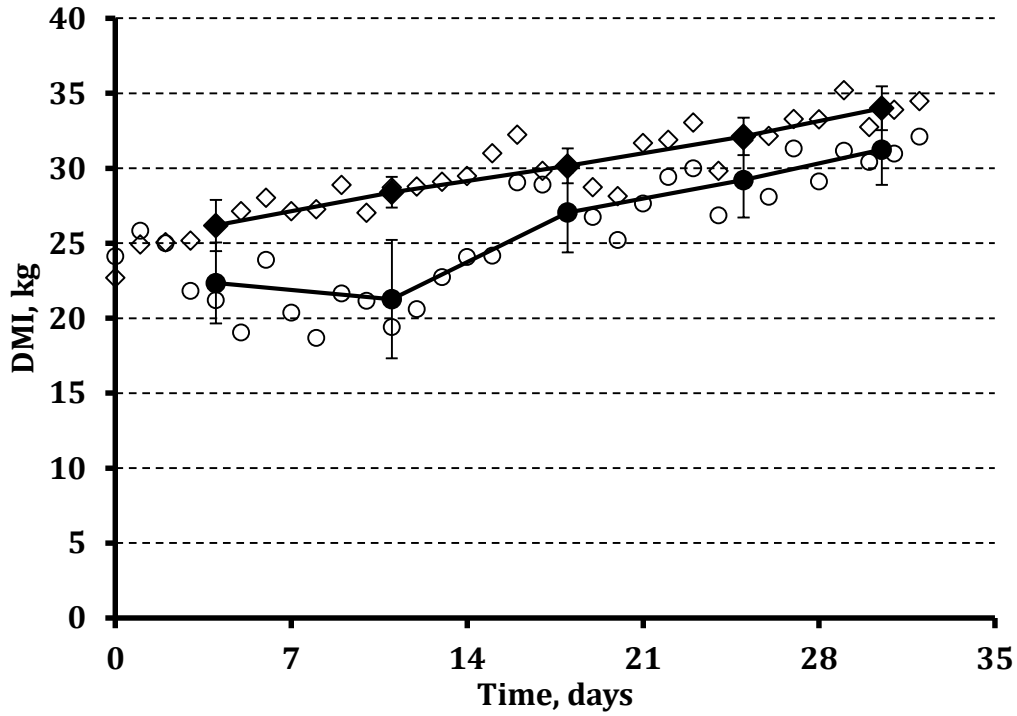


Figure I-2: Milk yield (kg) daily means (no fill) and covariate adjusted weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 0791) and treatment by time interaction (P = 0.0025). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

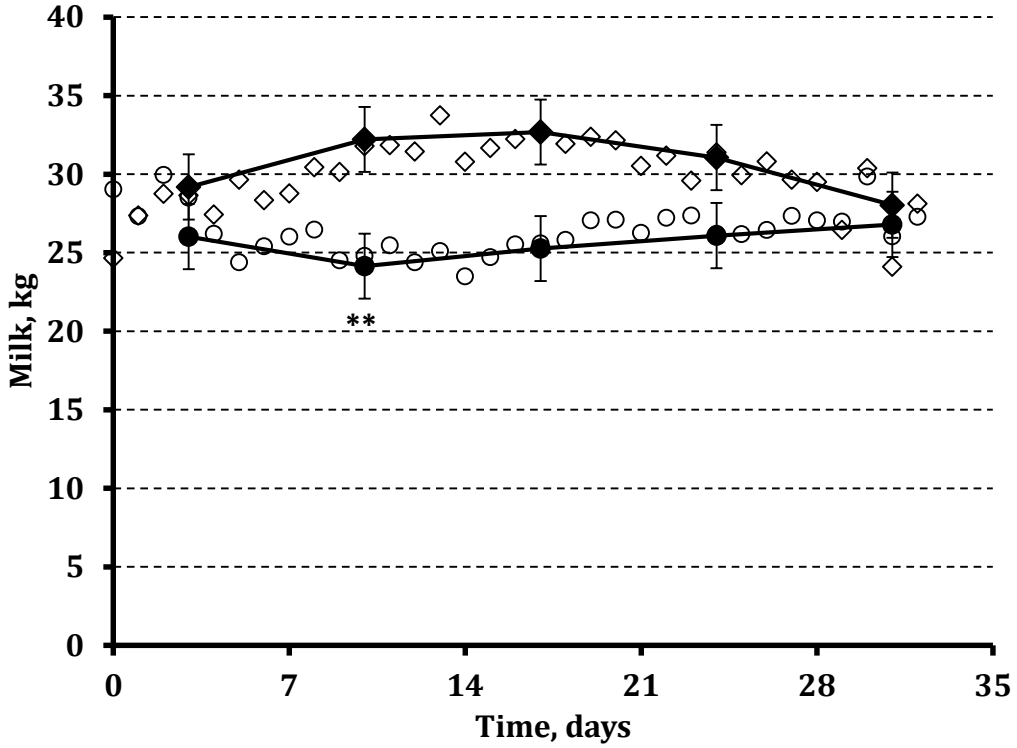


Figure I-3: Fat corrected milk yield (FCM, kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1883) and treatment by time interaction (P = 0.0026). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

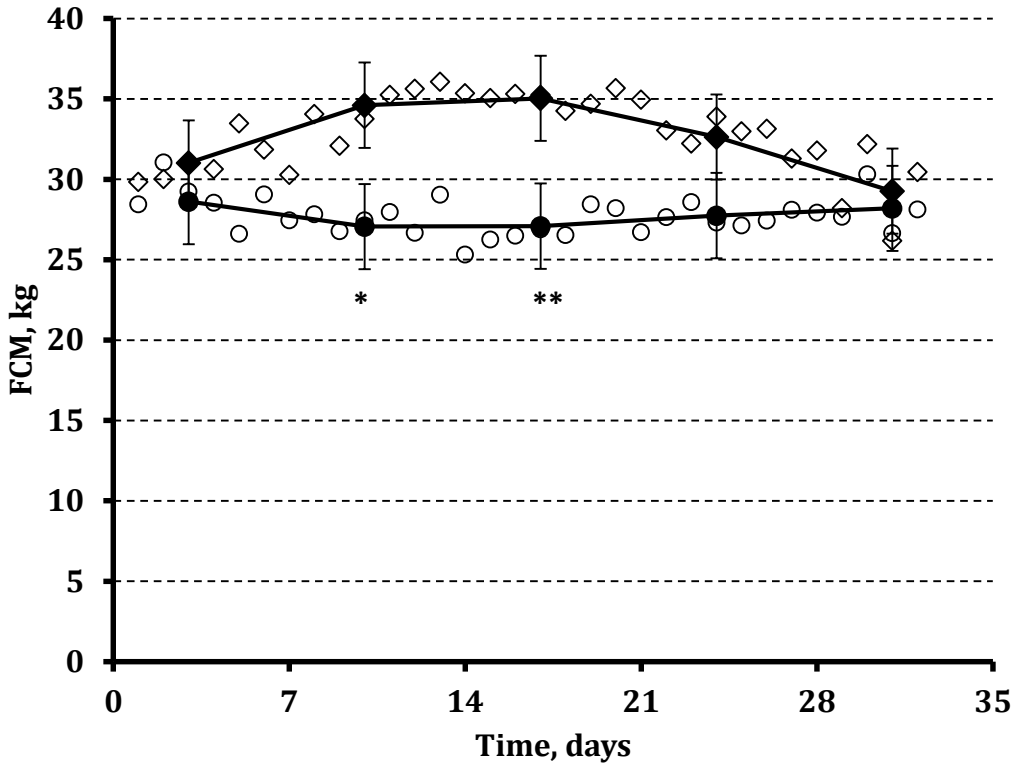


Figure I-4: Energy corrected milk yield (ECM, kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1669) and treatment by time interaction (P = 0.0019). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

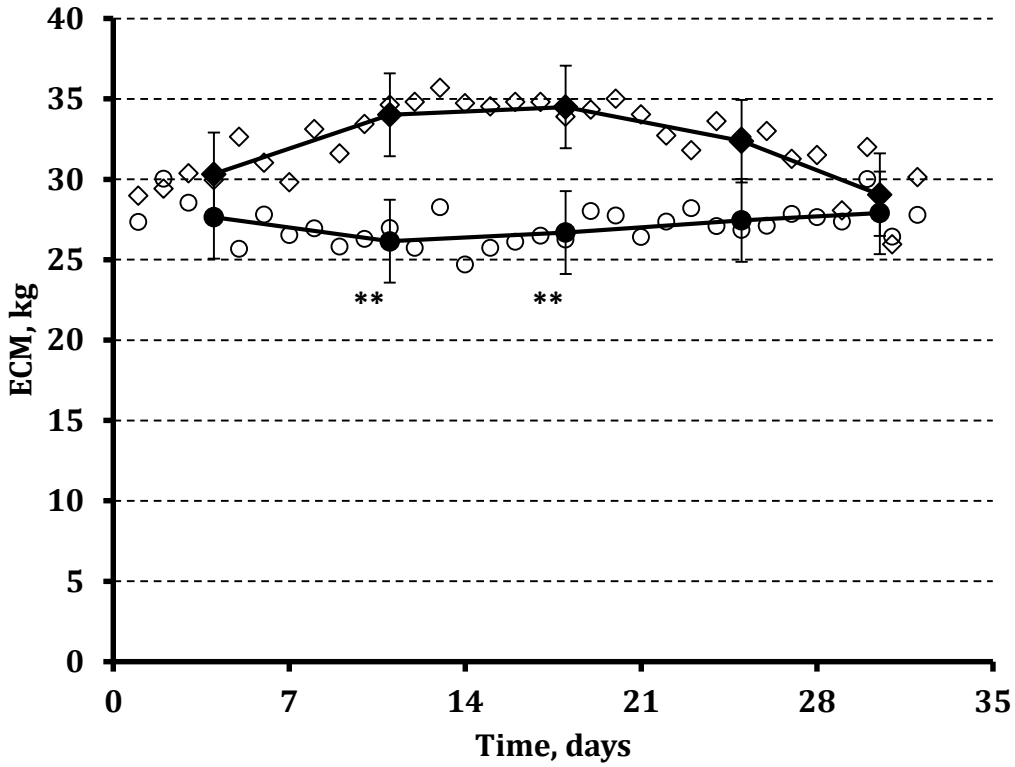


Figure I-5: Milk crude protein (CP, %) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1553$) and treatment by time interaction ($P = 0.3125$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).

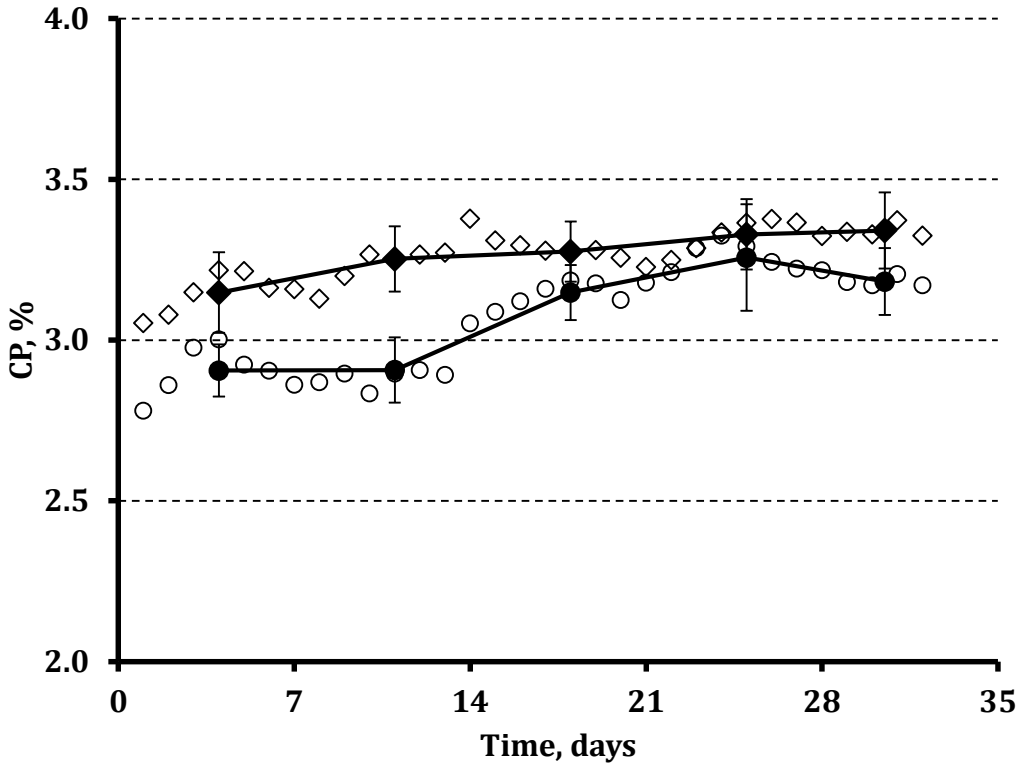


Figure I-6: Milk fat (%) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.0991) and treatment by time interaction (P = 0.2677). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

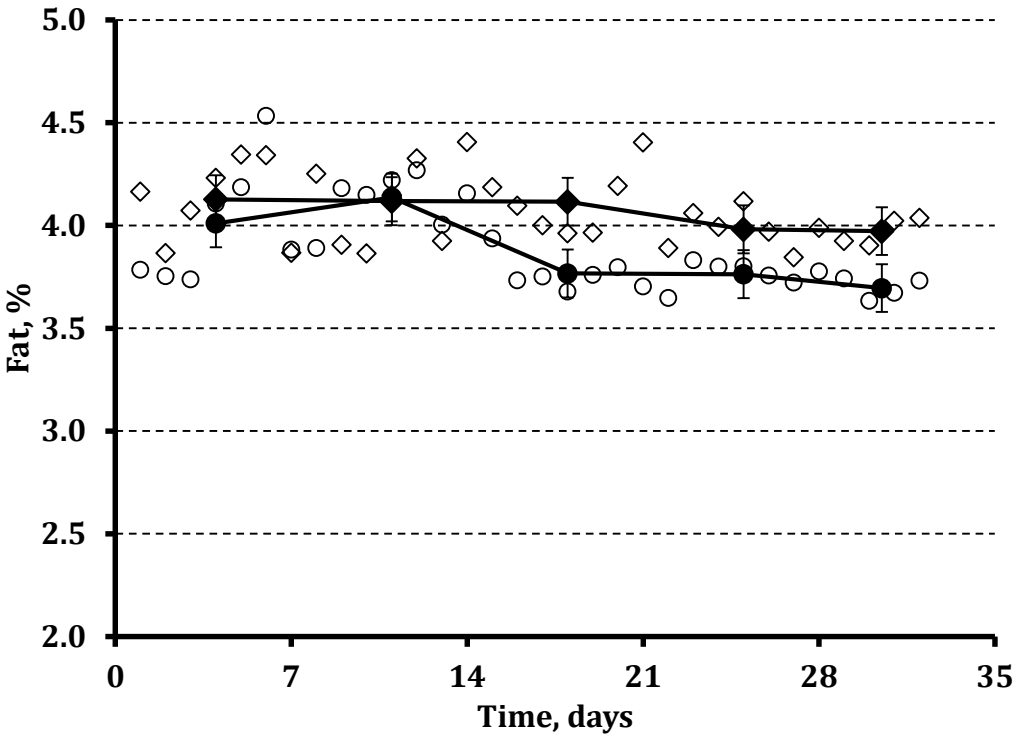


Figure I-7: Milk lactose (%) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.3787) and treatment by time interaction (P = 0.5016). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

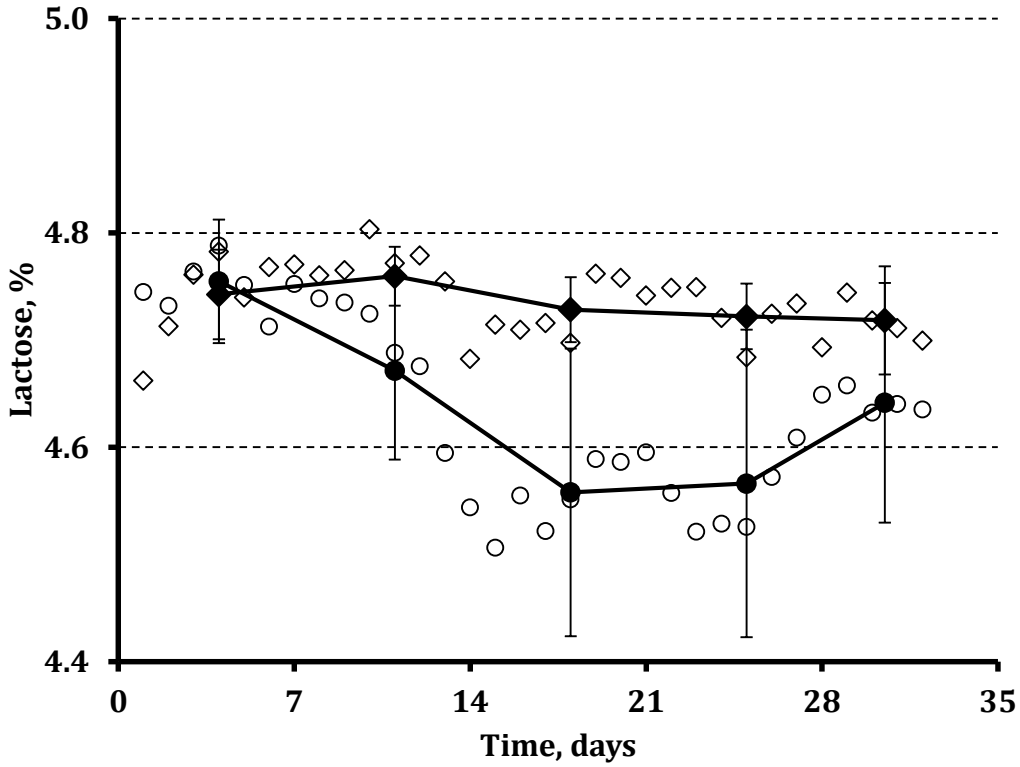


Figure I-8: Milk crude protein yield (CP, kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1183) and treatment by time interaction (P = 0.0012). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

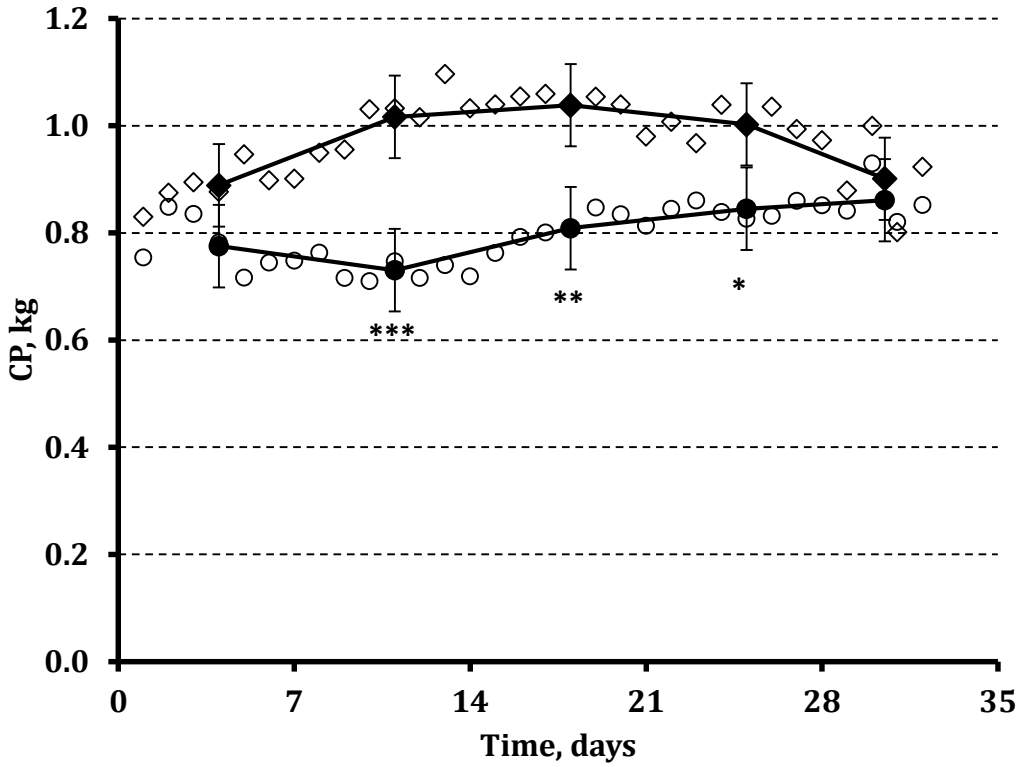


Figure I-9: Milk fat yield (kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1818) and treatment by time interaction (P = 0.0880). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

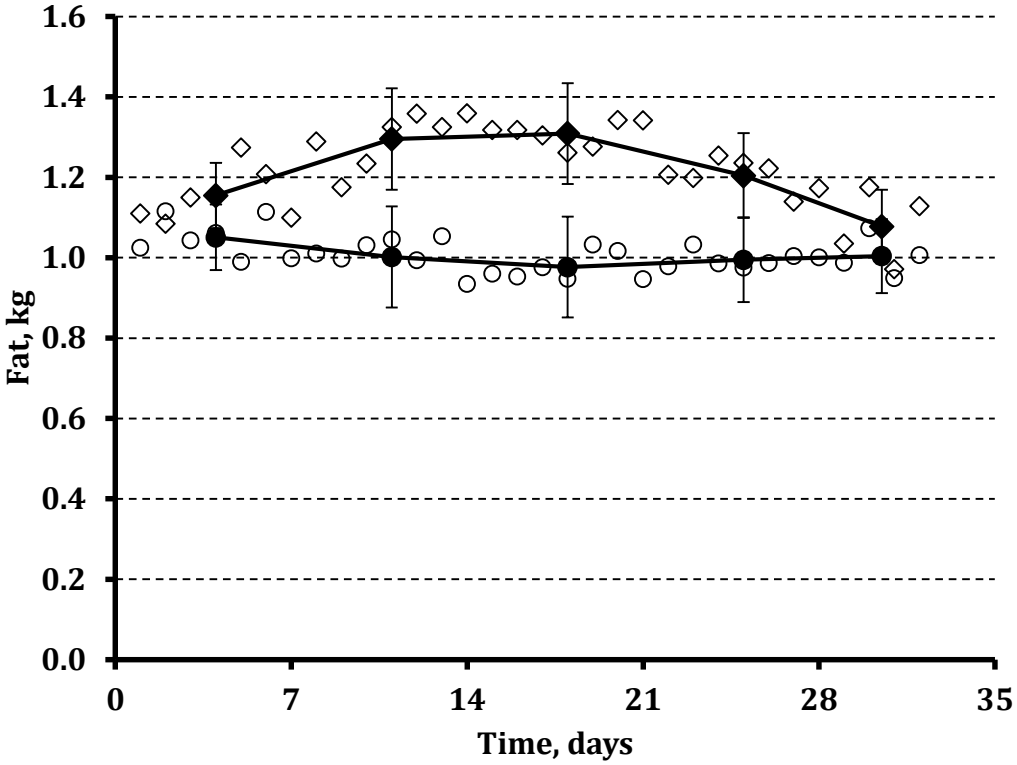


Figure I-10: Milk urea nitrogen (MUN, mg/dL) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1222) and treatment by time interaction (P = 0.3440). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

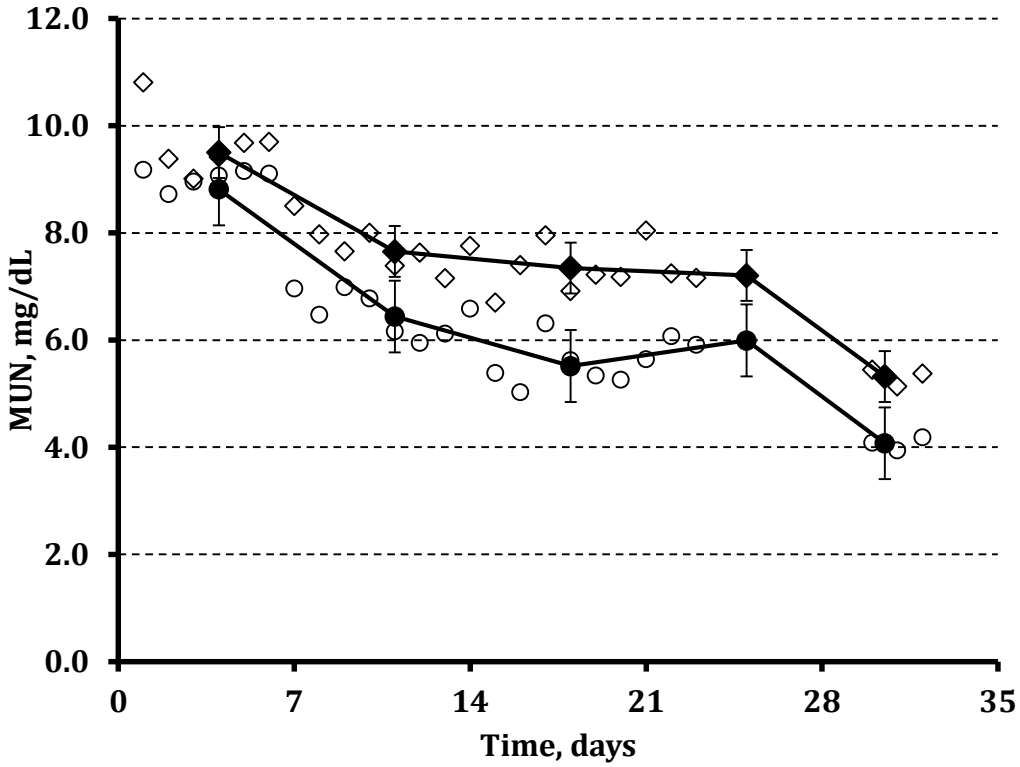


Figure I-11: Feed efficiency (FCM/DMI) means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.2835) and treatment by time interaction (P = 0.0671). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

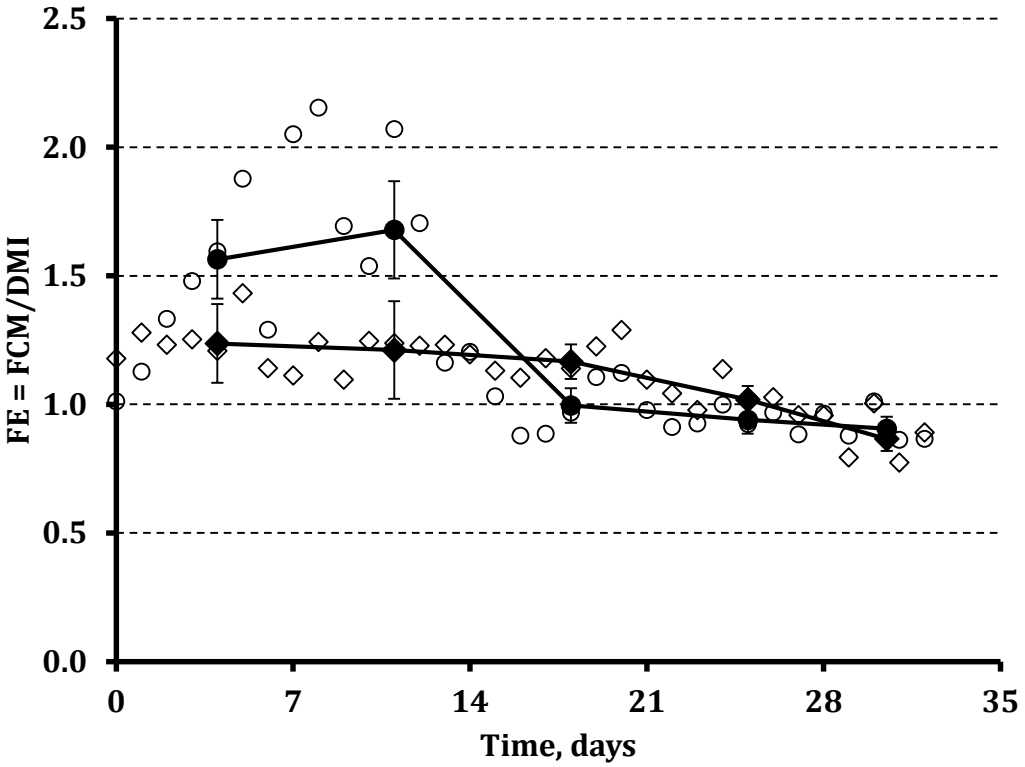


Figure I-12: BW gain (kg/day) weekly least square means± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.2838$) and treatment by time interaction ($P = 0.3335$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).

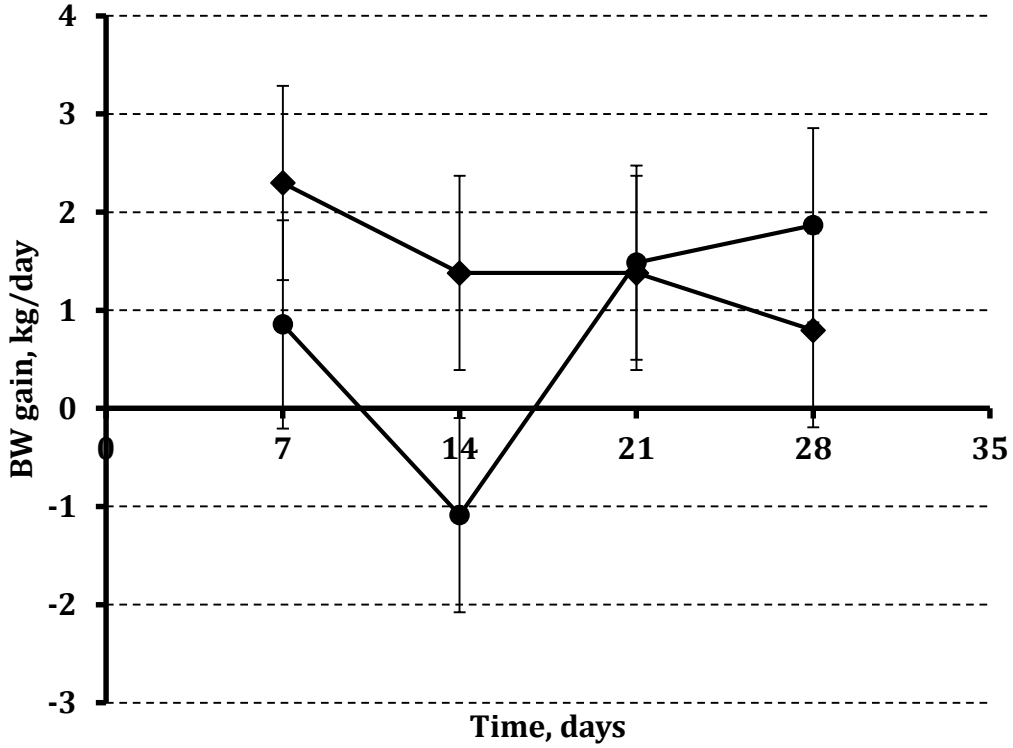
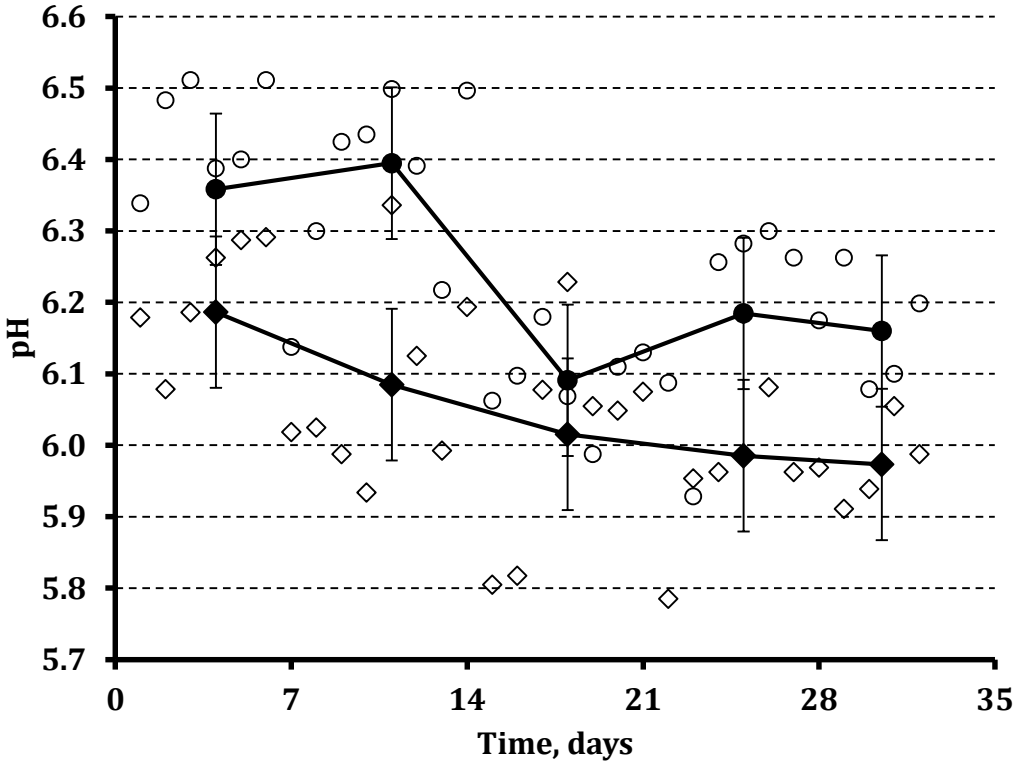


Figure I-13: Rumen pH daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1600) and treatment by time interaction (P = 0.0741). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).



SECTION II: Dry Matter Intake, Milk Production and Composition, Body Weight Gain and Rumen pH During the Intervention and Post-Intervention Periods

As previously stated, the following section reports SECTION 1 figures with added on a graphical representation of the production portion of the study once the supplementation ended.

Figure II-1: Dry matter intake (kg) daily means (no fill), covariate adjusted weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 2201) and treatment by time interaction (*P* = 0.1910). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01). The vertical line represents the end of the feeding trial.

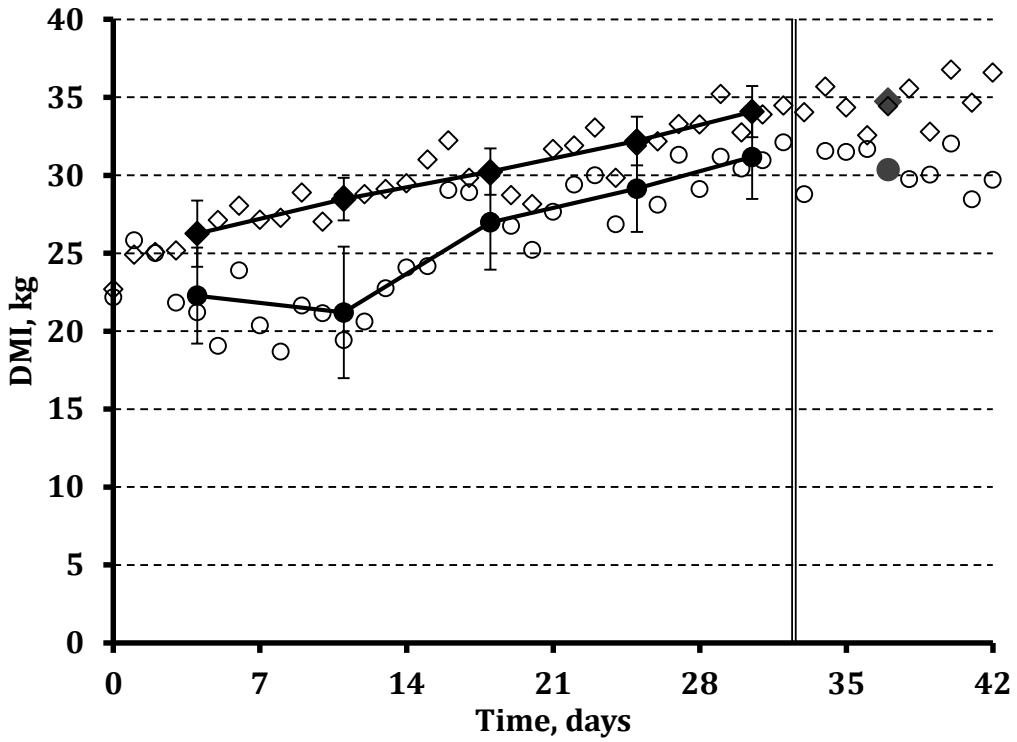


Figure II-2: Milk yield (kg) daily means (no fill), covariate adjusted weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 0791) and treatment by time interaction (*P* = 0.0025). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01). The vertical line represents the end of the feeding trial.

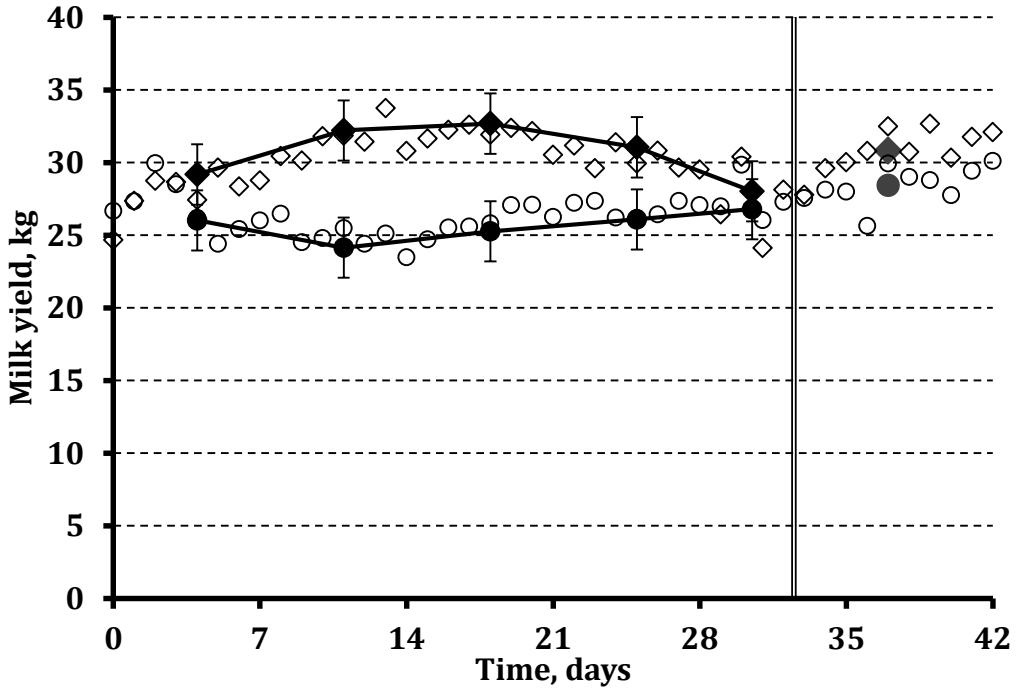


Figure II-3: Fat corrected milk yield (FCM, kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1883) and treatment by time interaction (P = 0.0026). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.

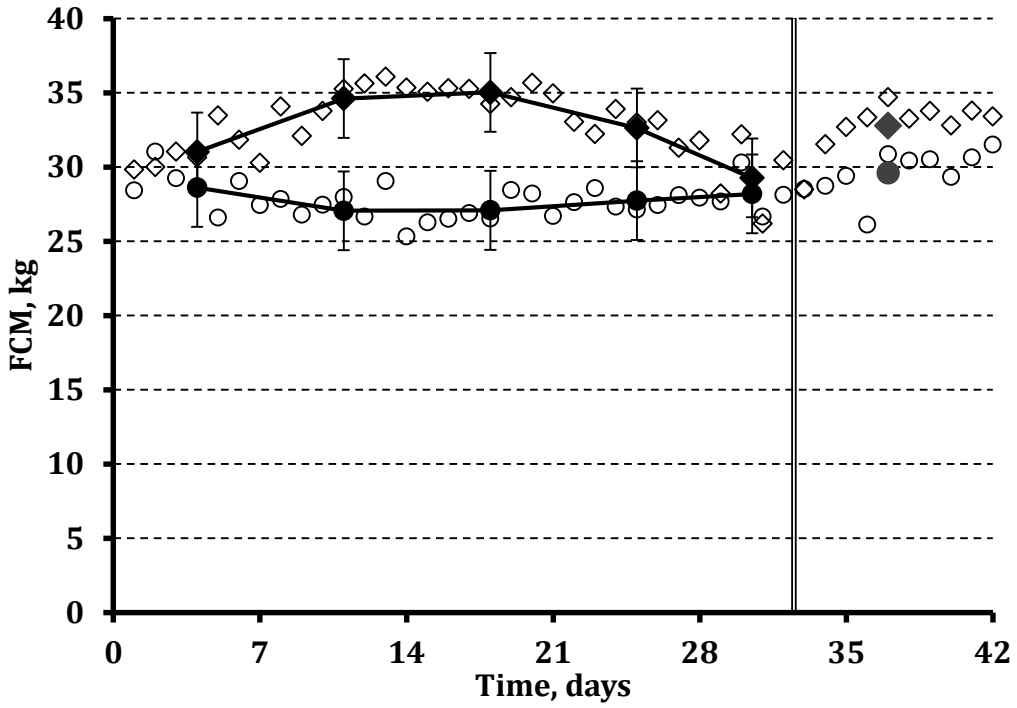


Figure II-4: Energy corrected milk yield (ECM, kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1669) and treatment by time interaction (P = 0.0019). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.

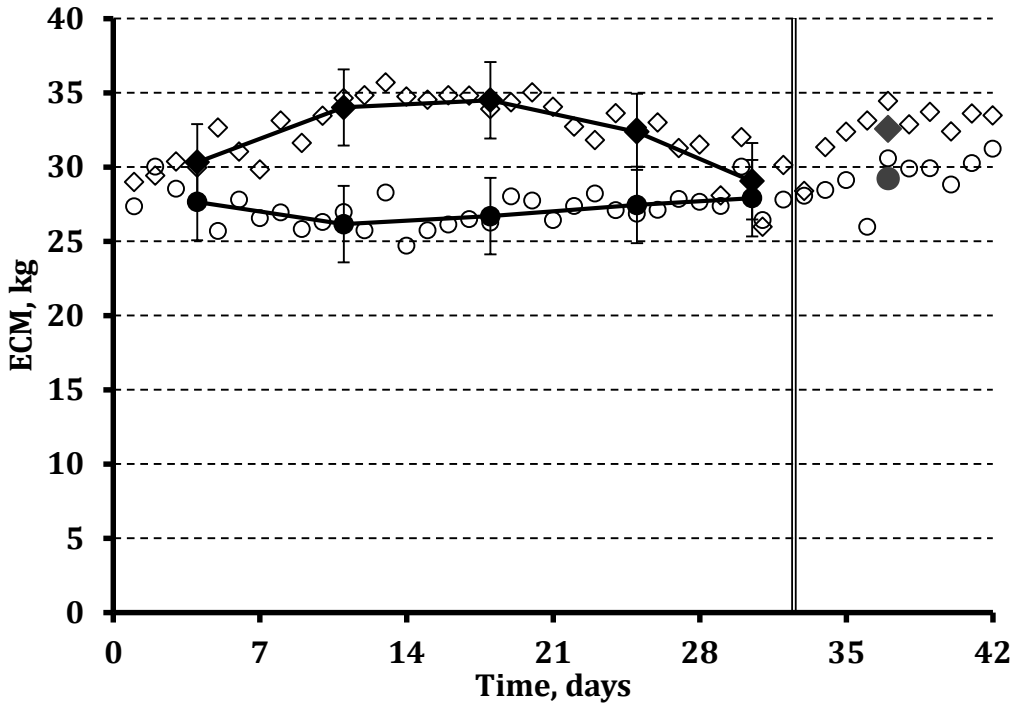


Figure II-5: Milk crude protein (CP, %) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1553$) and treatment by time interaction ($P = 0.3125$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

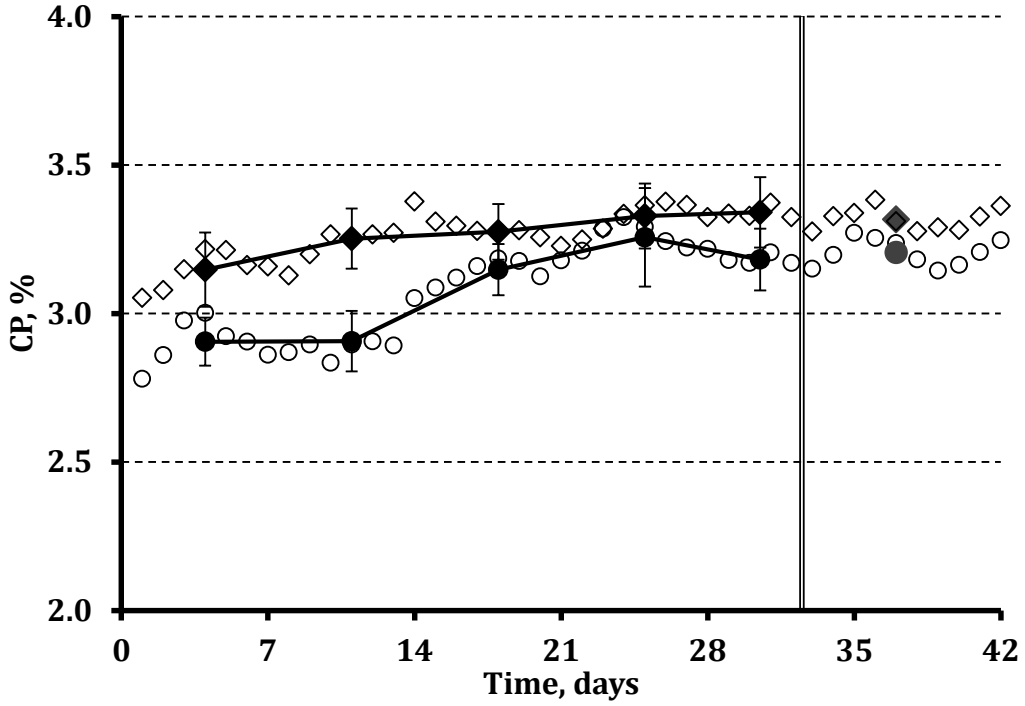


Figure II-6: Milk fat (%) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.0991) and treatment by time interaction (P = 0.2677). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.

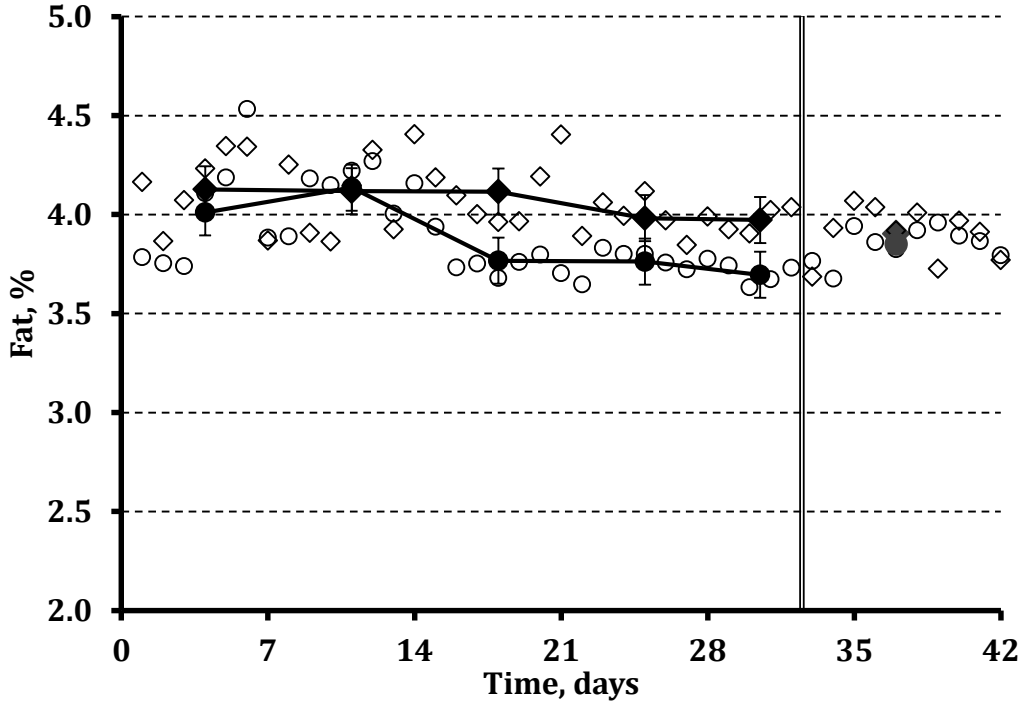


Figure II-7: Milk lactose (%) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.3787) and treatment by time interaction (P = 0.5016). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.

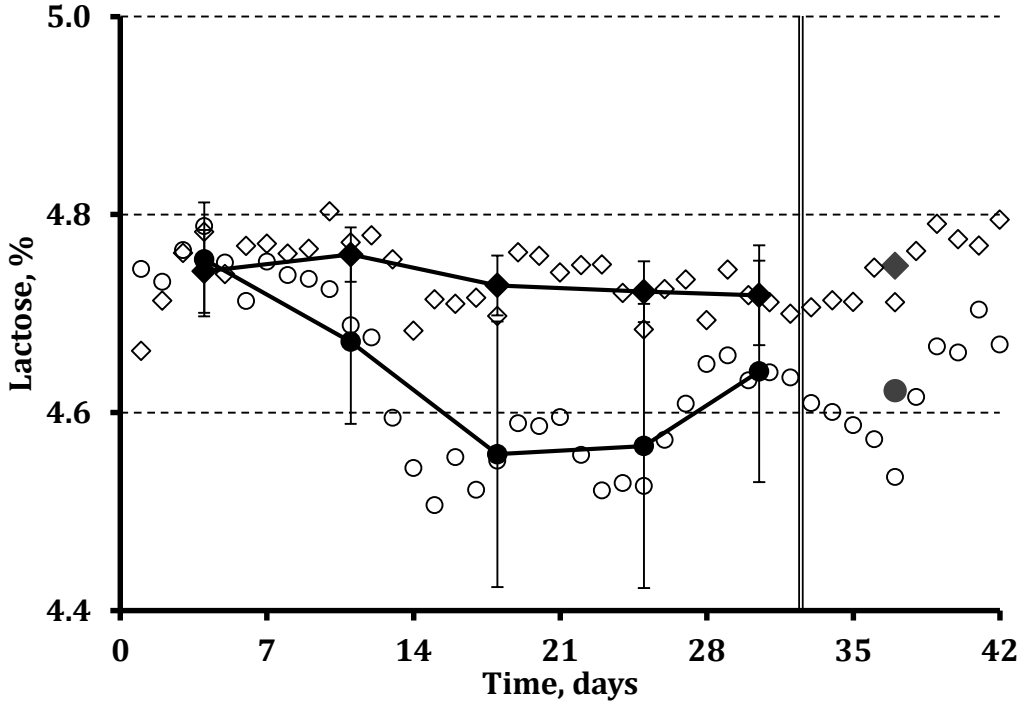


Figure II-8: Milk crude protein yield (CP, kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1183$) and treatment by time interaction ($P = 0.0012$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

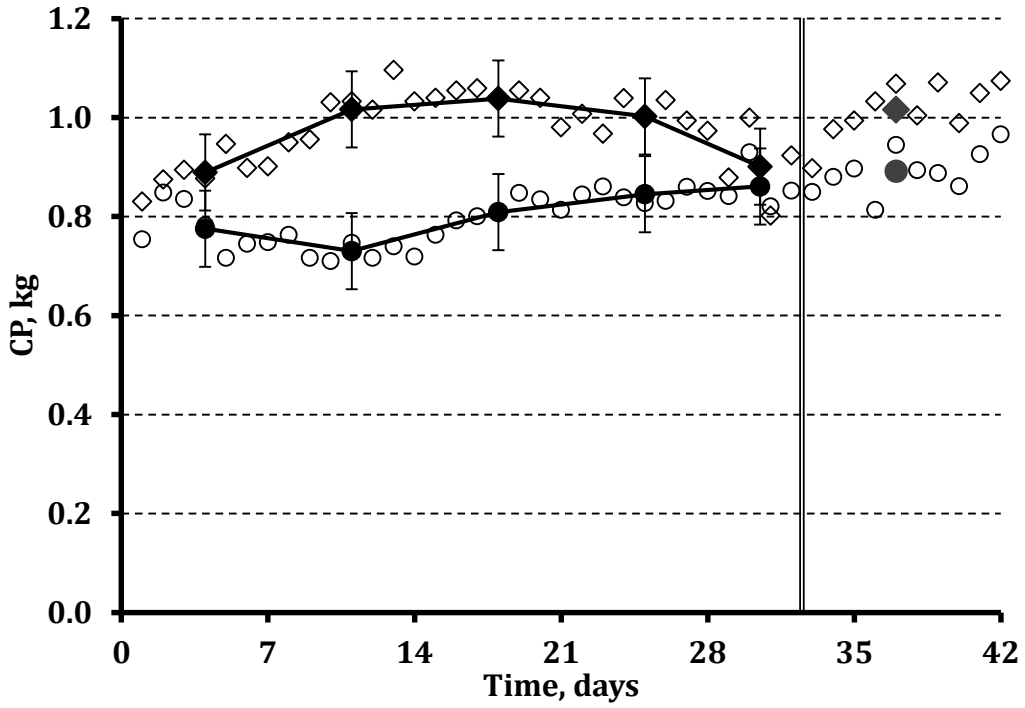


Figure II-9: Milk fat yield (kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0.1818) and treatment by time interaction (*P* = 0.0880). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01). The vertical line represents the end of the feeding trial.

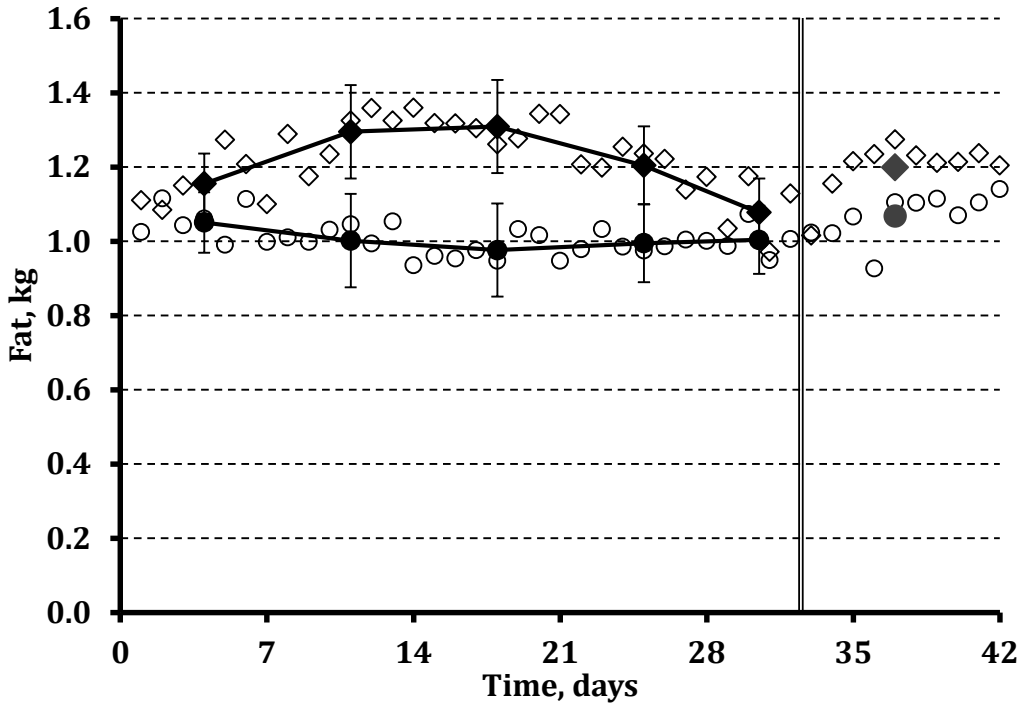


Figure II-10: Milk urea nitrogen (MUN, mg/dL) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0.1222) and treatment by time interaction (*P* = 0.3440). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

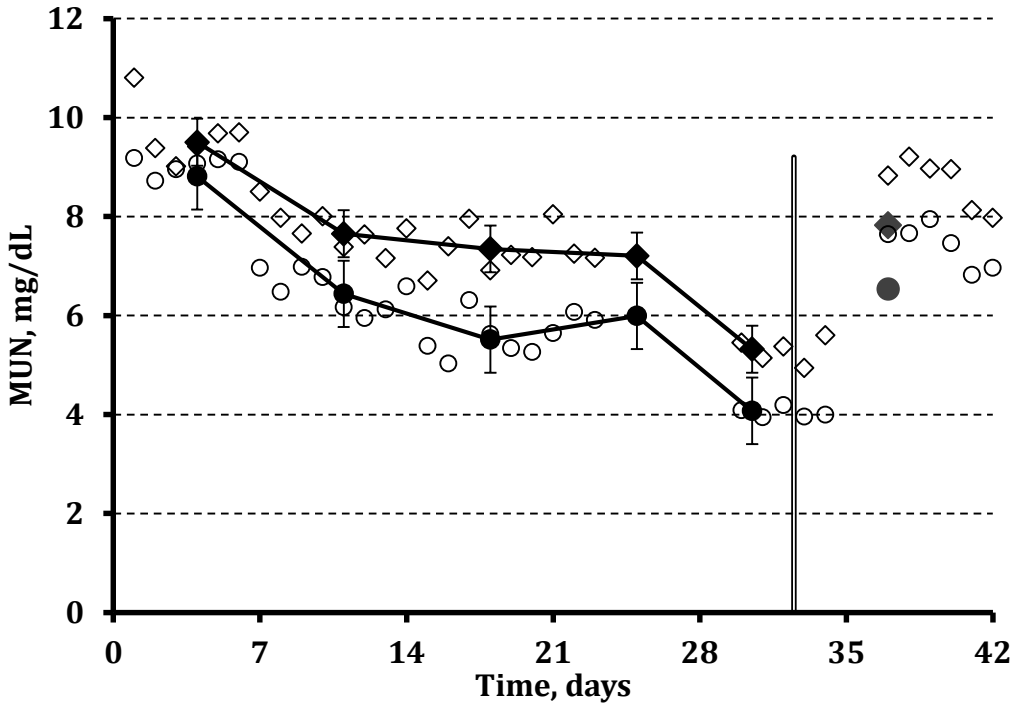


Figure II-11: Feed efficiency (FCM/DMI) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.2835$) and treatment by time interaction ($P = 0.0671$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

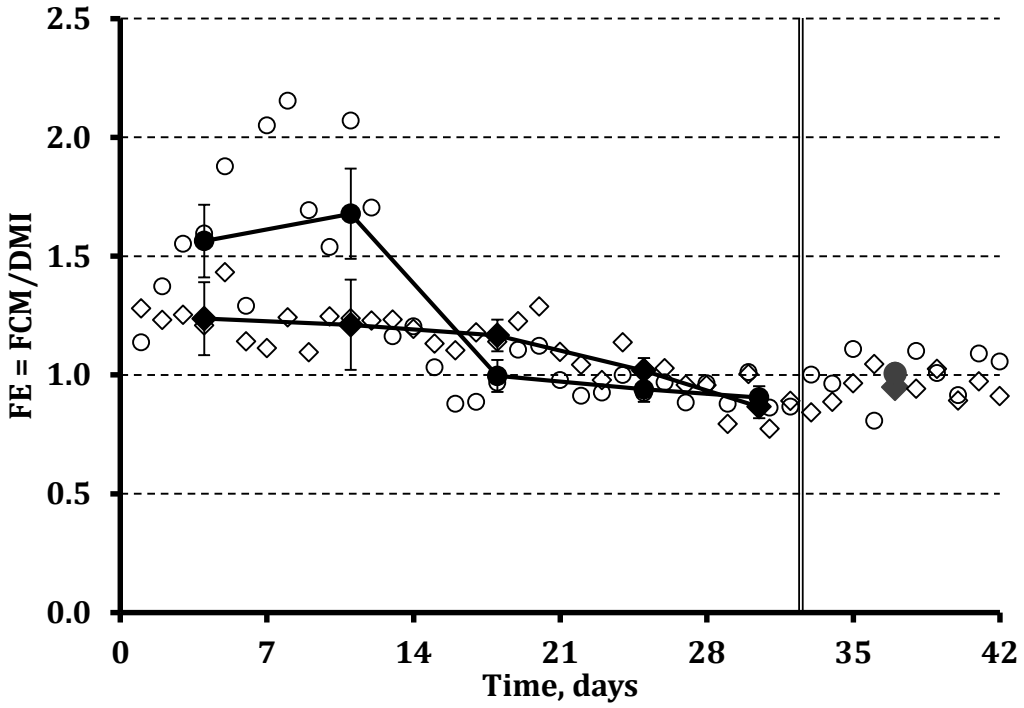


Figure II-12: BW gain (kg/day) weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) weekly least square means± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0.2838) and treatment by time interaction (*P* = 0.3335). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01). The vertical line represents the end of the feeding trial.

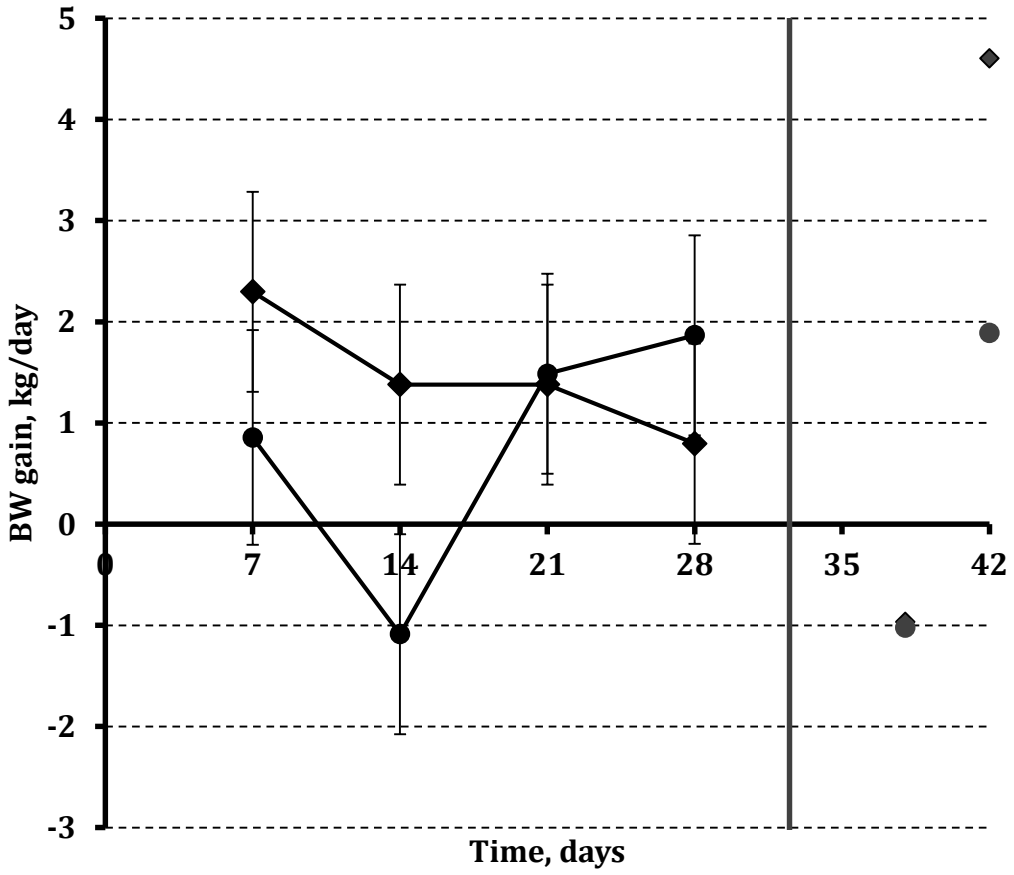
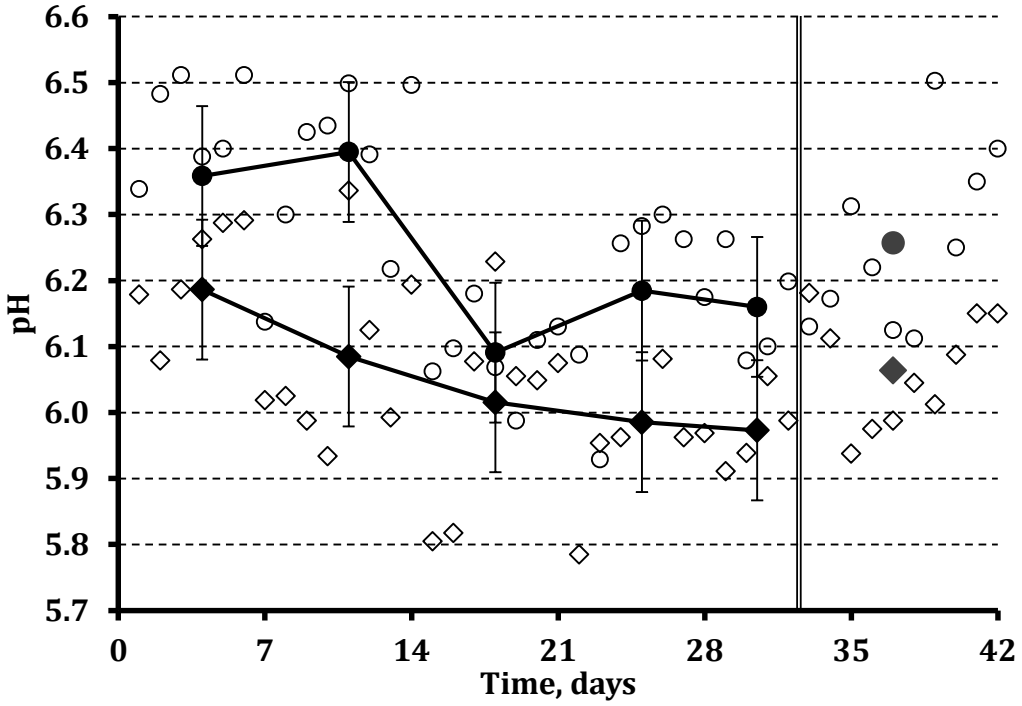


Figure II-13: Rumen pH daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0.1600) and treatment by time interaction (*P* = 0.0741). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01). The vertical line represents the end of the feeding trial.



SECTION III: Nutrient Composition of Feces and Digestibility

Statistical Analysis

Data was analyzed using the SAS/STAT software, Version 9.3 of the SAS System for PC. Copyright © 2014 SAS Institute Inc., Cary, NC, USA. Weekly fecal nutrients concentration and apparent nutrients digestibility were analyzed as repeated measures using the MIXED procedure available within SAS/STAT software. The model included the fixed effect of treatment (Control vs. Inoculated), time (week 1, 2, 3, 4 and 5) and their interaction. Measurements collected prior to treatment application were used as a covariate for the corresponding outcome variable. Unit ID within treatment was the subject of the repeated statement. The covariance structure that provided the best fit according to the Bayes Information Criterion (BIC) was chosen. The covariance structure employed consisted of compound symmetry for fecal percentage of DM, starch, NDF and protein and unstructured for the remaining outcomes. When a significant treatment by time interaction was observed, treatment means within week were compared using the SLICE option. Significance was declared at *p*-value <0.05 and tendency was declared at 0.05≤ *p*-value <0.10.

Results

Treatment least square means, fixed effects and covariance parameters estimates of the analysis including all units (analysis 1) are reported in Table III-1 and Figures III-1 to III-8. No significant treatment by week or main effect of treatment was observed on any of the outcomes measured. Fecal starch percentage tended to be higher for Inoculated vs Control (*P* = 0.0714) and consequently also a tendency for a lower starch digestibility for Inoculated was observed (*P* = 0. 0745).

Table III-1: Fecal matter concentration and digestibility least square means of cows assigned either to control or Inoculated.

	Treatment			Fixed Effects[1]			
Outcome	Control	Inoculated	SEM	Cov	Tx	Week	Tx*Week
Fecal matter, %					----------Pr > F----------		
DM	15.8	15.9	0.4	0.7429	0.9170	0.4837	0.6705
Starch	5.4	7.2	0.5	0.0356	0.0714	0.0004	0.2842
NDF	52.0	51.6	0.5	0.0677	0.5550	0.2417	0.5002
Protein	19.5	19.3	0.5	0.9404	0.7876	0.2909	0.6687
Lignin	11.6	10.8	0.4	0.0005	0.2080	0.0041	0.2597
Digestibility							
Starch	89.6	86.8	0.9	0.0010	0.0745	0.0014	0.6444
NDF	22.2	18.7	2.0	0.0053	0.2728	0.0934	0.6089
Protein	54.2	53.2	1.5	0.5947	0.6630	0.0631	0.2277

[1]Cov= covariate effect, Tx = treatment effect, Day = day effect; Tx*Day = treatment by day interaction.

Figure III-1: Fecal DM (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 9170) and treatment by time interaction (*P* = 0.6705). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

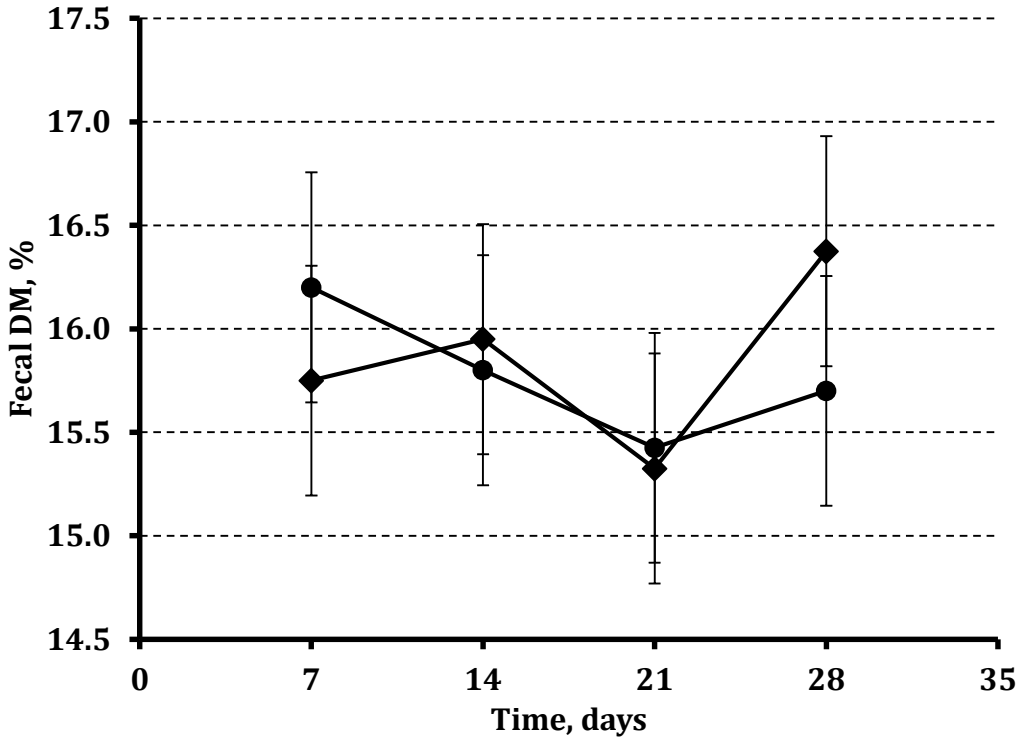


Figure III-2: Fecal Starch (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 0714) and treatment by time interaction (*P* = 0.2842). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

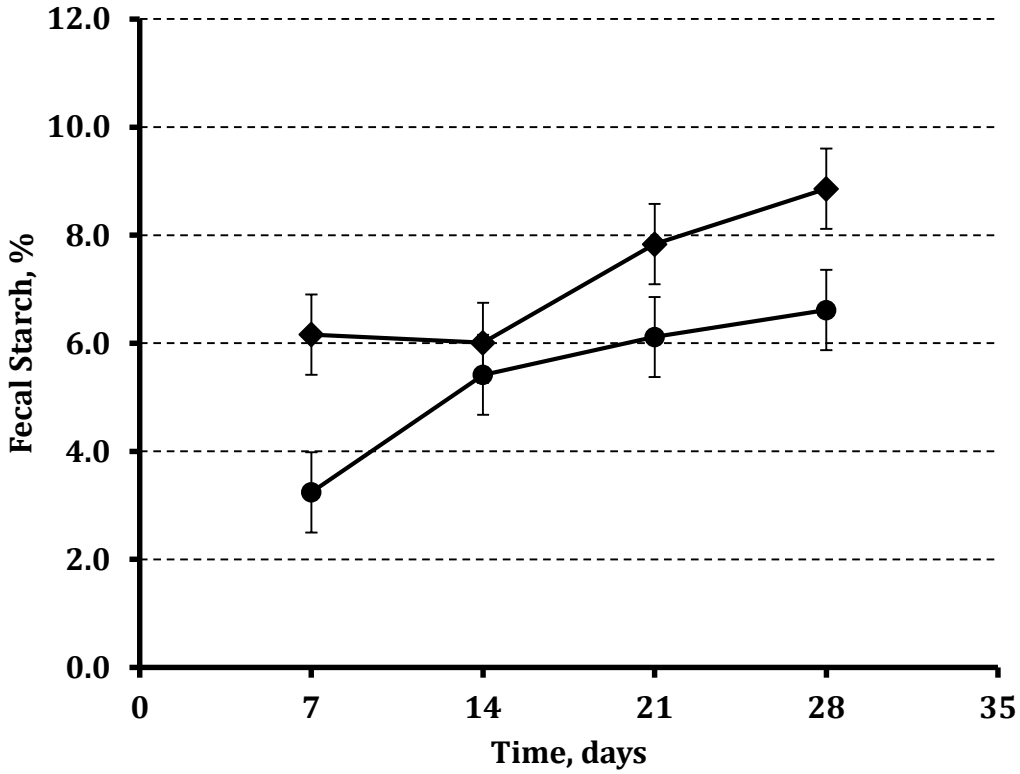


Figure III-3: Fecal NDF (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 5550) and treatment by time interaction (*P* = 0.5002). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

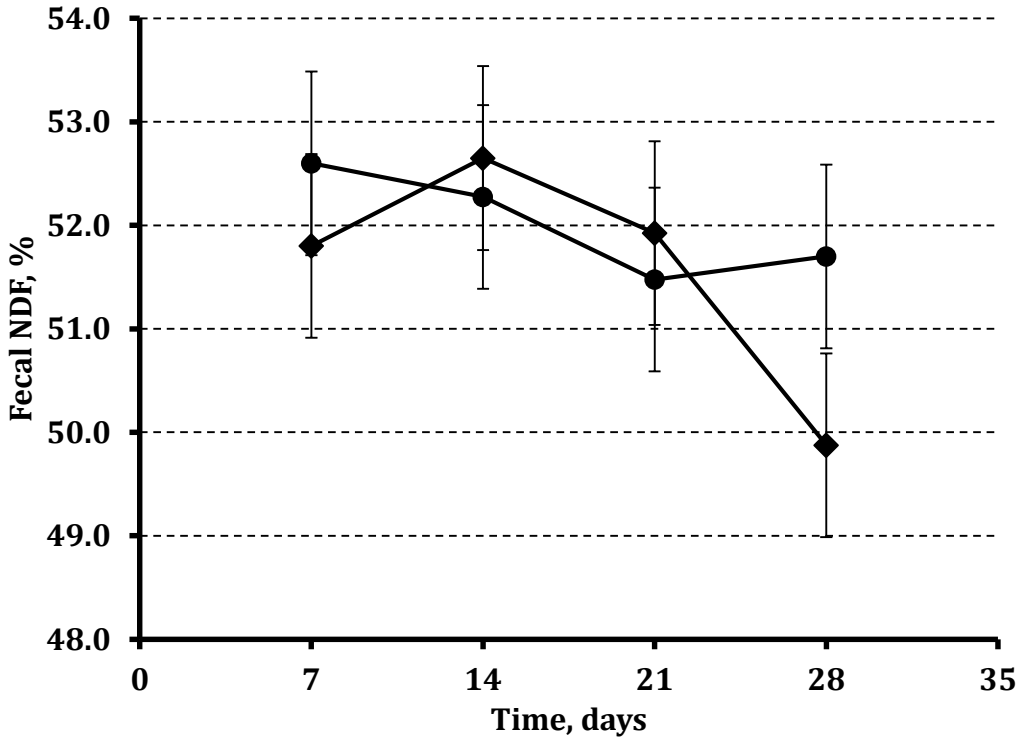


Figure III-4: Fecal protein (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 7876) and treatment by time interaction (*P* = 0.6687). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

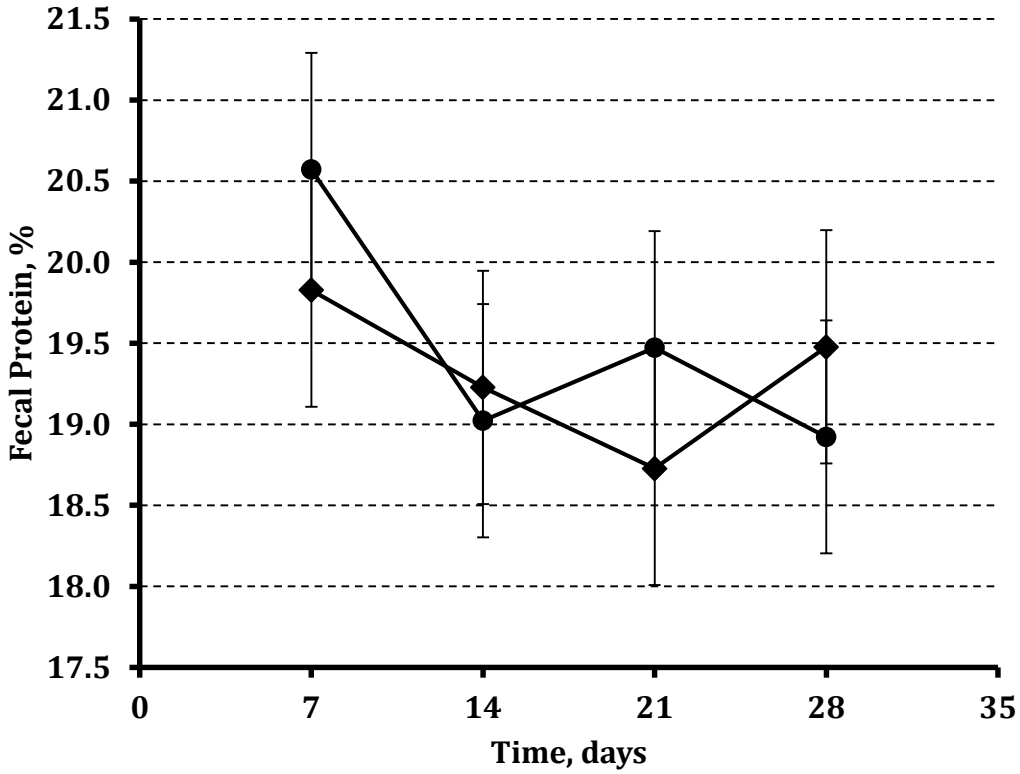


Figure III-5: Fecal lignin (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 2080) and treatment by time interaction (*P* = 0.2597). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

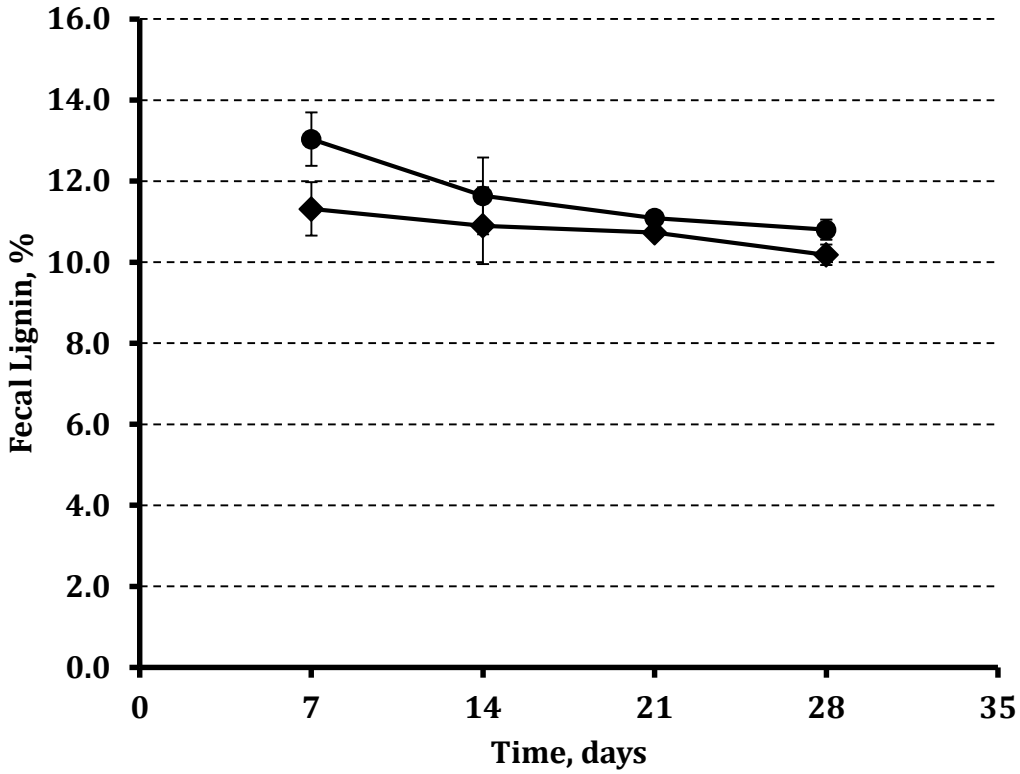


Figure III-6: Apparent starch digestibility (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 0745) and treatment by time interaction (P = 0.6444). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

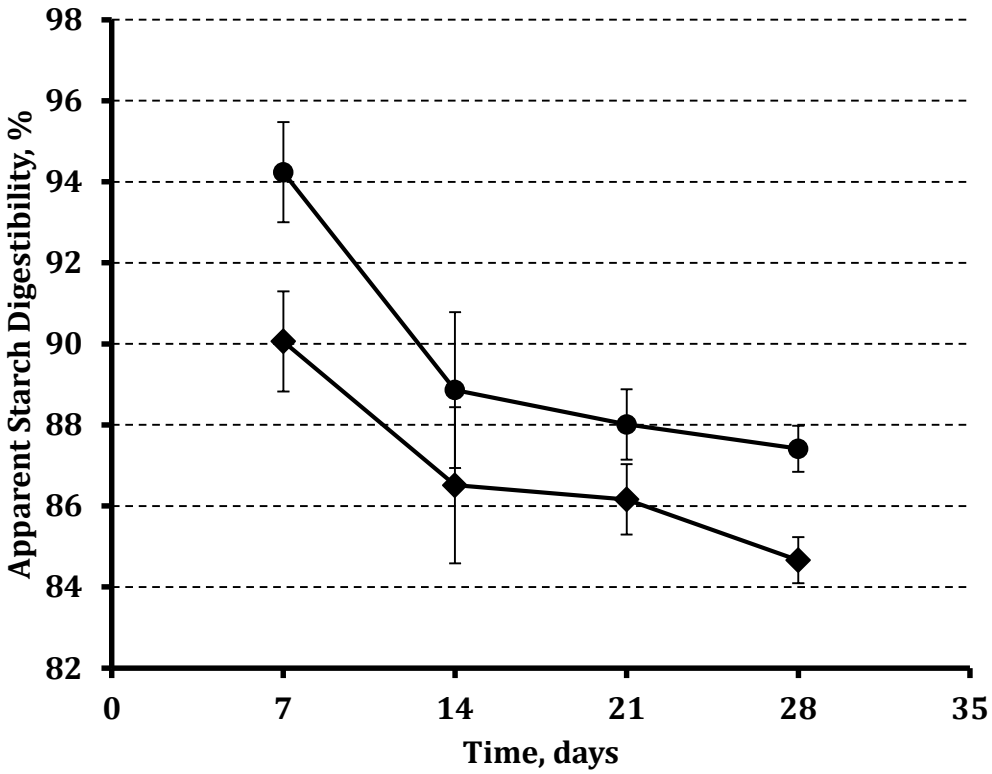


Figure III-7: Apparent NDF digestibility (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 2728) and treatment by time interaction (P = 0.6089). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

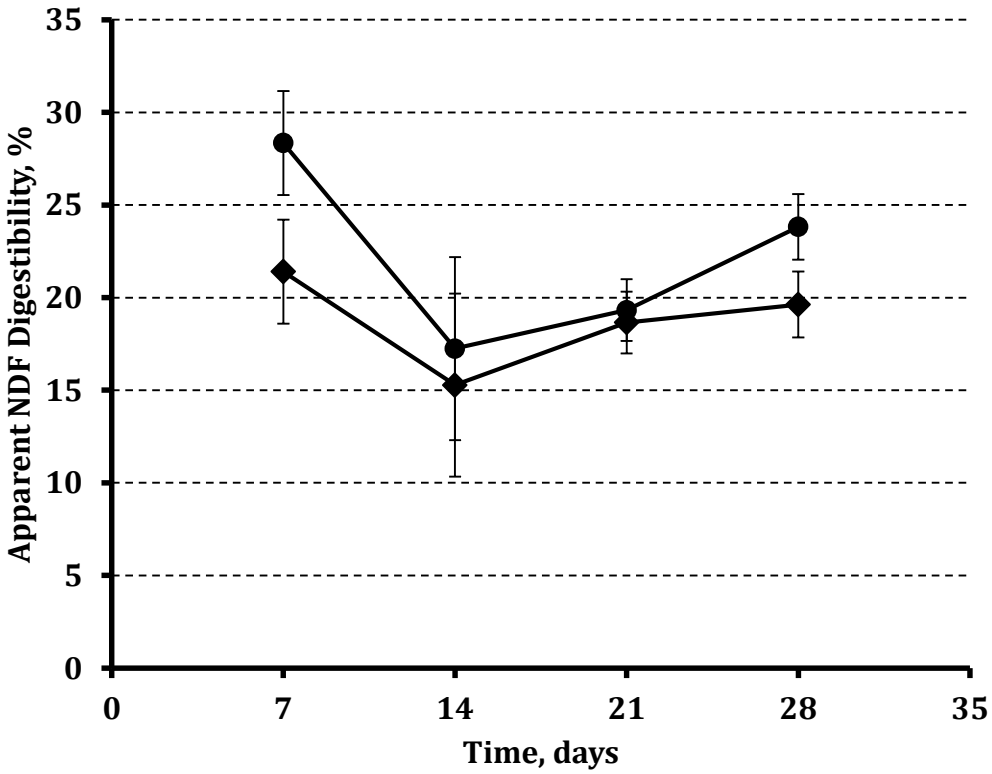
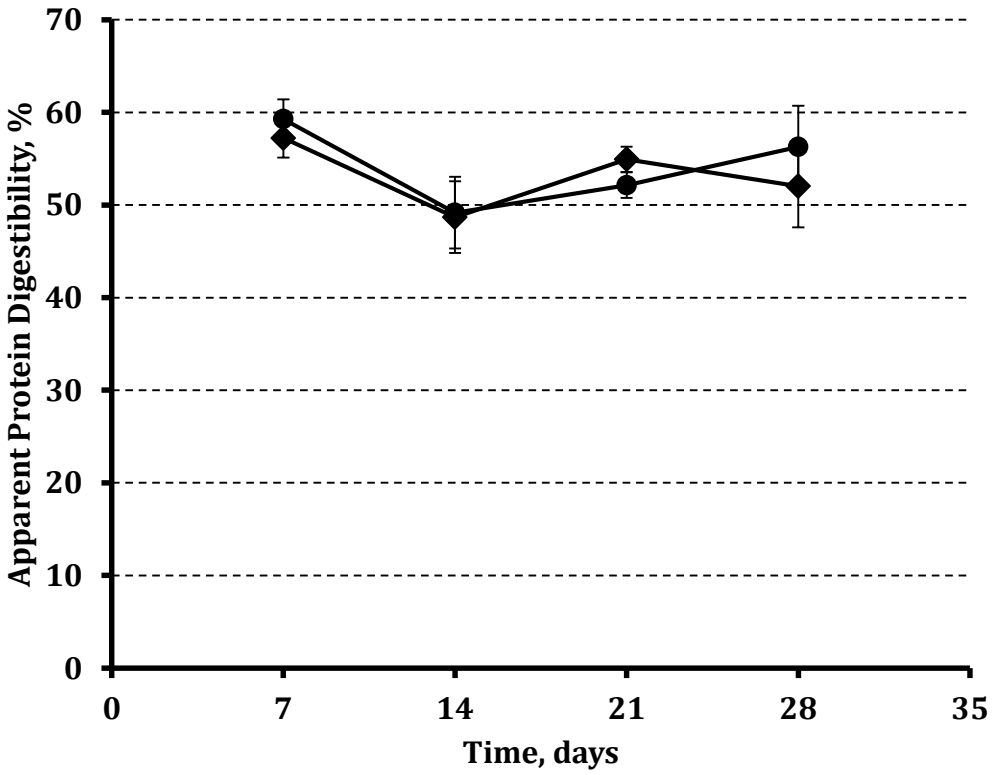


Figure III-8: Apparent protein digestibility (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 6630) and treatment by time interaction (P = 0.2277). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).



APPENDIX A (Materials and Methods)

Figure 0-1A: Schedule of events.

PRE-INTERVENTION PERIOD

Study Day	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
TMR Sampling									✓	
Fecal Sampling										✓
Digesta Sampling										
Cow Weight										✓
Feed Intake					✓	✓	✓	✓	✓	✓
Rumen pH										✓
Milk Yield					✓	✓	✓	✓	✓	✓
Milk Sampling										✓
Inoculation										

INTERVENTION PERIOD

Study Days	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
TMR Sampling						✓						
Fecal Sampling							✓					
Digesta Sampling	✓	✓	✓		✓			✓			✓	
Cow Weight							✓					
Feed Intake	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Rumen pH	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Milk Yield	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Milk Sampling	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Inoculation	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Study Days	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24
TMR Sampling	✓							✓				
Fecal Sampling		✓							✓			
Digesta Sampling		✓			✓			✓			✓	
Cow Weight		✓							✓			
Feed Intake	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Rumen pH	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Milk Yield	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Milk Sampling	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Inoculation	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Study Days	Day 25	Day 26	Day 27	Day 28	Day 29	Day 30	Day 31	Day 32
TMR Sampling			✓					
Fecal Sampling				✓				
Digesta Sampling		✓			✓			✓
Cow Weight				✓				
Feed Intake	✓	✓	✓	✓	✓	✓	✓	✓
Rumen pH	✓	✓	✓	✓	✓	✓	✓	✓
Milk Yield	✓	✓	✓	✓	✓	✓	✓	✓
Milk Sampling	✓	✓	✓	✓	✓	✓	✓	✓
Inoculation	✓	✓	✓	✓	✓	✓	✓	✓

POST-INTERVENTION PERIOD

Study Days	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
TMR Sampling	✓				✓				✓	
Fecal Sampling		✓				✓				✓
Digesta Sampling	✓			✓			✓			✓
Cow Weight	✓					✓				✓
Feed Intake	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Rumen pH	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Milk Yield	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Milk Sampling	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Inoculation										

Table 0-1A: Diet composition.

Ingredient	g/100g of DM
Alfalfa hay	7.79
Alfalfa green chop	5.98
Hay cubes	4.53
Corn silage	4.08
Wheat Silage	9.51
Almond Hulls	13.58
Citrus pulp	1.36
Wheat straw	0.89
Dry distillers grains	10.41
Steamed rolled corn	22.54
Canola	5.41
Cottonseed	5.33
Millrun	5.88
Salt	0.46
Molasses + Mineral and vitamin mix	2.26

Table 0-2A: Nutrient analysis of total mixed ration (TMR) offered to cows in both the control or microbial inoculation group during the Pre-Intervention (Pre), Intervention (I) and Post-Intervention (Post) periods.

Date	Study Day	Dry Matter (%)	Starch (% of DM)	NDF (% of DM)	Crude Protein (% of DM)	Lignin (% of DM)
1/26/16	Pre-9	66.7	21.0	28.9	17.6	6.1
2/2/16	I-6	64.2	22.5	25.4	17.7	5.0
2/9/16	I-13	66.5	17.9	28.7	17.2	5.5
2/16/16	I-20	66.8	20.6	26.7	17.2	5.1
2/23/16	I-27	67.8	21.6	26.8	17.5	5.4
2/29/16	Post-1	68.2	22.1	25.4	17.2	5.0
3/4/16	Post-5	69.3	21.2	26.7	17.1	5.3
3/8/16	Post-9	65.7	19.8	28.8	17.6	5.8

APPENDIX B (Section I)

Results

This analysis (n = 8) excluded cow IDs 3, 7, 8, 14 and 15. Treatment least square means, fixed effects and covariance parameters were estimated using the models described in Section I and are reported in Table I-1B and Figures I-1B to I-13B. Milk fat percentage was still numerically higher for Inoculated, but was neither significant nor tended to be significant. A treatment by time interaction was observed for milk yield (*P* = 0.0271, Figure I-2B) and milk protein yield (*P* = 0.0274, Figure I-8B). Milk and protein yields for Inoculated were higher on week 2 and lower on week 5 compared to the control group.

Table I-1B: Dry matter intake, milk production and composition, BW gain and rumen pH least square means (± SEM) of cows assigned to Control and Inoculated.

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	Treatment		Fixed Effect[1]			
Outcome	Control	Inoculated	Cov	Tx	Week	Tx* Week
				----------Pr > F----------		
DMI, kg	32.4 ± 1.1	32.0 ± 1.0	0.2657	0.8273	<0.0001	0.9269
Milk yield, kg	32.7 ± 0.8	33.1 ± 0.7	0.0047	0.7282	0.0031	0.0271
FCM, kg	34.5 ± 1.3	35.4 ± 1.2	--	0.6267	0.0002	0.0948
ECM, kg	33.8 ± 1.2	34.9 ± 1.1	--	0.5339	0.0002	0.0670
Milk components, %			--			
Crude Protein	3.04 ± 0.11	3.22 ± 0.10	--	0.2352	0.0033	0.0971
Fat	3.77 ± 0.10	4.00 ± 0.10	--	0.1346	0.0122	0.4820
Lactose	4.76 ± 0.06	4.72 ± 0.06	--	0.6333	0.2797	0.3795
Milk components yield, kg			--			
Crude Protein	1.00 ± 0.03	1.05 ± 0.03	--	0.3111	0.0004	0.0274
Fat	1.24 ± 0.05	1.31 ± 0.05	--	0.3727	0.0002	0.2287
MUN, mg/dL	7.00 ± 0.55	7.46 ± 0.50	--	0.5513	<0.0001	0.7861
FCM/DMI	1.11 ± 0.05	1.12 ± 0.04	--	0.8765	0.0013	0.0810
BW gain, kg/day	1.68 ± 0.38	1.33 ± 0.32		0.4919	0.2239	0.9799
Rumen pH	6.16 ± 0.11	6.04 ± 0.10	--	0.4334	0.0017	0.3331

[1]Cov= covariate effect, Tx = treatment effect, Day = day effect; Tx*Day = treatment by day interaction.

Figure I-1B: Dry matter intake (kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.8273$) and treatment by time interaction ($P = 0.9269$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).

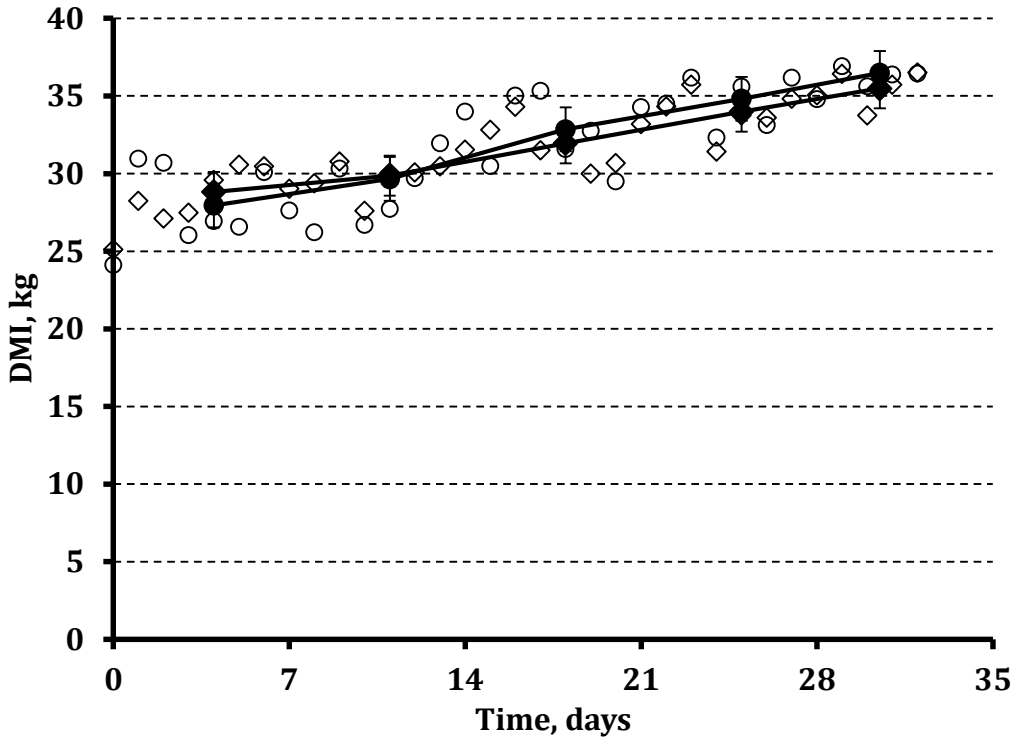


Figure I-2B: Milk yield (kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.7282) and treatment by time interaction (P = 0.0271). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

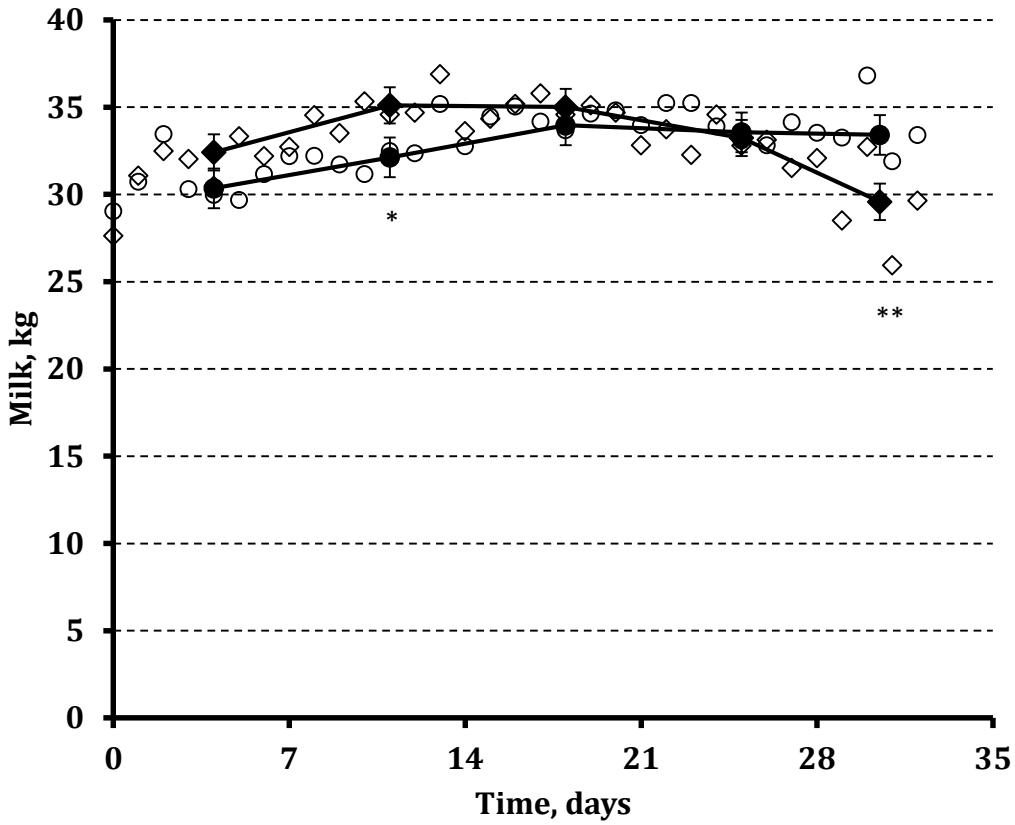


Figure I-3B: Fat corrected milk yield (FCM, kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.6267) and treatment by time interaction (P = 0.0948). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

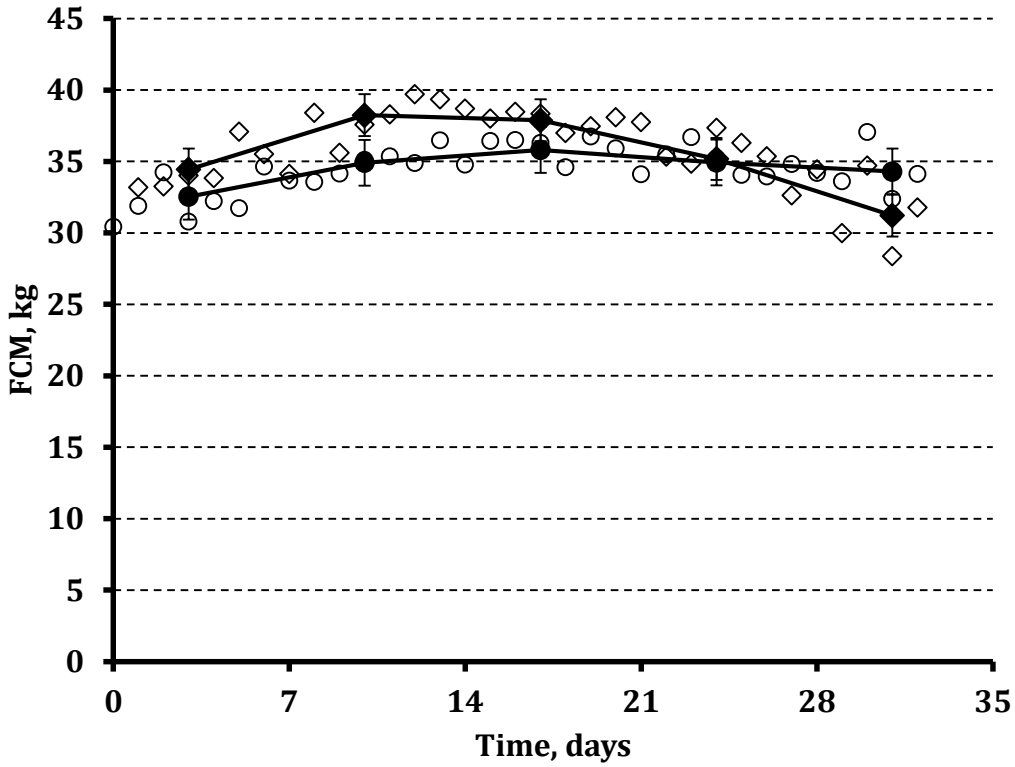


Figure I-4B: Energy corrected milk yield (ECM, kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.5339) and treatment by time interaction (P = 0.0670). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

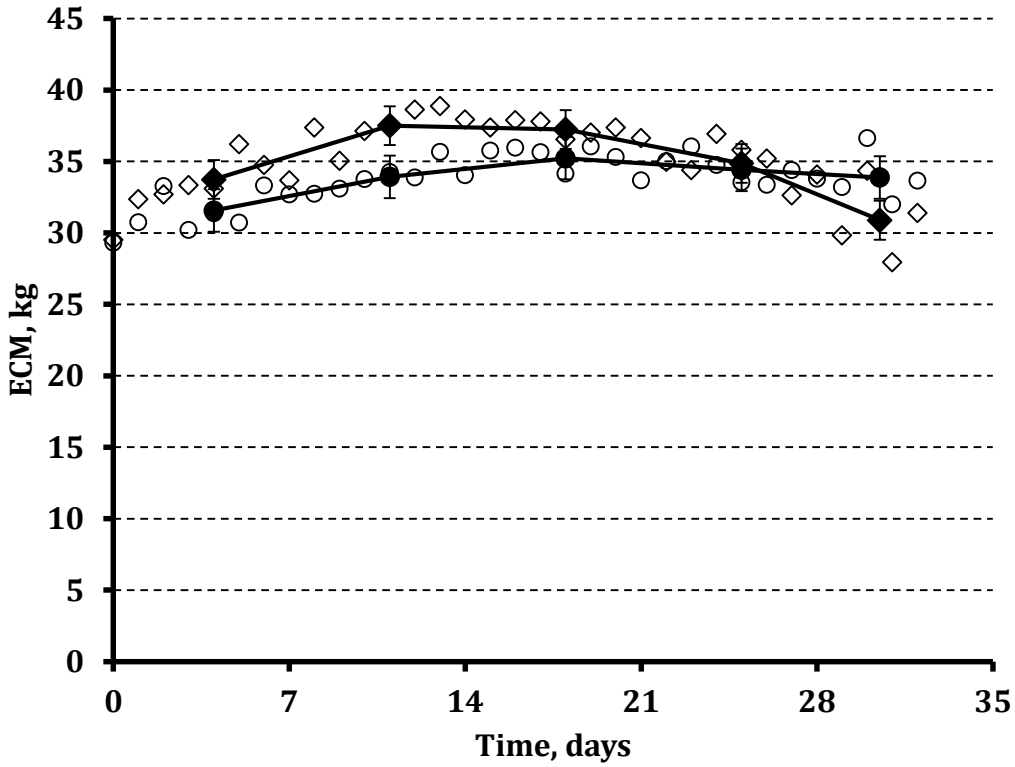


Figure I-5B: Milk crude protein (CP, %) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.2352$) and treatment by time interaction ($P = 0.0971$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).

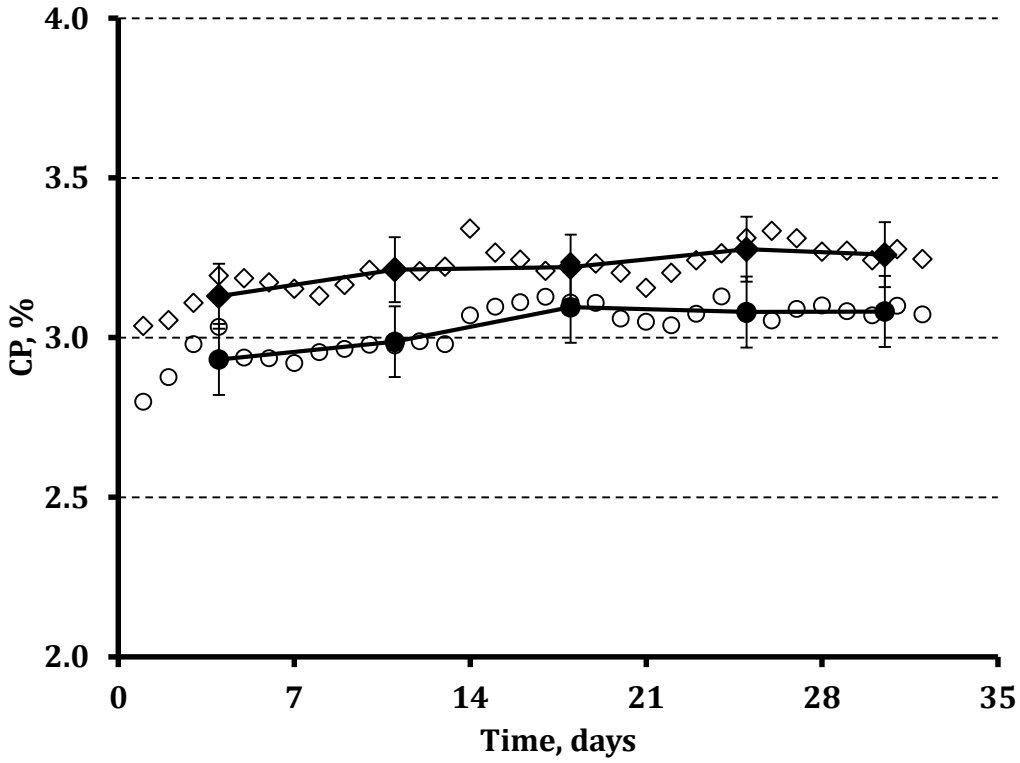


Figure I-6B: Milk fat (%) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0.1346) and treatment by time interaction (*P* = 0.4820). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

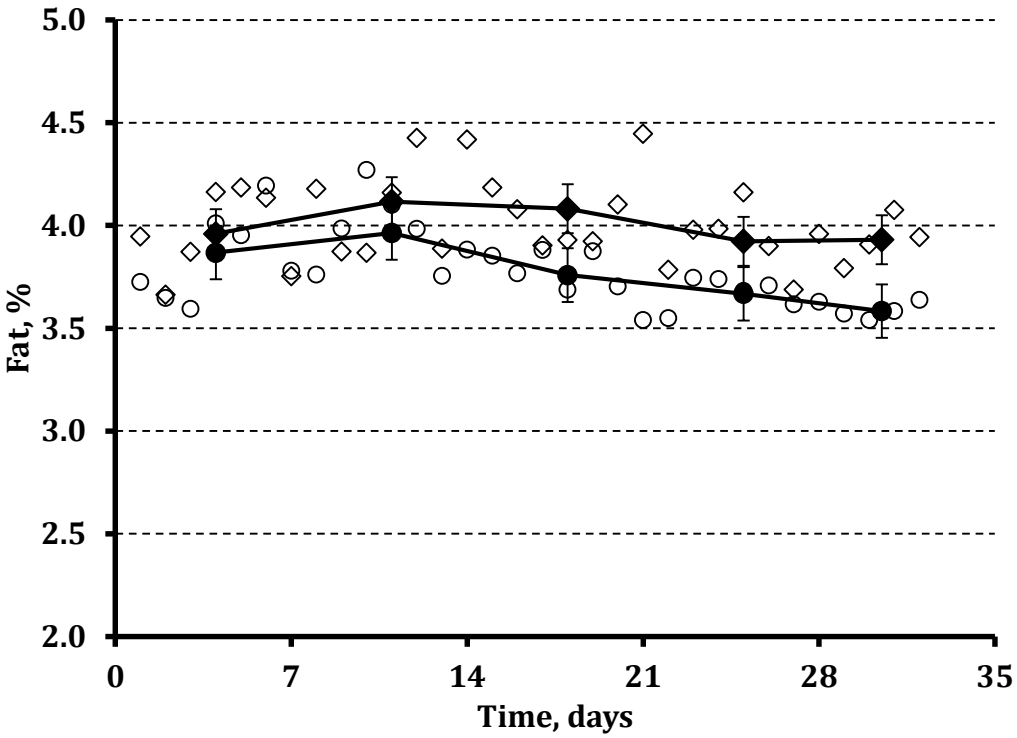


Figure I-7B: Milk lactose (%) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.6333$) and treatment by time interaction ($P = 0.3795$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).

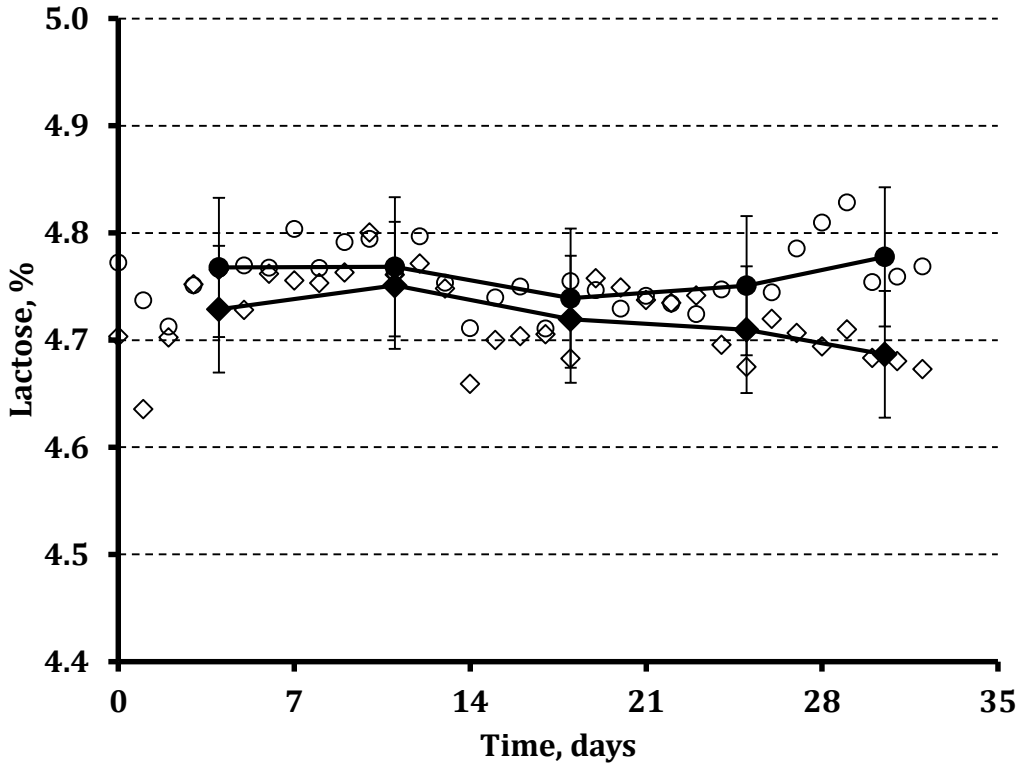


Figure I-8B: Milk crude protein yield (CP, kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.3111) and treatment by time interaction (P = 0.0274). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

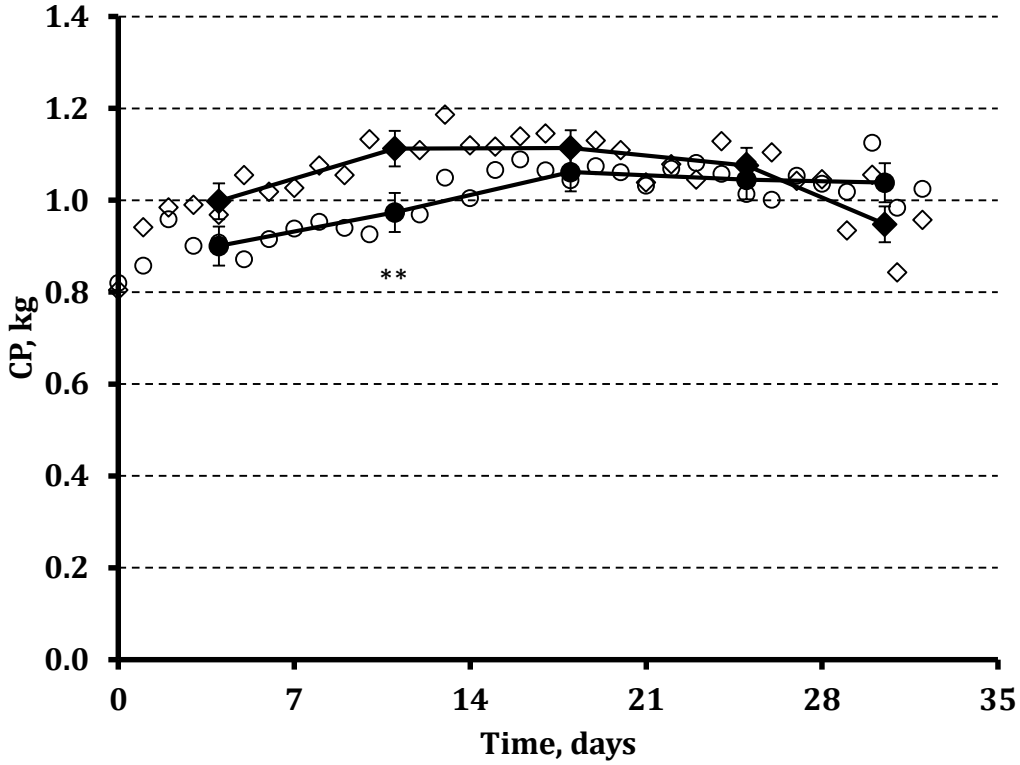


Figure I-9B: Milk fat yield (kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.3727) and treatment by time interaction (P = 0.2287). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

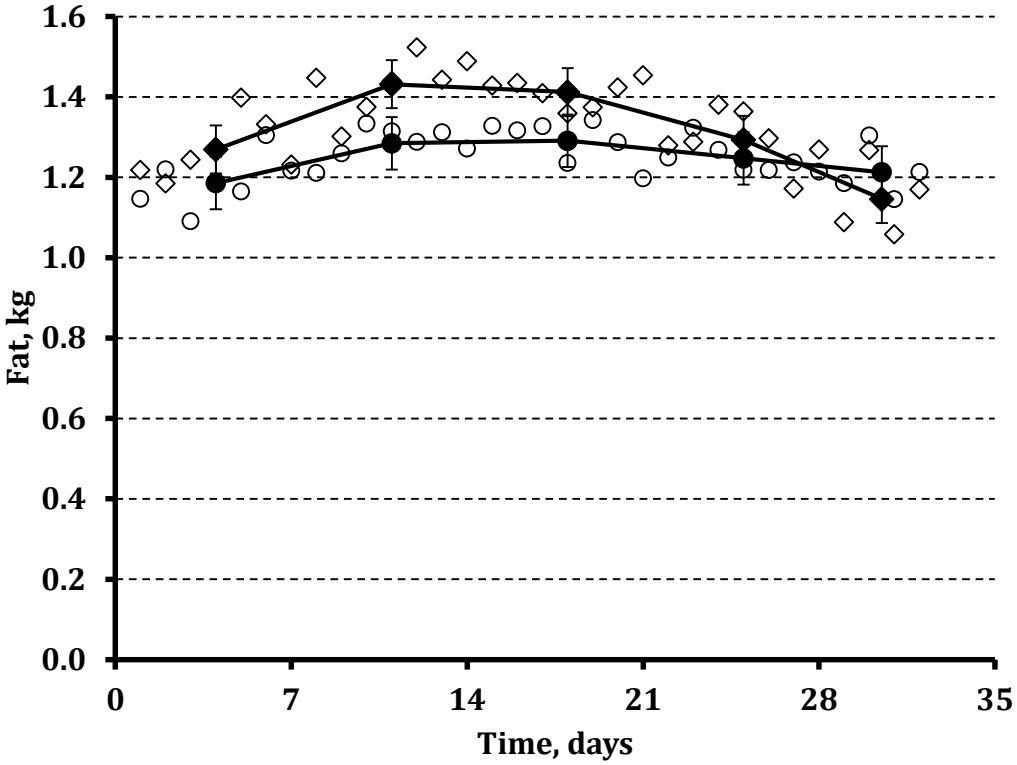


Figure I-10B: Milk urea nitrogen (MUN, mg/dL) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.5513) and treatment by time interaction (P = 0.7861). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

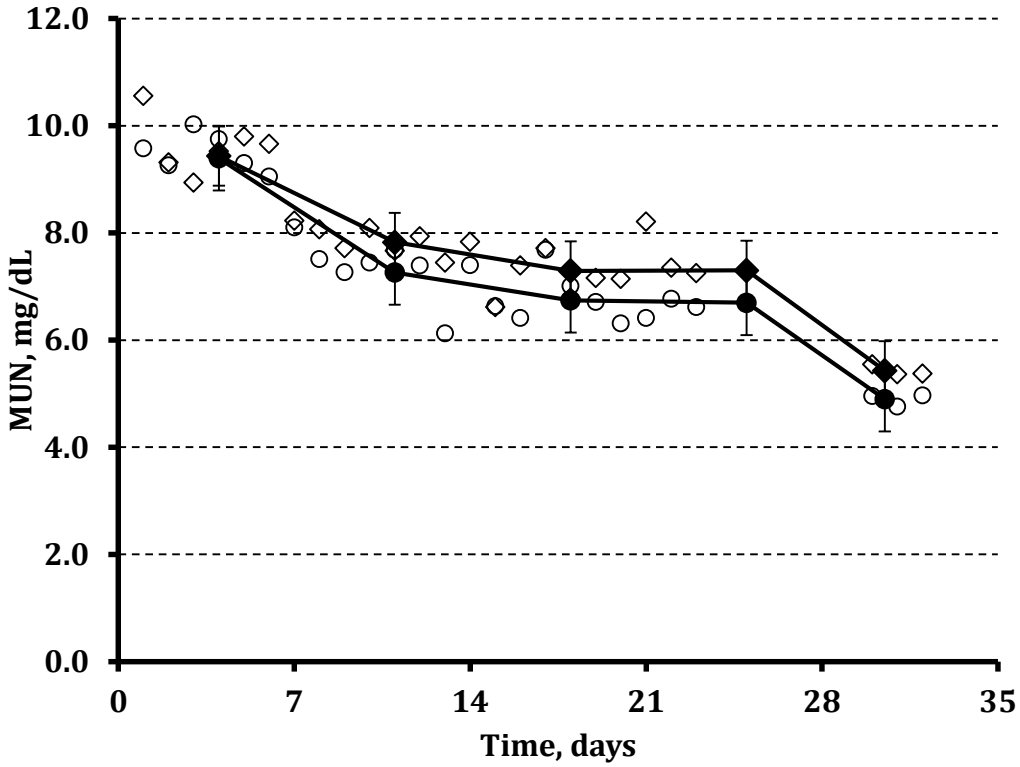


Figure I-11B: Feed efficiency (FCM/DMI) means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.8765) and treatment by time interaction (P = 0.0810). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

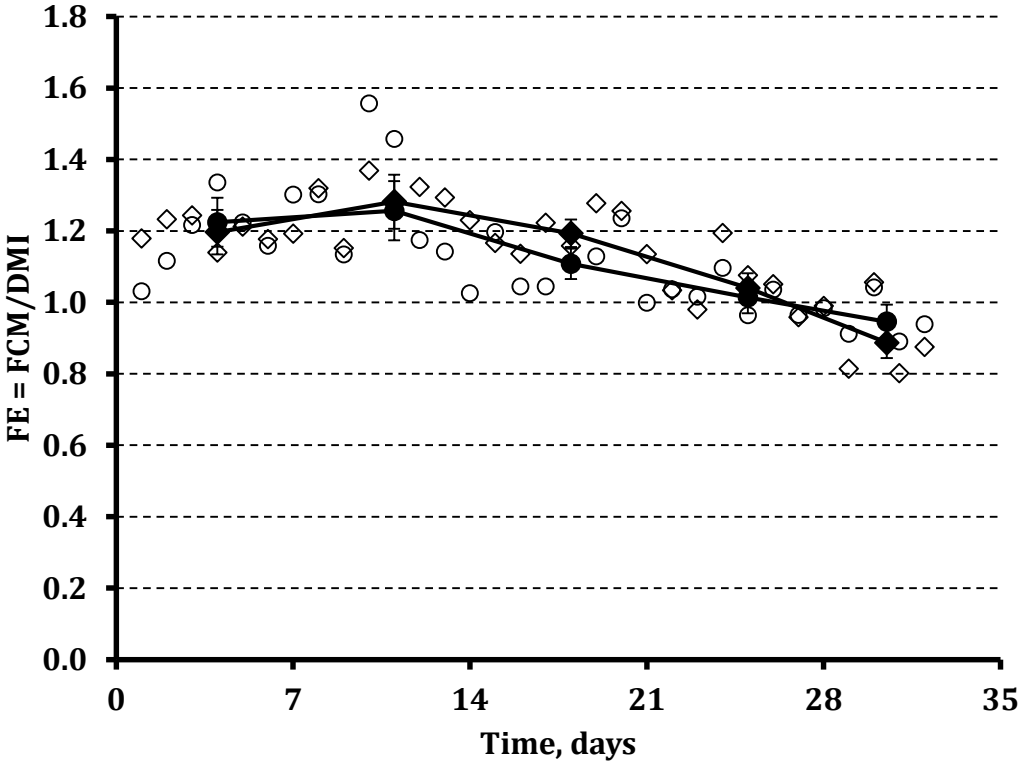


Figure I-12B: BW gain (kg/day) weekly least square means± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 4919) and treatment by time interaction (*P* = 0.9799). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

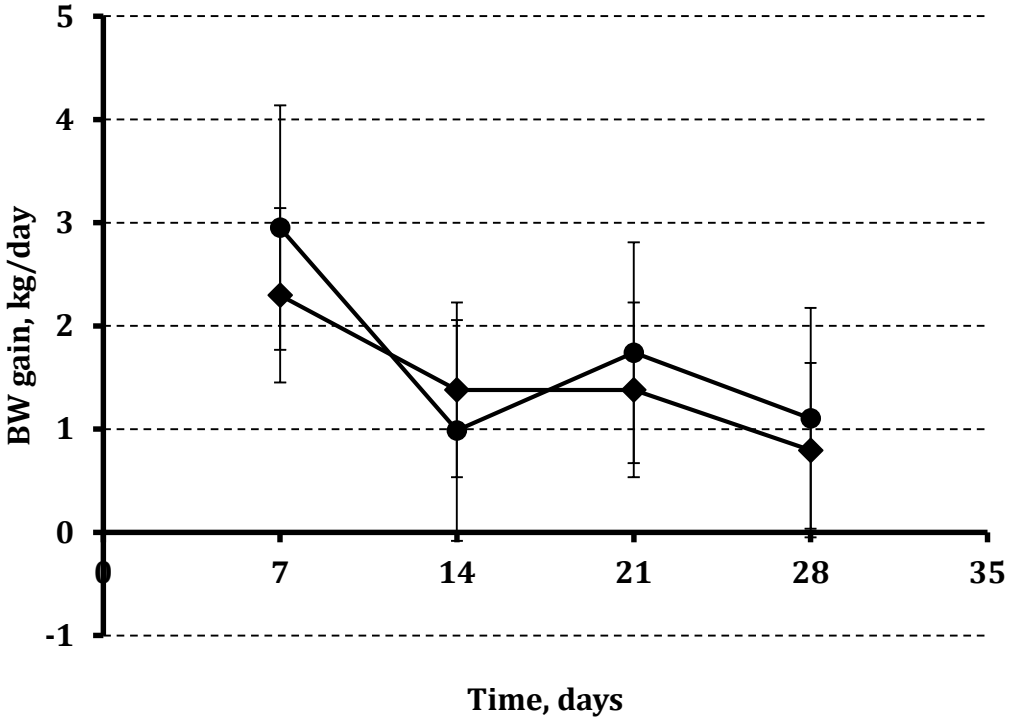
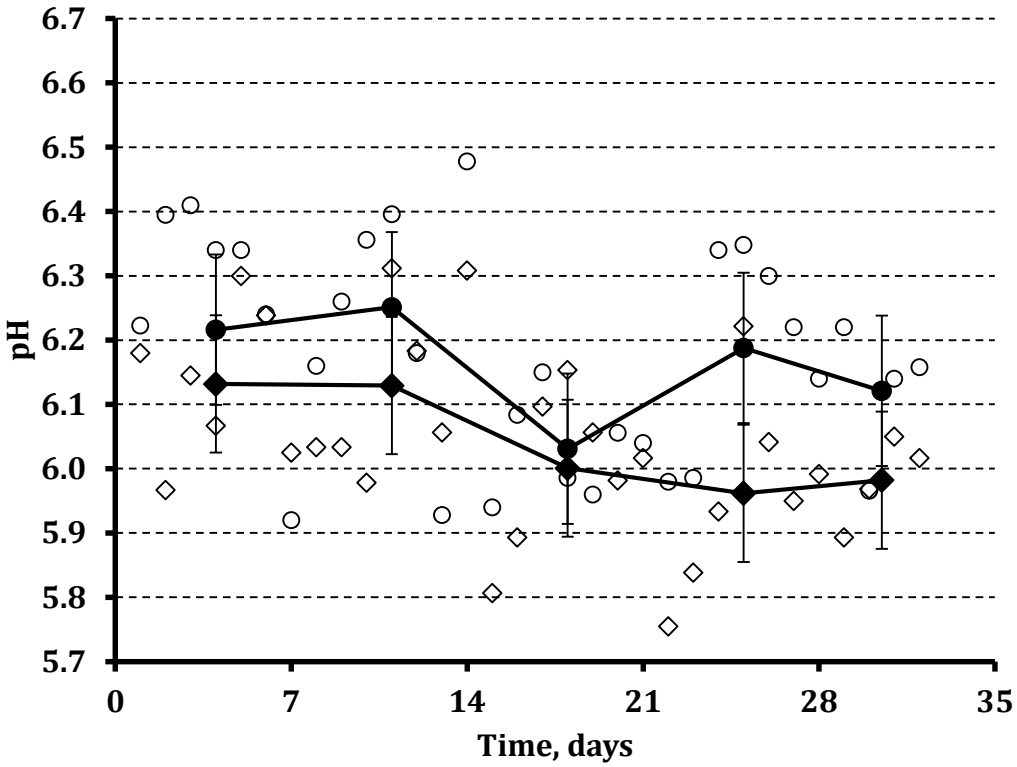


Figure I-13B: Rumen pH daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.4334) and treatment by time interaction (P = 0.3331). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).



APPENDIX C (Section II)

This analysis (n = 8) excluded cow IDs 3, 7, 8, 14 and 15. Treatment least square means, fixed effects and covariance parameters were estimated using the models described in Section II.

Figure II-1C: Dry matter intake (kg) daily means (no fill), covariate adjusted weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 2201) and treatment by time interaction (*P* = 0.1910). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01). The vertical line represents the end of the feeding trial.

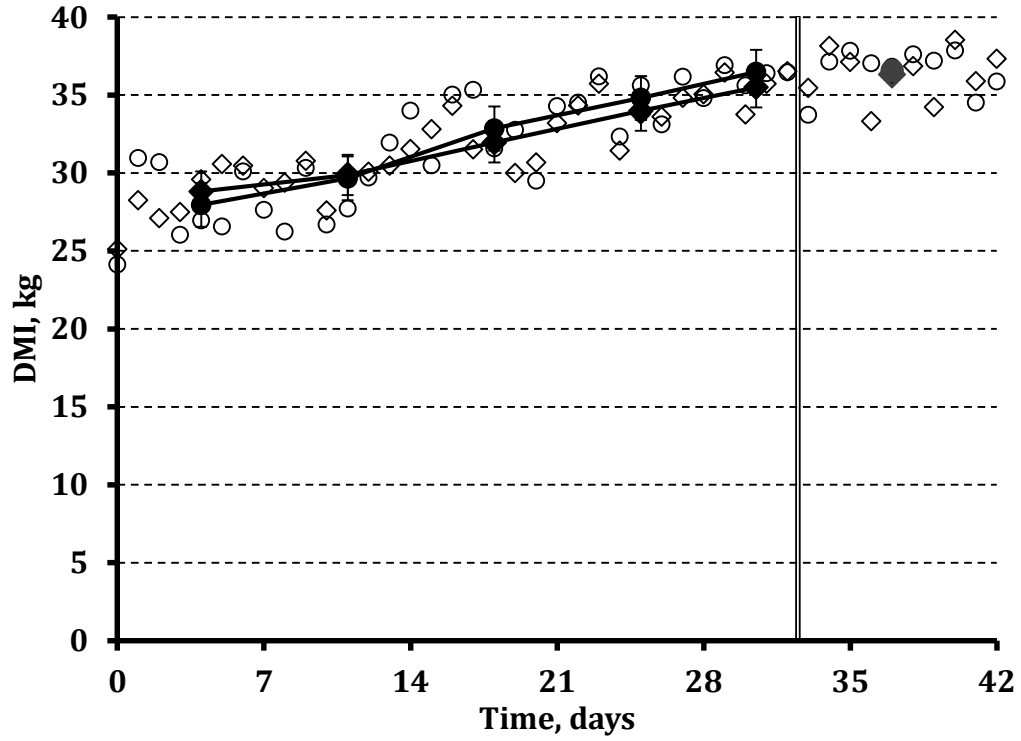


Figure II-2C: Milk yield (kg) daily means (no fill), covariate adjusted weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 0791) and treatment by time interaction (P = 0.0025). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.

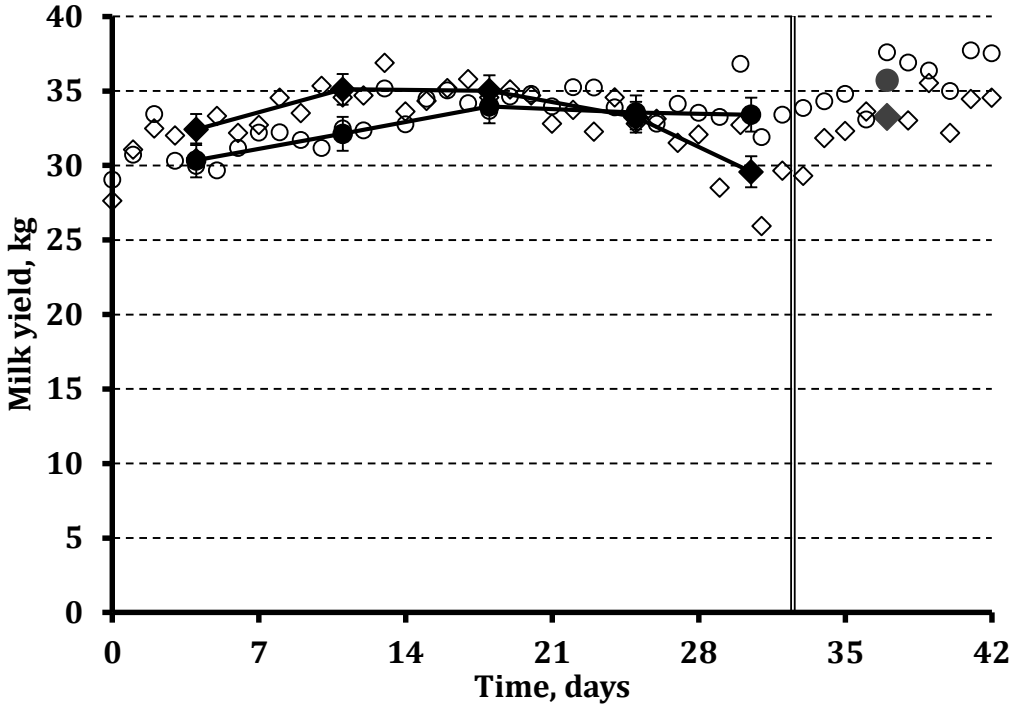


Figure II-3C: Fat corrected milk yield (FCM, kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1883$) and treatment by time interaction ($P = 0.0026$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

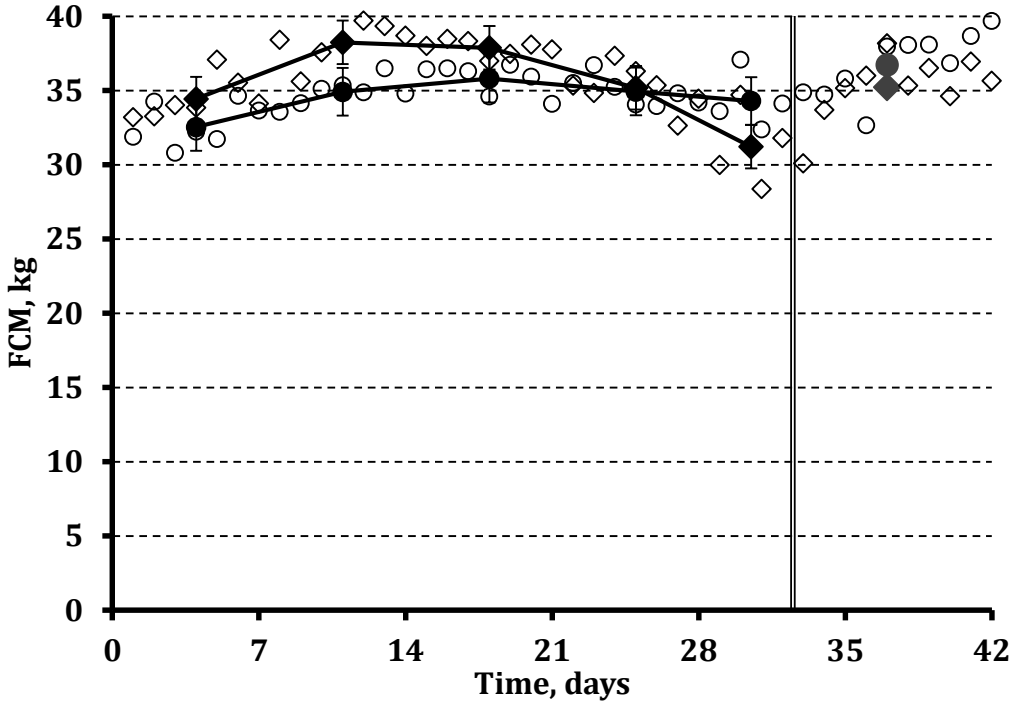


Figure II-4C: Energy corrected milk yield (ECM, kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1669$) and treatment by time interaction ($P = 0.0019$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

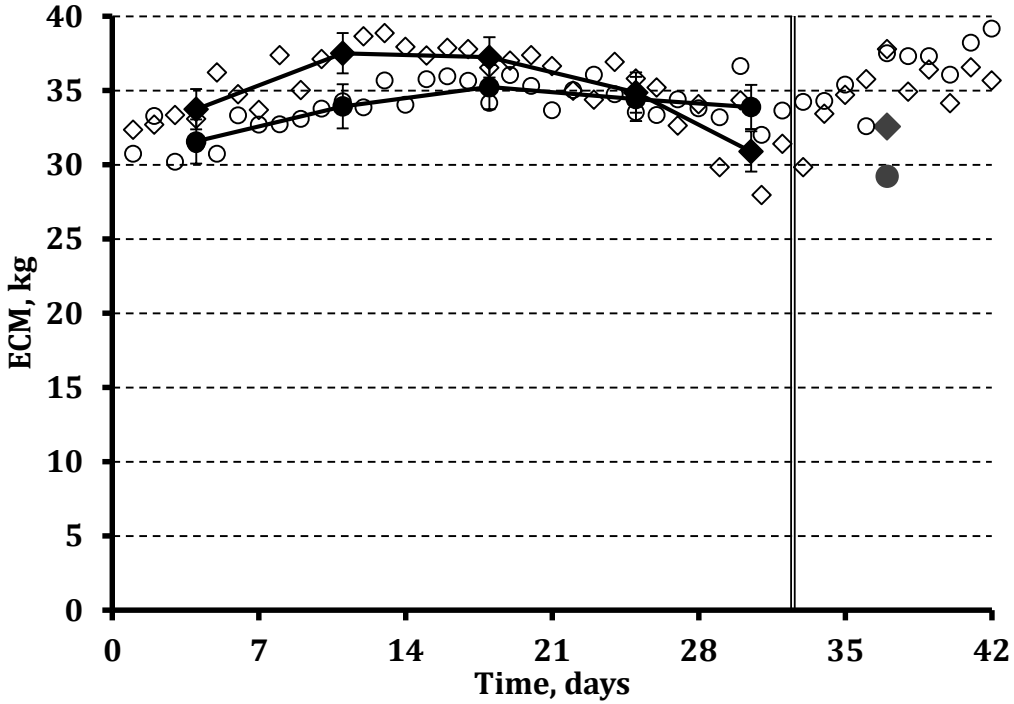


Figure II-5C: Milk crude protein (CP, %) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1553) and treatment by time interaction (P = 0.3125). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.

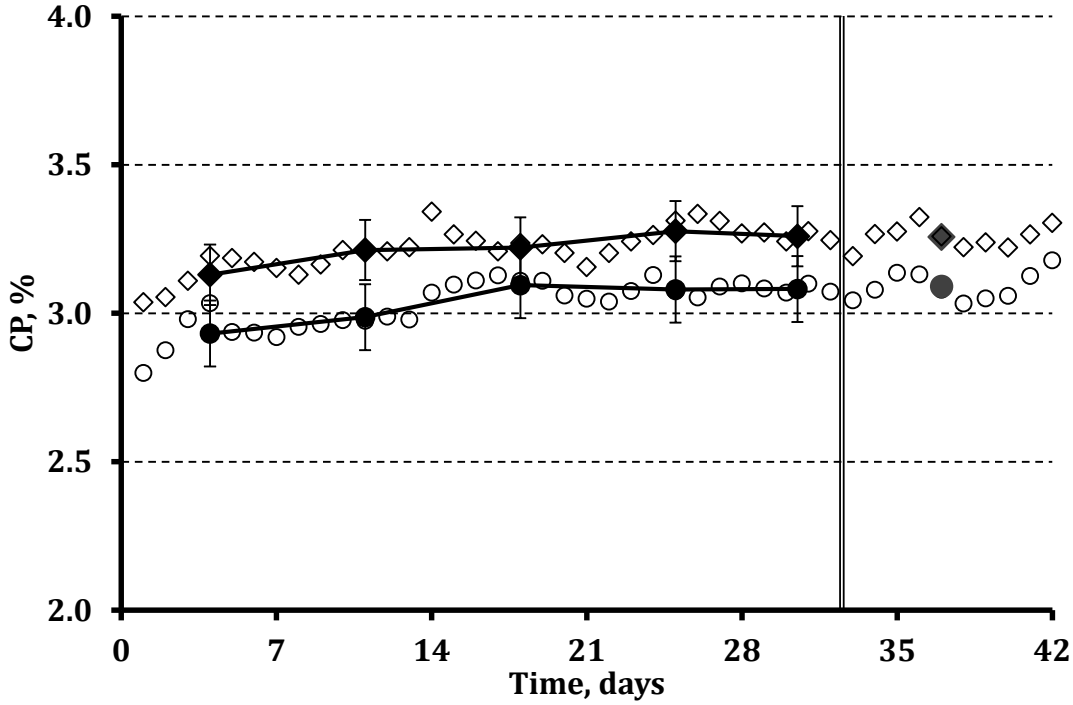


Figure II-6C: Milk fat (%) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0.0991) and treatment by time interaction (*P* = 0.2677). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01). The vertical line represents the end of the feeding trial.

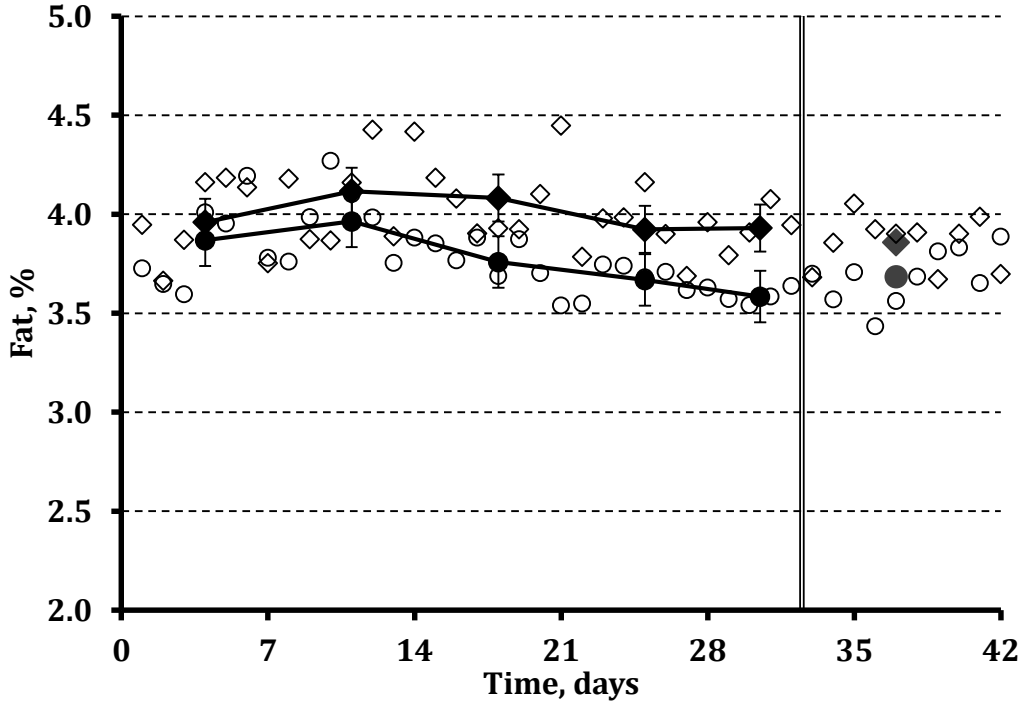


Figure II-7C: Milk lactose (%) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0.3787) and treatment by time interaction (*P* = 0.5016). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01). The vertical line represents the end of the feeding trial.

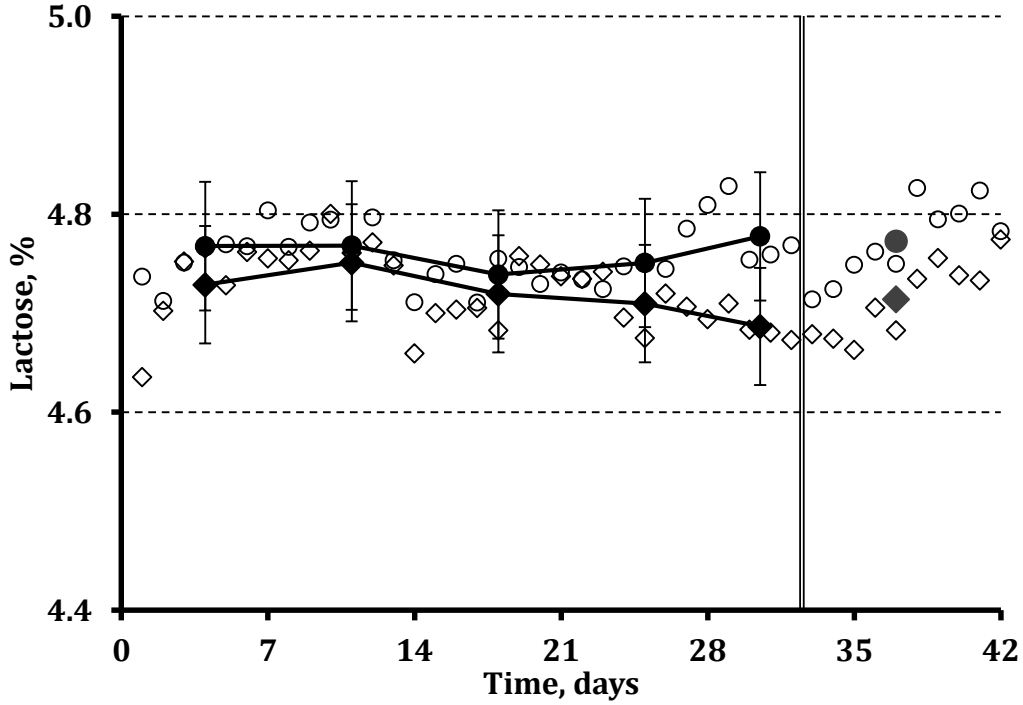


Figure II-8C: Milk crude protein yield (CP, kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1183$) and treatment by time interaction ($P = 0.0012$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

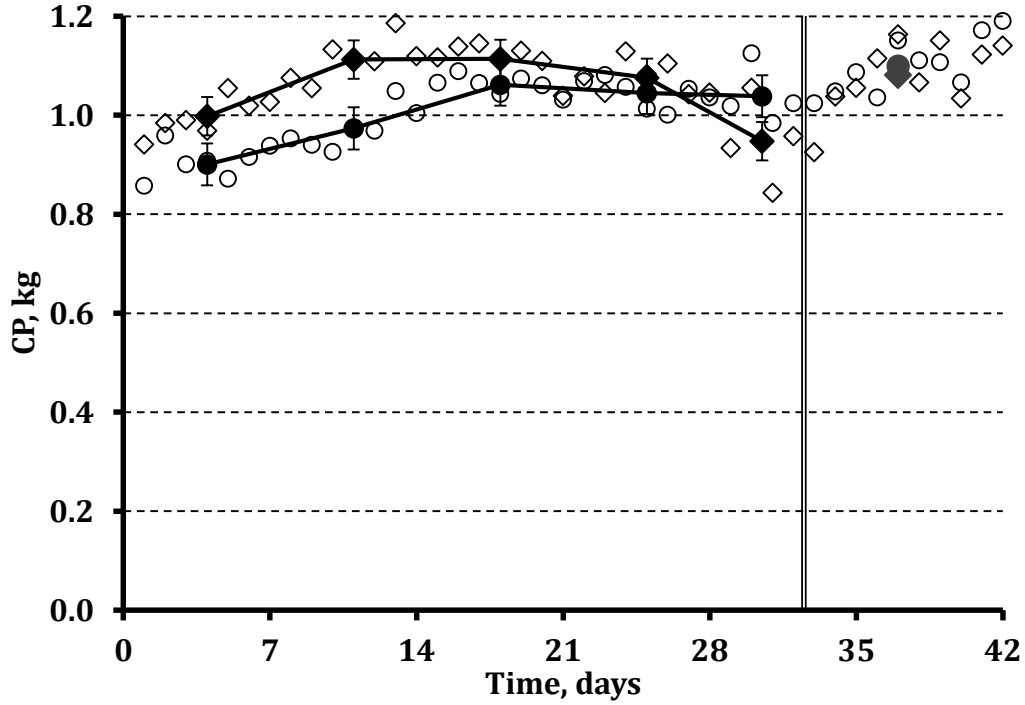


Figure II-9C: Milk fat yield (kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0.1818) and treatment by time interaction (*P* = 0.0880). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01). The vertical line represents the end of the feeding trial.

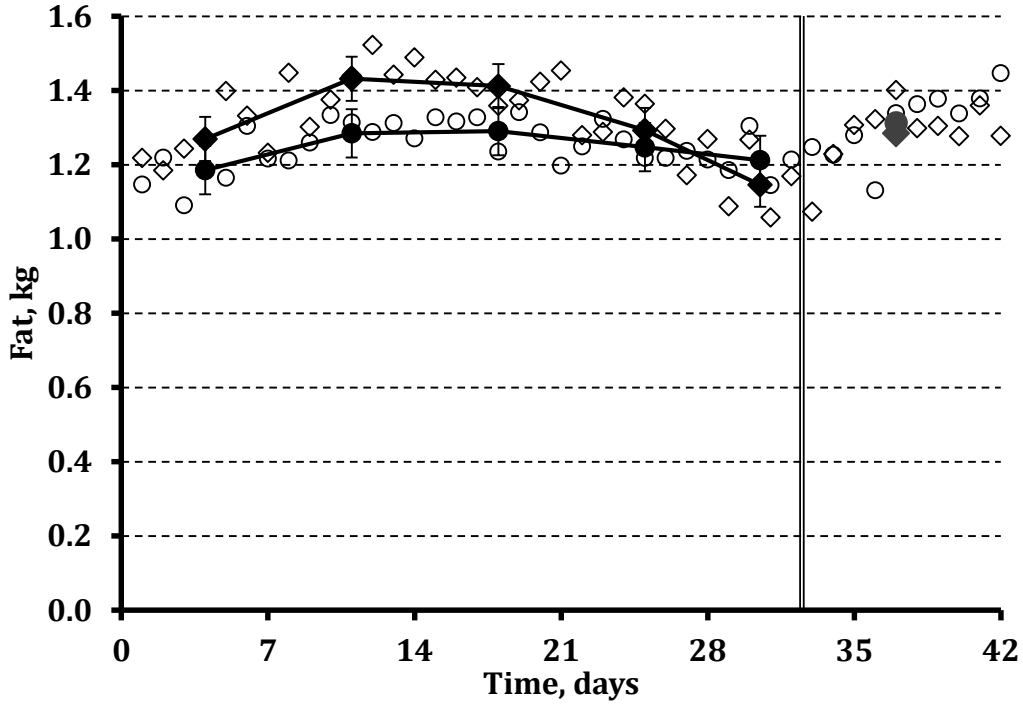


Figure II-10C: Milk urea nitrogen (MUN, mg/dL) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1222$) and treatment by time interaction ($P = 0.3440$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

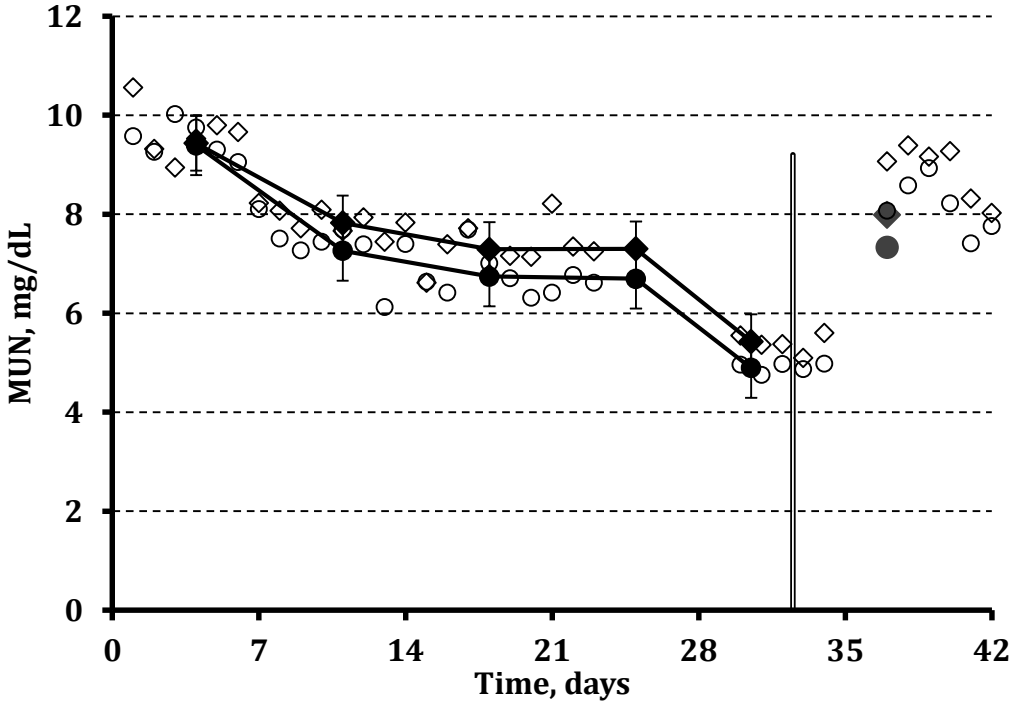


Figure II-11C: Feed efficiency (FCM/DMI) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.2835$) and treatment by time interaction ($P = 0.0671$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

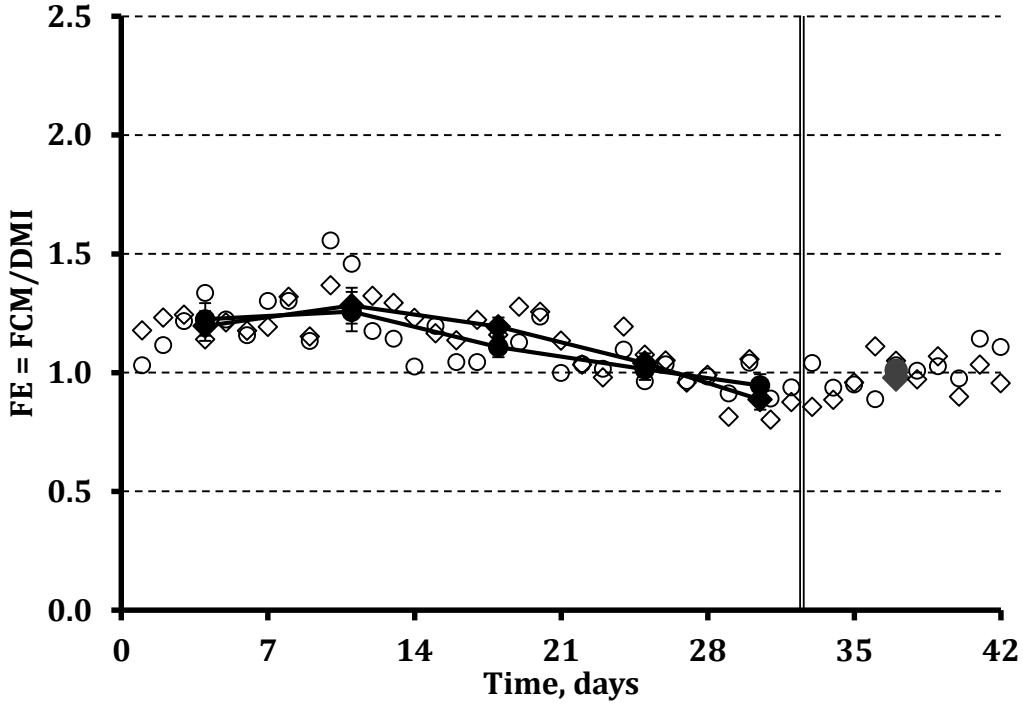


Figure II-12C: BW gain (kg/day) weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) weekly least square means± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.2838) and treatment by time interaction (P = 0.3335). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.

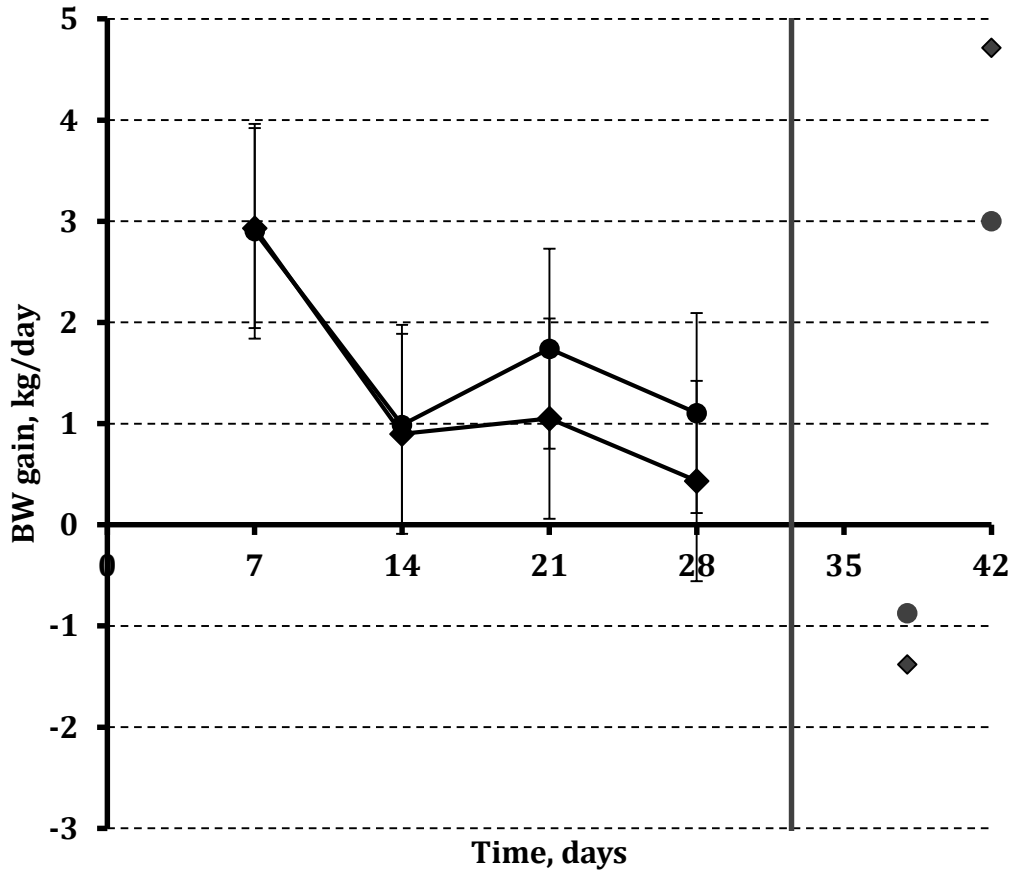
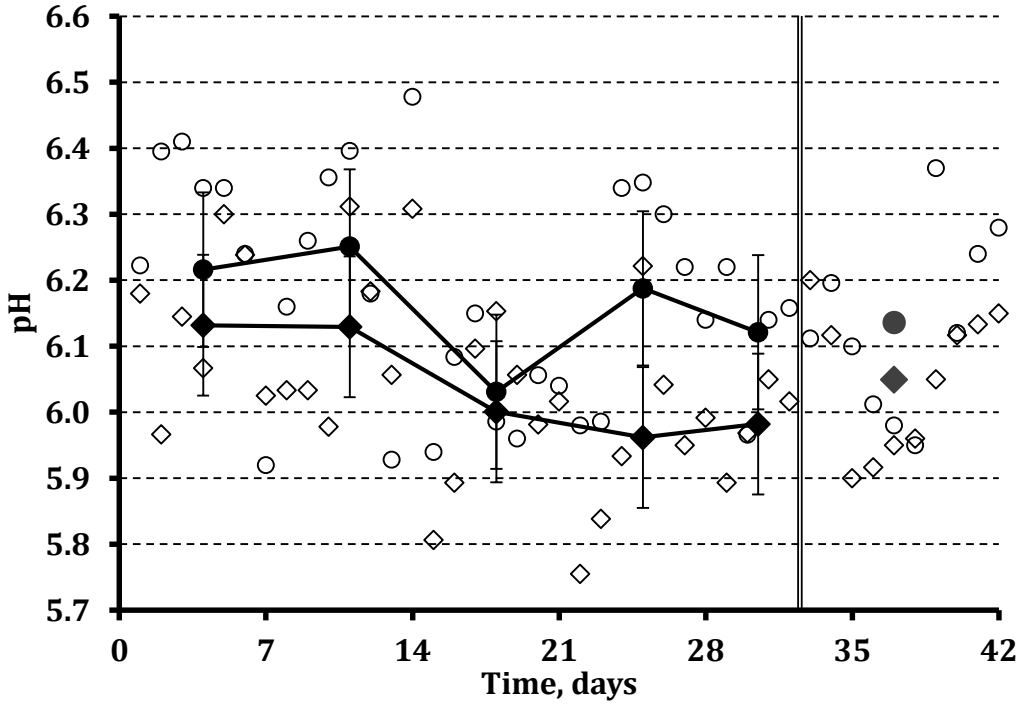


Figure II-13C: Rumen pH daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0.1600) and treatment by time interaction (*P* = 0.0741). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01). The vertical line represents the end of the feeding trial.



APPENDIX D (Section III)

Results

This analysis (n = 4) excluded 4 fecal pools that included cows IDs 3, 7, 8, 14 and 15. Treatment least square means, fixed effects and covariance parameters were estimated using the models described in Section III and are reported in Table III-1D and Figures III-1D to III-13D. No significant treatment by week interaction was observed on any of the outcomes measured. Apparent protein digestibility was higher for Inoculated vs Control (*P* = 0.0143).

Table III-1D: Fecal matter concentration and digestibility least square means of cows assigned either to Control or Inoculated.

	Treatment			Fixed Effects[1]			
Outcome	Control	Inoculated	SEM	Cov	Tx	Week	Tx*Week
Fecal matter, %					----------Pr > F----------		
DM	15.7	15.8	1.0	0.9171	0.9640	0.8255	0.2329
Starch	6.7	7.2	0.8	0.4476	0.7626	0.0500	0.1666
NDF	50.5	51.4	0.1	0.3030	0.1121	0.7716	0.5054
Protein	19.5	19.0	0.3	0.6937	0.4977	0.6587	0.8478
Lignin	10.6	10.8	0.3	0.8202	0.6845	0.0776	0.2826
Digestibility							
Starch	87.5	86.7	1.4	0.7876	0.8063	0.0095	0.6958
NDF	17.5	19.2	3.3	0.9898	0.7936	0.0216	0.4751
Protein	50.8	53.4	0.1	0.0281	0.0143	0.0669	0.8853

[1]Cov= covariate effect, Tx = treatment effect, Day = day effect; Tx*Day = treatment by day interaction.

Figure III-1D: Fecal DM (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 9640) and treatment by time interaction (*P* = 0.2329). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

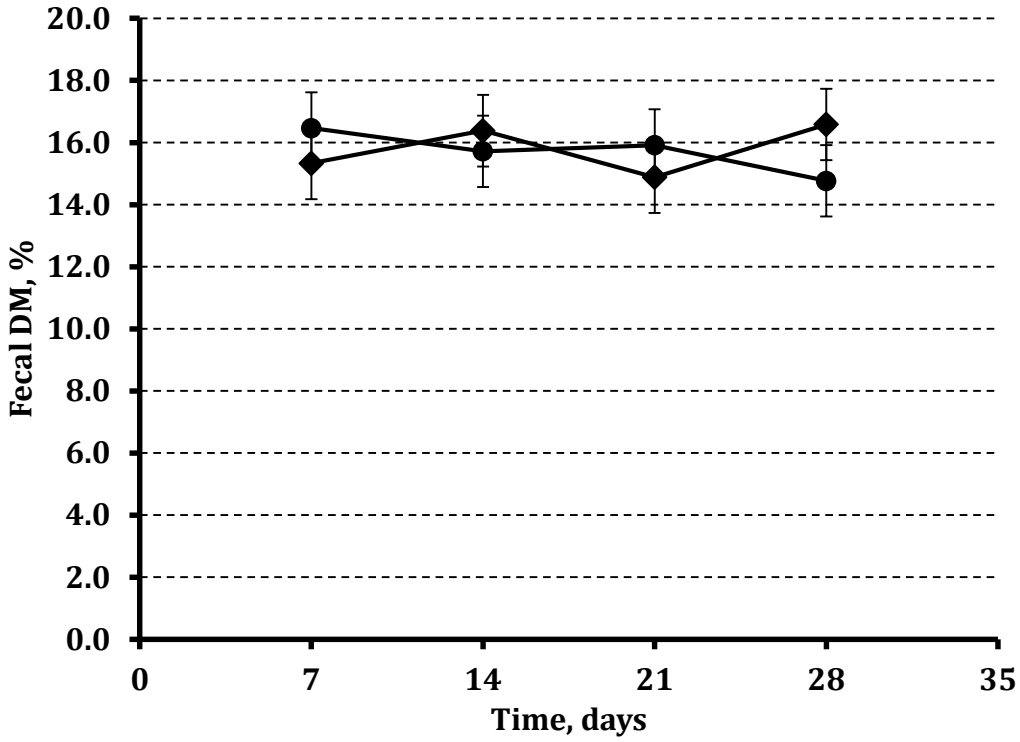


Figure III-2D: Fecal Starch (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 7626) and treatment by time interaction (*P* = 0.1666). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

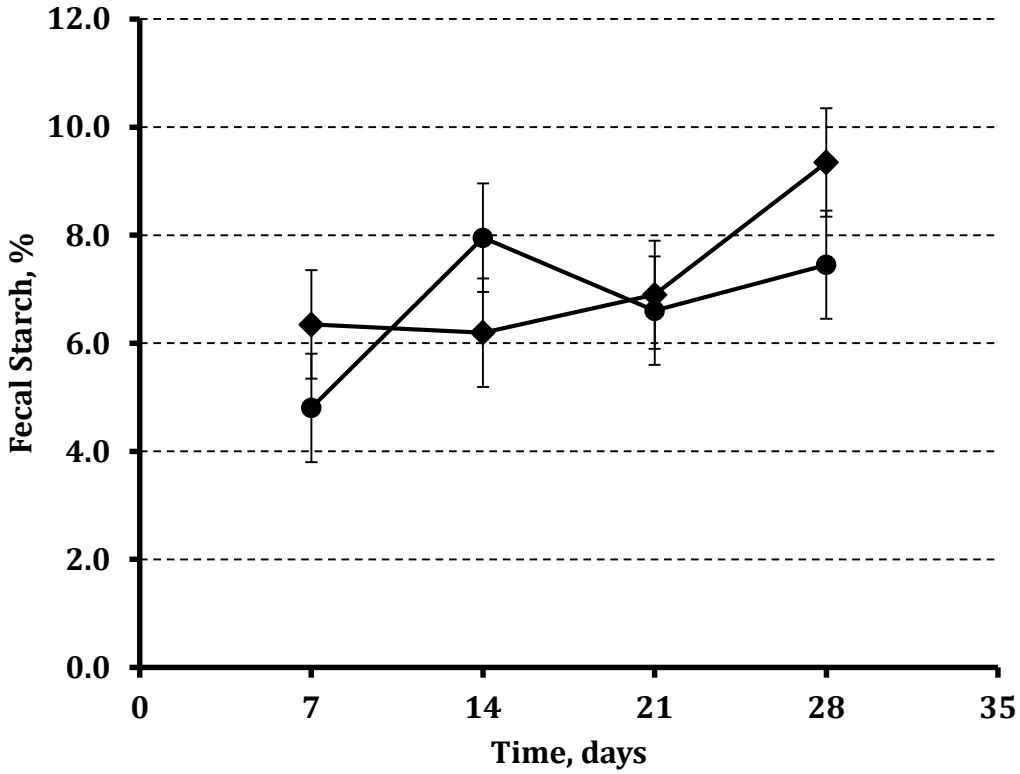


Figure III-3D: Fecal NDF (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 1121) and treatment by time interaction (*P* = 0.5054). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

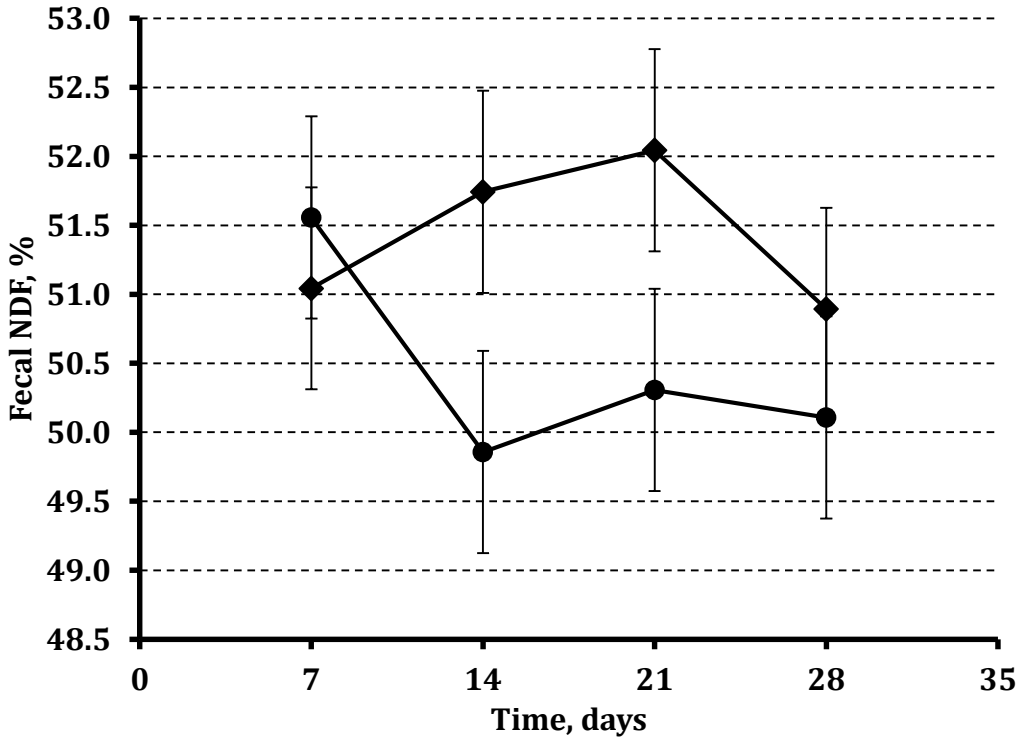


Figure III-4D: Fecal protein (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 4977) and treatment by time interaction (*P* = 0.8478). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

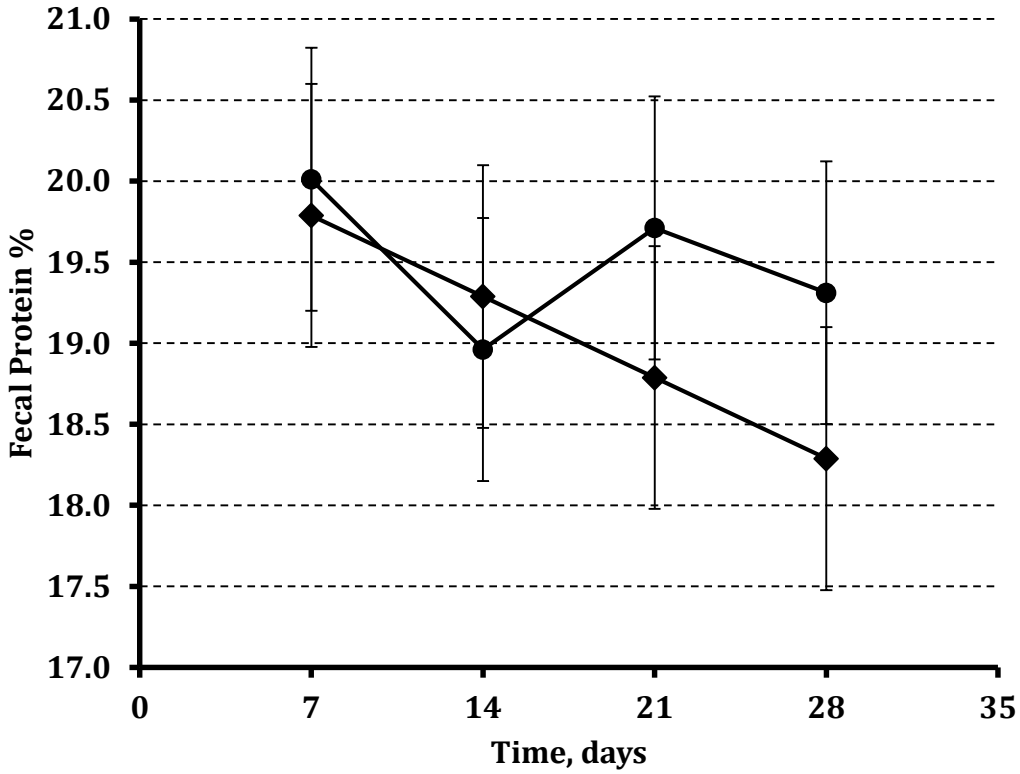


Figure III-5D: Fecal lignin (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 6845) and treatment by time interaction (*P* = 0.2826). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

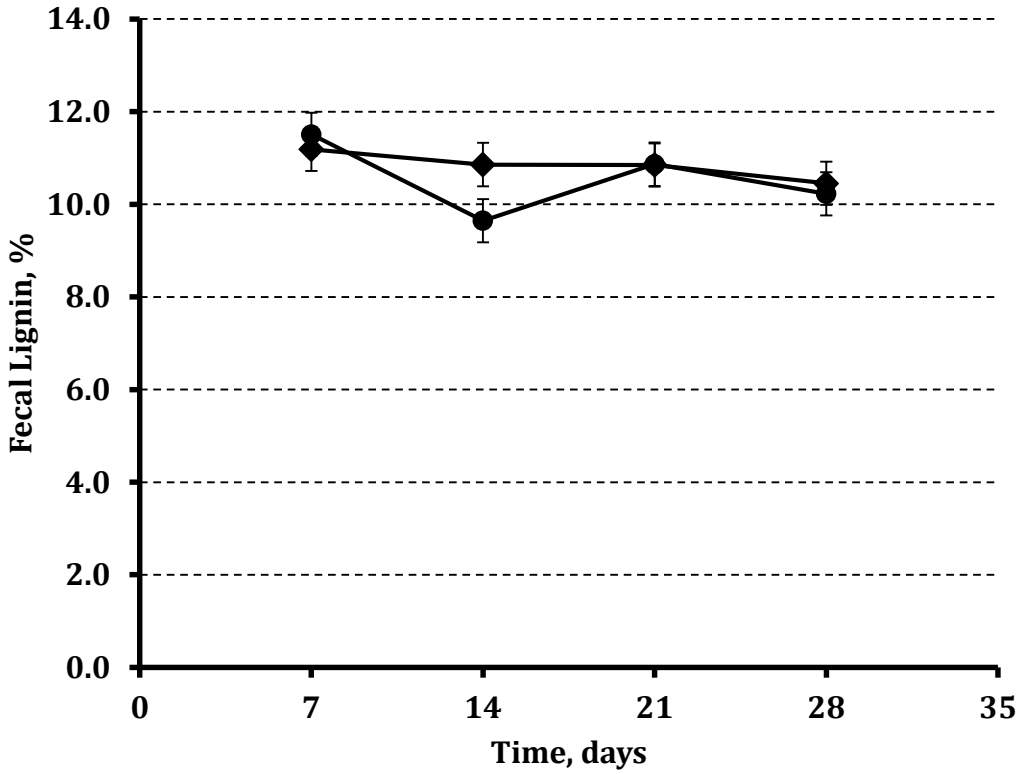


Figure III-6D: Apparent starch digestibility (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0.8063) and treatment by time interaction (*P* = 0.6958). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

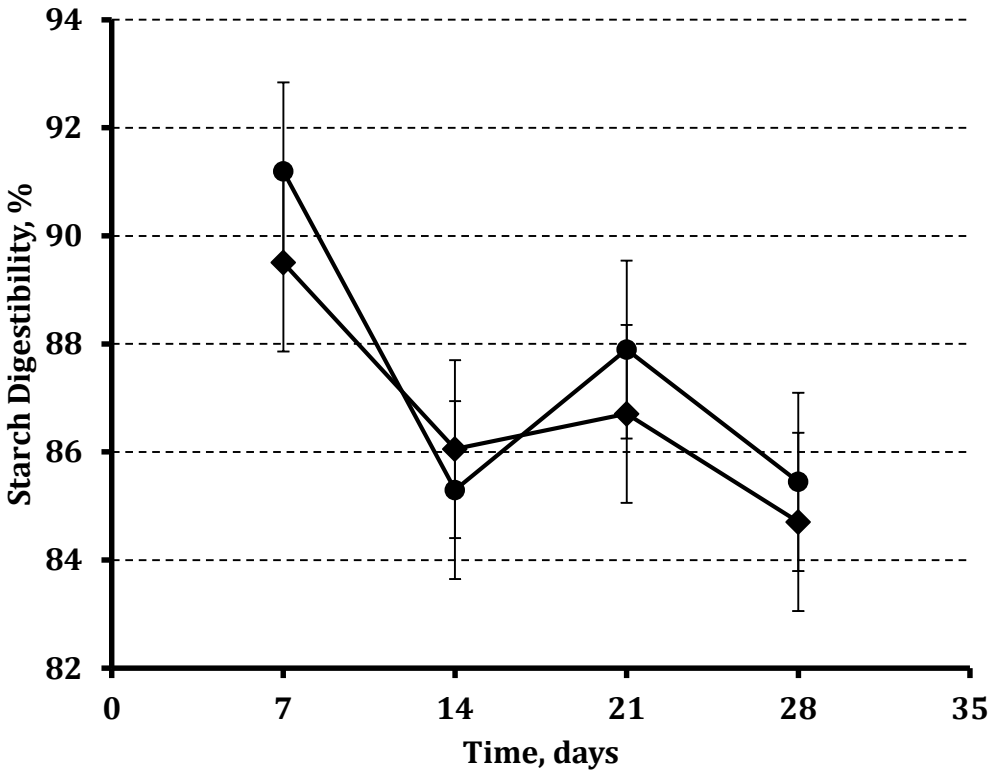


Figure III-7D: Apparent NDF digestibility (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.7936$) and treatment by time interaction ($P = 0.4751$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).

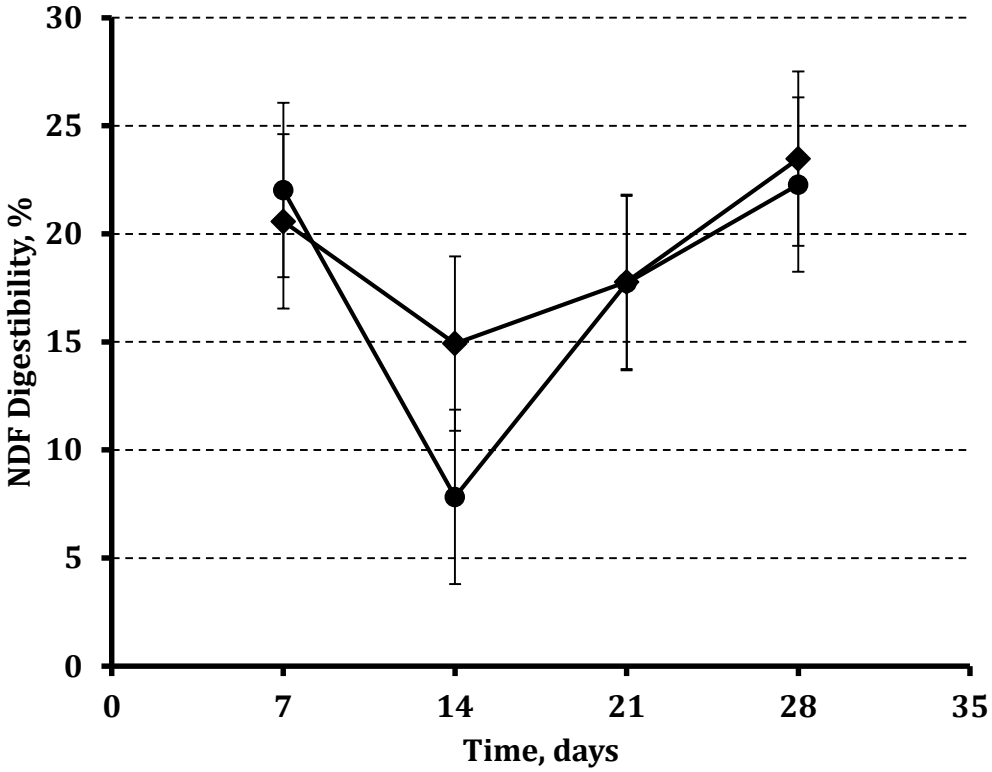
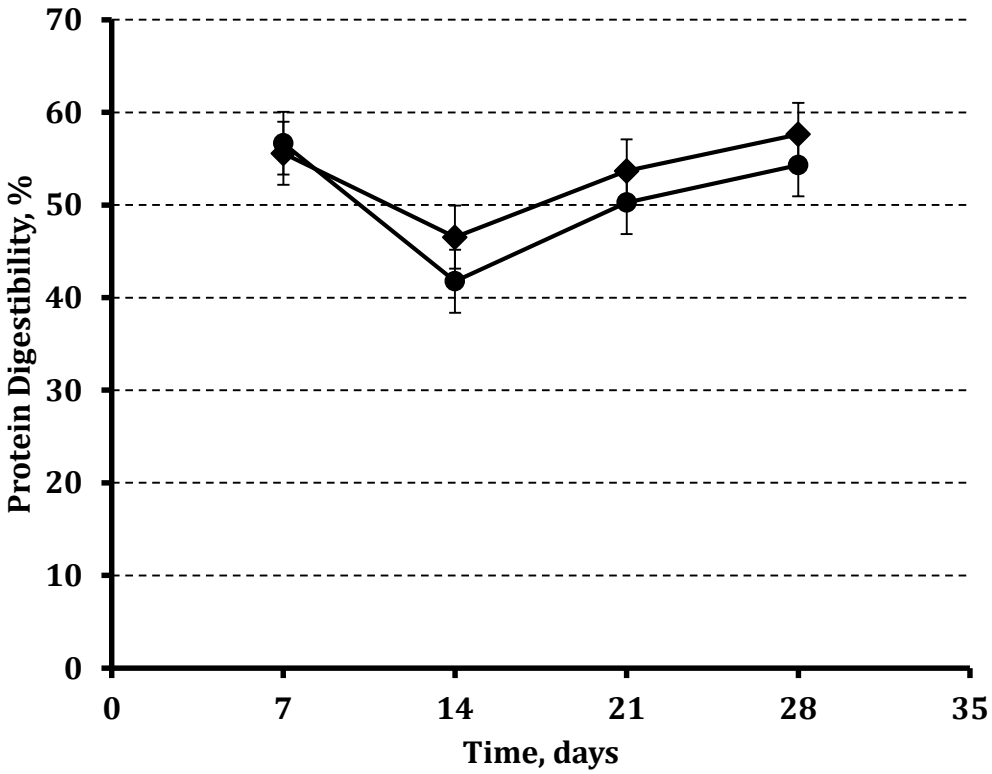


Figure III-9D: Apparent protein digestibility (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.0143$) and treatment by time interaction ($P = 0.8853$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).



Final In-Life Phase Report

"Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms in Live Lactating Dairy Holstein Heifers via Direct Injection into the Rumen"

Study Number (b) (4)

Study Sponsor
Ascus Biosciences, Inc.
6450 Lusk Blvd
Suites E109 / 209
San Diego, CA 92121

In-Life Test Facility
(b) (4)

Signature Page

"Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms in Live Lactating Dairy Holstein Heifers via Direct Injection into the Rumen"

(b) (4)

In-Life Phase Report

(b) (4)

29 Aug 17

Date

Study Director

Table of Contents

Experiment Overview:

There were 3 treatment groups in the study. 8 experimental Holstein cows (average ~100 days in milk) received 2 microbes via injection into the rumen (Treatment Group 1: Dairy-20 & Dairy-21). 8 experimental Holstein cows (average ~100 days in milk) received 3 microbes via injection into the rumen (Treatment Group 2: Dairy- 10, Dairy-20 & Dairy-21). 8 experimental Holstein cows (average ~100 days in milk) received 3 basal suspension medias (no microbes) via injection into the rumen (Treatment Group 3: Control).

The cows were inoculated daily after the morning milking for 28 days. Fecal contents were sampled from each cow on study day 1 (prior to inoculation), and on study day 8, study day 16, study day 24, and study day 28. Samples had NDF and ADF determined. Feed samples were collected on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, and Study Day 28. Samples had NDF and ADF determined. Rumen contents were sampled from each cow on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, Study Day 28, Study Day 35 and Study Day 38. Twice daily milking, milk production measurements and clinical udder evaluations by quarter were performed every day from Study Day -7 to Study Day 38 for every individual animal, except for Cow 54027, which was not enrolled until Study Day 10, and for Cow 51005, which was removed from the study after Study Day 9. Both of these cows were removed from the statistical analyses. Cows were observed daily for overall clinical health from Study Day -7 to Study Day 38. Milk component measurements were taken on Study Days -7 to 38 in the AM and on Study Days 8 to 38 in the PM. Cows 54027 and 51005 were not included in the analysis.

Each individual cow was the experimental unit. The parameters statistically analyzed include the following:

- Fecal ADF, NDF, NDFom, and Dry Matter Percentage
- Feed ADF, NDF, NDFom, and Dry Matter Percentage
- Milk Production (Milk Production, Milk Fat Yield, Milk Protein Yield, Energy-Corrected Milk Yield, 3.5% Fat Corrected Milk Yield
- Milk Component Data: Milk Fat Percentage, Milk Protein Percentage, Milk Somatic Cell Count

Methods:

Research Candidate Evaluation

On Study Day -7, twenty-four lactation Holstein cows were evaluated for age, breed, parity, days in milk, duplicate ear tags with the same number, health, previous treatment history, disposition, udder with four good quarters.

Ration

The composition and calculated nutrient analysis for the ration fed during the study is in Attachment 1.

Ascus Representatives and Dairy Rumen Associated Microorganisms

Ascus Biosciences Laboratory provided the following:

- Sponsor Representatives
  Justin Wong
  Jordan Embree
- Ascus processed all microbes "in house", concentrations and re-suspensions were held in anaerobic vials on ice ready for administration.
- All negative control solutions were produced in the same manner.

Intra-Rumen Injection Administration

- An (b) (4) employee ((b) (4)) administered the daily intra-rumen injections to each cow.
- The intra-rumen injection site location was on the left side of the animal behind the last ribs in the paralumbar fossa. Prior to injection, each site was disinfected with isopropyl alcohol and allowed to dry.
- A 12-gauge 2-inch hypodermic needle was inserted through the abdominal wall and into the rumen. Afterwards, an 18-gauge, 6-inch spinal needle was inserted through the 12-gauge needle into the dorsal rumen.
- After needle insertion, intra-rumen location was confirmed by aspiration using a dose syringe.
- The Ascus representative gave syringes containing the appropriate microbes, or no microbes, to the dose administrator.

- After administration both needles inserted were removed and no further procedures were required.

Data and Samples Collected:

Measurements and Clinical Observations:

1. Twice daily milking, milk production measurements and clinical udder evaluations by quarter were performed every day for the full 38-day study period of the study for every individual animal.

Milk measurements collected were as follows:

Milk yield in pounds (Measured twice daily from Study Day -7 to 38).

*Milk fat percentage. (Daily from Study Day -7 to 7 from the A.M. milking, and then twice daily to Study Day 38). Note: The Sponsor requested A.M. and P.M. milk sampling and measurements starting on Study Day 8.

*Milk protein percentage.

*Milk lactose percentage.

*Milk solid percentage.

*SCC (Somatic Cell Count).

*Samples and measurements were daily from Study Day -7 to 7 from the A.M. milking, and then twice daily to Study Day 38. Note: The Sponsor requested A.M. and P.M. milk sampling and measurements starting on Study Day 8.

Clinical udder evaluations were scored as follows:

1=Normal Quarter/Normal Milk

2=Normal Quarter/Questionable Milk

3=Normal Quarter/Abnormal Milk

4=Swollen Quarter/Abnormal Milk

5= Swollen Quarter/Abnormal Milk/Systemic Abnormal Clinical Observations

2. Overall Clinical Health Observations:

Cows were observed daily for overall clinical health from Study Day -7 to Study Day 38.

3. Feed sampling:

Feed samples were collected on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, and Study Day 28. Samples had NDF and ADF determined.

4. Fecal sampling:
Fecal contents were sampled from each cow on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, and Study Day 28. Samples had NDF and ADF determined.

5. Rumen sampling:
Rumen contents were sampled from each cow on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, Study Day 28, Study Day 35 and Study Day 38.

Rumen samples were collected via an orally inserted rumen tube.

Approximately 10 mL of rumen content was added to a conical containing Stop solution (prepared at Ascus, 15mL conicals were prefilled with 3 mL of stop solution)

Stop solution composition: 3 mL of ethanol containing 5% Trizol™

Hold sample conicals containing stop solution were stored at 4^0C until used.

At the time of sampling, each tube was sealed, then shake vigorously to disperse stop solution throughout rumen sample.

All tubes were stored at -20°C prior to shipment to Ascus Biosciences.

Statistical Analysis Methods:

All statistical comparisons of the treatment main effect and two-way interactions with the treatment main effect were performed at the 0.10 level of significance. Statistical analyses were performed using R statistical software version 3.4.0.

Fecal ADF and NDF

Fecal ADF (% DM), NDF (% DM), NDFom (% DM), and Dry Matter Percentage values from Study Days 1, 8, 16, 24, 28 were analyzed using the R package "nlme" and the lme function for linear mixed models, with treatment, study day, and treatment by study day interaction as fixed effects and Cow ID as a random effect (where appropriate).

```
fit <- lme (Response ~ Treatment_Group*Day, random = ~ 1 | ID, data=fecal_data)
```

Each model was compared to a fixed-effect only model using a Chi-squared test. Least square means were used to compare treatment groups using the unadjusted p-values and Satterthwaite degrees of freedom was used to test for significant differences. Tukey's method was used to adjust the p-values for multiple comparisons.

Milk Production

The daily total milk production data was transformed into four additional variables: Milk Fat Yield, Milk Protein Yield, Energy-Corrected Milk Yield, and 3.5% Fat Corrected Milk Yield. Milk Fat Yield was obtained using the following formula:

$$Milk\ Fat\ Yield = Milk\ Production\ (lbs) \times Milk\ Fat\ Percentage$$

Daily total milk production measurements were transformed into Milk Fat Yield using the average of the AM and PM Milk Fat Percentages for each cow on the corresponding Study Day. There were no PM Milk Fat Percentage measurements on Study Days 1 through 7, so the AM measurement was used for the calculation of Milk Fat Yield on these Study Days instead of the average.

Milk Protein Yield was obtained using the following formula:

$$Milk\ Protein\ Yield = Milk\ Production\ (lbs) \times Milk\ Protein\ Percentage$$

Daily total milk production measurements were transformed into Milk Protein Yield using the average of the AM and PM Milk Protein Percentages for each cow on the corresponding Study Day. There were no PM Milk Protein Percentage measurements on Study Days 1 through 7, so the AM measurement was used for the calculation of Milk Protein Yield on these Study Days instead of the average.

Energy-Corrected Milk Yield was obtained using the following formula:

$$ECM = 0.327 \times Milk\ Production\ (lbs) + 12.95 \times Milk\ Fat\ Yield + 7.2 \times Milk\ Protein\ Yield$$

Daily total milk production measurements were transformed into Energy-Corrected Milk Yield using the average of the AM and PM Milk Protein Percentages and the average of the AM and PM Milk Fat Percentages for each cow on the corresponding Study Day. There were no PM Milk

Protein Percentage or Milk Fat Percentage measurements on Study Days 1 through 7, so the AM measurements were used for the calculation of Energy-Corrected Milk Yield on these Study Days instead of the averages.

3.5% Fat Corrected Milk Yield was obtained using the following formula:

$$FCM = 0.432 \times Milk\ Production\ (lbs) + 16.23 \times Milk\ Fat\ Yield$$

Daily total milk production measurements were transformed into 3.5% Fat Corrected Milk Yield using the average of the AM and PM Milk Protein Percentages and the average of the AM and PM Milk Fat Percentages for each cow on the corresponding Study Day. There were no PM Milk Protein Percentage or Milk Fat Percentage measurements on Study Days 1 through 7, so the AM measurements were used for the calculation of 3.5% Fat Corrected Milk Yield on these Study Days instead of the averages.
Milk Production, Milk Fat Yield, Milk Protein Yield, Energy-Corrected Milk Yield, and 3.5% Fat Corrected Milk Yield measurements from Study Days 1 to 38 were analyzed using the R package "nlme" and the lme function for linear mixed models, with treatment, week (time period), and the treatment by week interaction term as fixed effects and Cow ID as a random effect (where appropriate).

```
fit <- lme (Response ~ Trt_Group*Time_Period + (1 | Cow_ID), data=milk_data_prod, na.action = na.omit)
```

Each model was compared to a fixed-effect only model using a Chi-squared test. Least square means were used to compare treatment groups using the unadjusted p-values and Satterthwaite degrees of freedom was used to test for significant differences. Tukey's method was used to adjust the p-values for multiple comparisons.

Milk Component Data

Milk data (Milk Fat Percentage, Milk Protein Percentage, Milk Somatic Cell Count) measurements from Study Days 1 to 38 AM and 8 to 38 PM were analyzed using the R package "nlme" and the lme function for linear mixed models, with treatment, week (time period), and the treatment by week interaction terms as fixed effects and Cow ID as a random effect (where appropriate). AM and PM measurements were averaged per study day per cow for analysis. The data for Study Days 1 through 7 were only AM measurements.

```
fit <- lme(Response ~ Trt_Group*Time_Period + (1 | Cow_ID), data=milk_data, na.action = na.omit)
```

Each model was compared to a fixed-effect only model using a Chi-squared test. Least square means were used to compare treatment groups using the unadjusted p-values and Satterthwaite degrees of freedom was used to test for significant differences. Tukey's method was used to adjust the p-values for multiple comparisons.

Feed Data

The feed data was a set of Dry Matter Percentage, ADF (% DM), NDF (% DM), and NDFom (% DM) values for samples taken on Study Days 1, 8, 16, 24, and 28. A summary table was produced for this data set.

Results:

Research Candidate Evaluation

On Study Day -7, twenty-four lactation Holstein cows that were 3-6 years-old, with a parity range of 2-4 lactations, 89-111 days in milk, with duplicate ear tags with the same number, good health, no previous medical treatment history within the previous 30 days, good disposition, and udder with four good quarters were selected for the study.

Ascus Representatives and Dairy Rumen Associated Microorganisms

Justin Wong and/or Jordan Embree were present on each day of dosing and presented the individual administering the intra-rumen injection with syringes containing the appropriate microbes or no microbes. Ascus processed all microbes "in house"; and each of the microbes was delivered at the dose of 1×10^9 CFUs/day.

Intra-Rumen Injection Administration

In general, the daily intra-rumen injections were administered uneventfully. Only small (<1 cm) injection site swellings were observed and were considered incidental.

Milk Production

Milk production (AM, PM and daily total) and milk component data (milk fat percentage, milk protein percentage, milk lactose percentage, milk solids percentage and milk somatic cell counts) measurements were taken on Study Days -7 to 38, but only the measurements from Study Days 1 to 38 were analyzed. The AM and PM measurements were pooled for analysis.

Variables are grouped by model outcome (where appropriate): non-significant TRT effect, significant TRT effect, significant TRT*Time Period (week) or TRT*Day effect. Statistically significant results for variables follow, when necessary. Only the appropriate differences are listed and significant differences are denoted with an asterisk (*).

Table 1 contains the Milk Production Data: Means by Study Day by Treatment Group. Table 2 contains the Milk Production (Prod) Data: Means by Time Period by Treatment Group. Table 3 contains Model Information for Milk Production Data. Table 4 contains Milk Production Data Differences for Treatment Effects. For Milk Production, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0185. Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 3, p=0.0754. Figure 1 shows the Graph of Weekly Least Square Means for Milk Production. Although the Treatment Group by Week interaction was significant, there were no significant individual Treatment Group LSMean differences within week for Milk Fat Yield. The adjustment for multiple comparisons created this disparity. Figure 2 shows the Graph of Weekly Least Square Means for Milk Fat Yield. For Milk Protein Yield, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0302. Figure 3 shows the Graph of Weekly Least Square Means for Milk Protein Yield. For Energy-corrected Milk Yield, Treatment Group 1 had significantly higher values than Treatment Group 3 during Week 2, p=0.0942. Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0303. Figure 4 shows the Graph of Weekly Least Square Means for Energy-Corrected Milk Yield. For 3.5% Fat-Corrected Milk Yield, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0405. Figure 5 shows the Graph of Weekly Least Square Means for 3.5% Fat Corrected Milk Yield.

Table 1 Milk Production (Prod:lbs) Data: Means by Study Day by Treatment Group

	Average of AM Milk Prod			Average of PM Milk Prod			Average of Daily Total Milk Prod		
Study Day	T1	T2	T3	T1	T2	T3	T1	T2	T3
-7	56.875	62.250	57.750	33.500	30.875	31.750	90.375	93.125	89.500
-6	51.750	46.750	50.500	45.125	47.000	45.625	96.875	93.750	96.125
-5	57.000	58.250	60.625	45.500	47.875	43.375	102.500	106.125	104.000
-4	55.625	56.750	55.375	46.000	47.500	42.750	101.625	104.250	98.125
-3	54.500	55.000	55.375	47.625	46.250	45.500	102.125	101.250	100.875
-2	56.625	58.000	57.750	46.750	45.750	40.500	103.375	103.750	98.250
-1	63.875	61.375	56.000	41.375	47.875	47.625	105.250	109.250	103.625
1	55.250	56.750	54.375	41.500	48.750	47.500	96.750	105.500	101.875
2	45.000	53.375	53.000	44.250	46.500	43.250	89.250	99.875	96.250
3	55.500	60.125	50.375	42.000	47.500	43.125	97.500	107.625	93.500
4	54.375	60.500	56.750	42.500	49.750	45.875	96.875	110.250	102.625
5	48.125	60.375	56.000	45.000	52.250	46.500	93.125	112.625	102.500
6	53.750	57.000	54.875	45.750	53.250	43.500	99.500	110.250	98.375
7	51.750	60.000	58.125	43.750	50.500	45.750	95.500	110.500	103.875
8	52.750	59.750	50.000	47.625	50.375	41.750	100.375	110.125	91.750
9	50.000	57.750	51.125	45.625	50.250	41.500	95.625	108.000	92.625
10	56.875	58.000	45.750	52.500	52.500	41.250	109.375	110.500	87.000
11	54.250	56.250	48.125	47.625	52.500	43.500	101.875	108.750	91.625
12	53.625	50.500	46.250	45.875	47.875	39.875	99.500	98.375	86.125
13	54.875	56.125	45.500	43.500	49.000	43.375	98.375	105.125	88.875
14	53.000	56.500	44.875	46.375	50.000	43.250	99.375	106.500	88.125
15	56.750	55.500	50.500	48.000	53.625	45.500	104.750	109.125	96.000
16	55.750	58.500	48.125	48.125	51.375	44.625	103.875	109.875	92.750
17	54.750	55.000	46.500	49.875	46.750	43.000	104.625	101.750	89.500
18	56.500	58.750	50.375	46.000	47.250	42.250	102.500	106.000	92.625
19	54.500	53.375	47.750	47.250	51.500	46.000	101.750	104.875	93.750
20	57.875	55.500	47.750	48.375	51.000	43.875	106.250	106.500	91.625
21	53.500	57.125	51.875	46.125	48.500	41.750	99.625	105.625	93.625
22	55.125	56.375	49.250	42.125	47.000	44.375	97.250	103.375	93.625
23	56.625	56.375	52.625	46.625	47.500	41.750	103.250	103.875	94.375
24	52.625	54.125	52.250	45.750	49.500	45.875	98.375	103.625	98.125
25	48.625	55.250	46.875	46.750	50.625	50.000	95.375	105.875	96.875
26	50.375	54.500	50.000	44.250	47.250	45.250	94.625	101.750	95.250
27	53.625	56.375	54.375	43.125	43.375	45.375	96.750	99.750	99.750
28	50.125	52.000	51.875	43.250	45.000	41.875	93.375	97.000	93.750
29	47.125	47.500	49.125	42.500	41.625	42.000	89.625	89.125	91.125
30	49.500	49.625	49.625	43.750	46.500	44.125	93.250	96.125	93.750
31	51.000	48.250	48.125	43.375	40.625	40.000	94.375	88.875	88.125
32	51.625	50.250	49.000	42.750	45.250	41.500	94.375	95.500	90.500
33	56.125	49.500	51.250	46.125	47.500	45.125	102.250	97.000	96.375
34	52.750	51.375	51.625	47.875	45.250	43.625	100.625	96.625	95.250
35	51.125	53.000	52.000	43.500	44.250	45.750	94.625	97.250	97.750
36	44.125	50.625	54.250	41.750	42.000	46.500	85.875	92.625	100.750
37	49.625	54.625	52.750	40.500	45.000	43.875	90.125	99.625	96.625
38	51.125	49.250	52.875	38.375	42.750	42.375	89.500	92.000	95.250

Table 2 Milk Production (Prod:lbs) Data: Means by Time Period by Treatment Group

	Average of AM Milk Prod			Average of PM Milk Prod			Average of Daily Total Milk Prod		
Time Period	T1	T2	T3	T1	T2	T3	T1	T2	T3
Baseline	56.607	56.911	56.196	43.696	44.732	42.446	100.304	101.643	98.643
Treatment	53.424	56.491	50.545	45.696	49.330	43.982	99.121	105.821	94.527
Post-Treatment	50.413	50.400	51.063	43.050	44.075	43.488	93.463	94.475	94.550

Table 3 Model Information for Milk Production Data, Study Day Model

Variable	Model Type	P-values		Decision
		Treatment_Group	Treatment_Group*Week	
Milk Production	Mixed	0.3233	<0.0001	(3)
Milk Fat Yield	Mixed	0.637	0.022	(3)
Milk Protein Yield	Mixed	0.5017	<0.0001	(3)
Energy-Corrected Milk Yield	Mixed	0.4284	<0.0001	(3)
3.5% Fat-Corrected Milk Yield	Mixed	0.4348	<0.0001	(3)

Decisions:

(1) There were no significant terms involving Treatment Group. No further evaluation is needed.

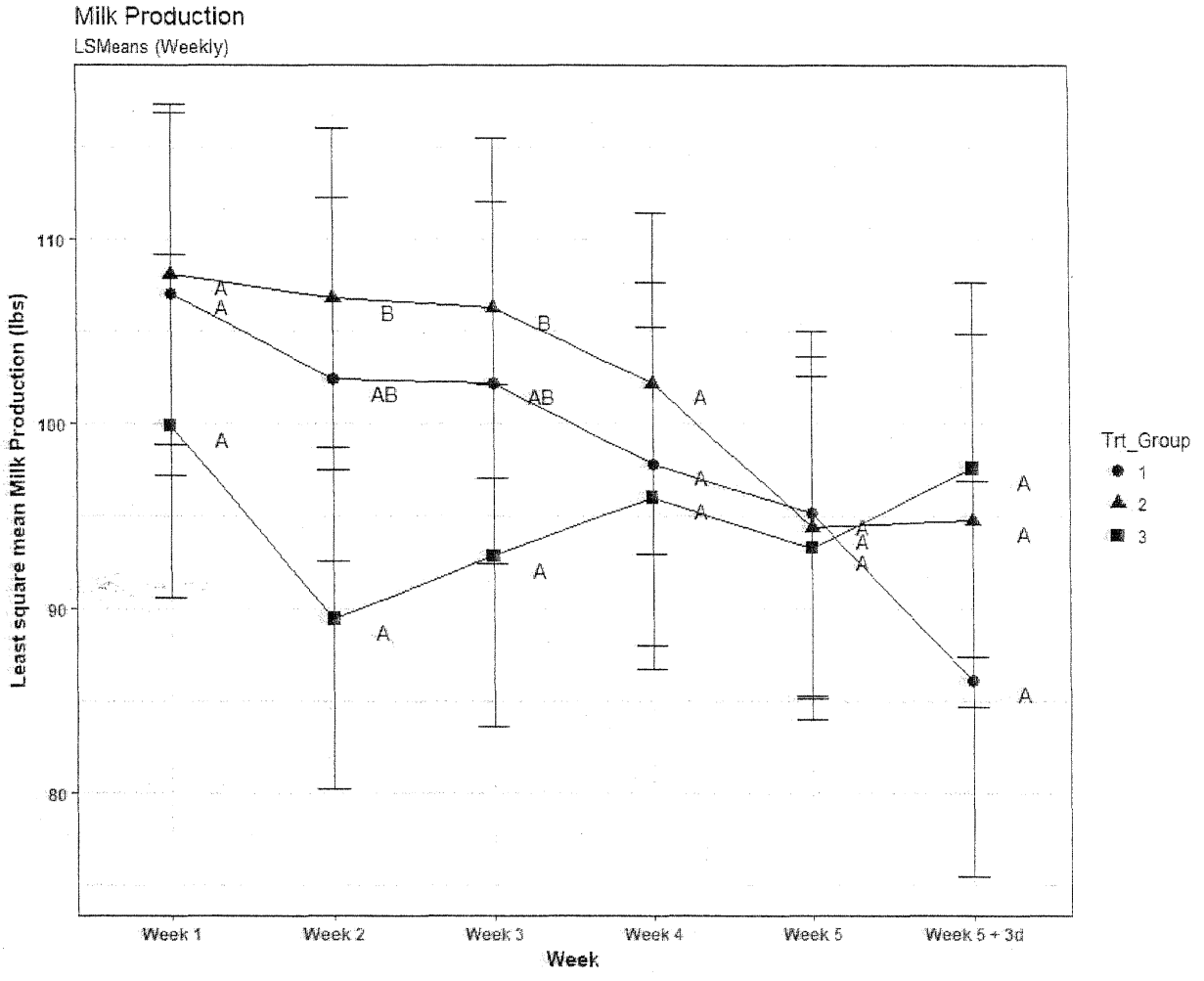
(2) The Treatment Group main effect is significant at α=0.10, and the 2-way interaction is not significant. Compare treatment means from the main effect of Treatment Group.

(3) The Treatment Group by Week interaction is significant at α=0.10. Compare treatment means within each week.

Table 4 Decision (3) Milk Production Data Differences and Standard Deviations for Treatment Effects

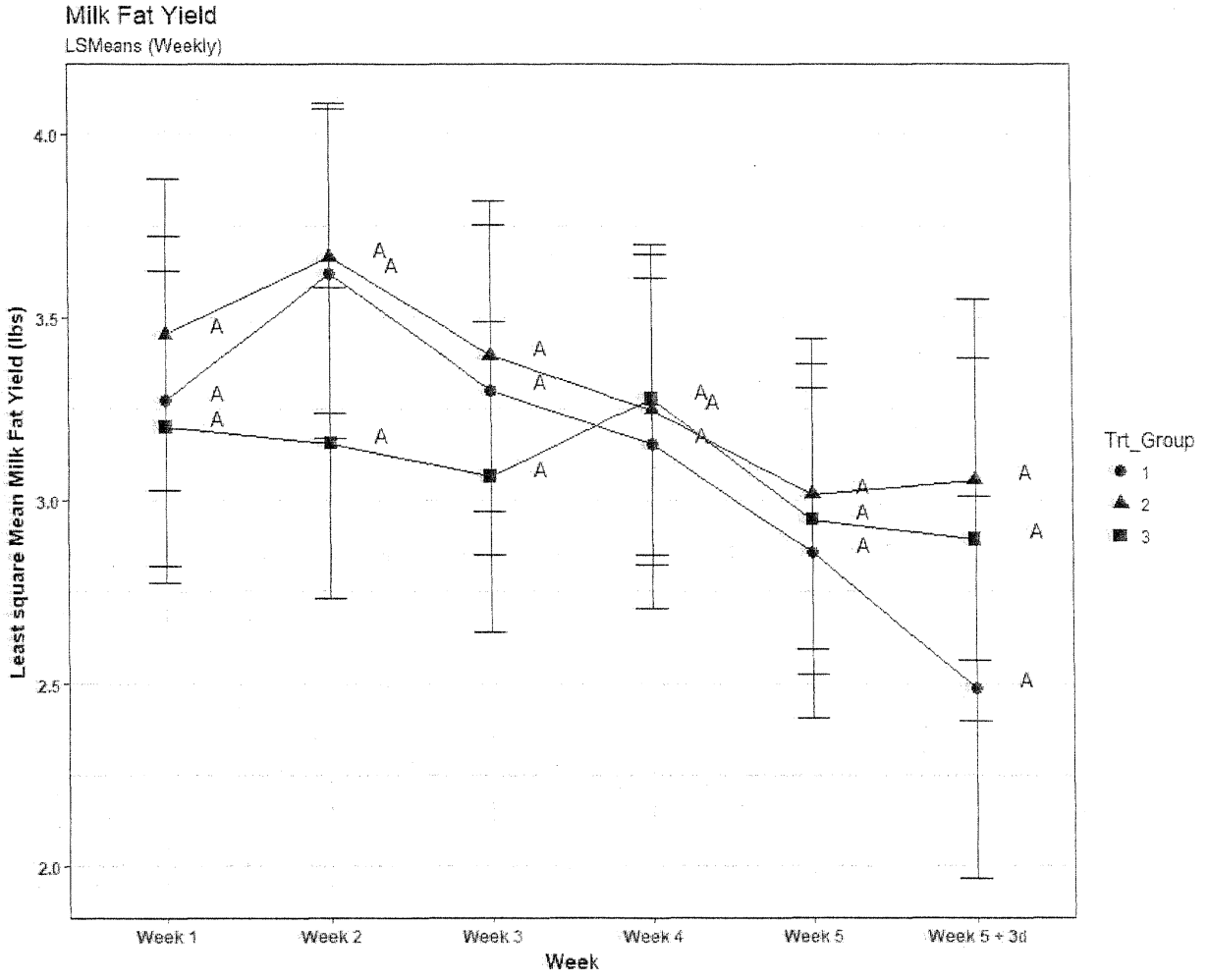
Variable	Week	Compare	Difference	Standard Error	P-value
Milk Production	2	Treatment Group 1 vs. Treatment Group 3	6.4707	2.9723	0.0998
Milk Production	2	Treatment Group 2 vs. Treatment Group 3	8.6607	2.8715	0.0179
Milk Production	3	Treatment Group 2 vs. Treatment Group 3	6.7054	2.8715	0.0737
Milk Fat Yield	1	Treatment Group 1 vs Treatment Group 2	26.5311	11.9021	0.0905
Milk Protein Yield	1	Treatment Group 1 vs Treatment Group 2	21.5625	7.5398	0.0251
Milk Protein Yield	1	Treatment Group 2 vs Treatment Group 3	-16.1260	7.2853	0.0932
Energy-Corrected Milk Yield	2	Treatment Group 2 vs Treatment Group 3	520.3163	214.2768	0.0614
3.5% Fat-Corrected Milk Yield	1	Treatment Group 1 vs Treatment Group 2	433.2352	193.6081	0.0890

Figure 1: Graph of Weekly Least Square Means for Milk Production



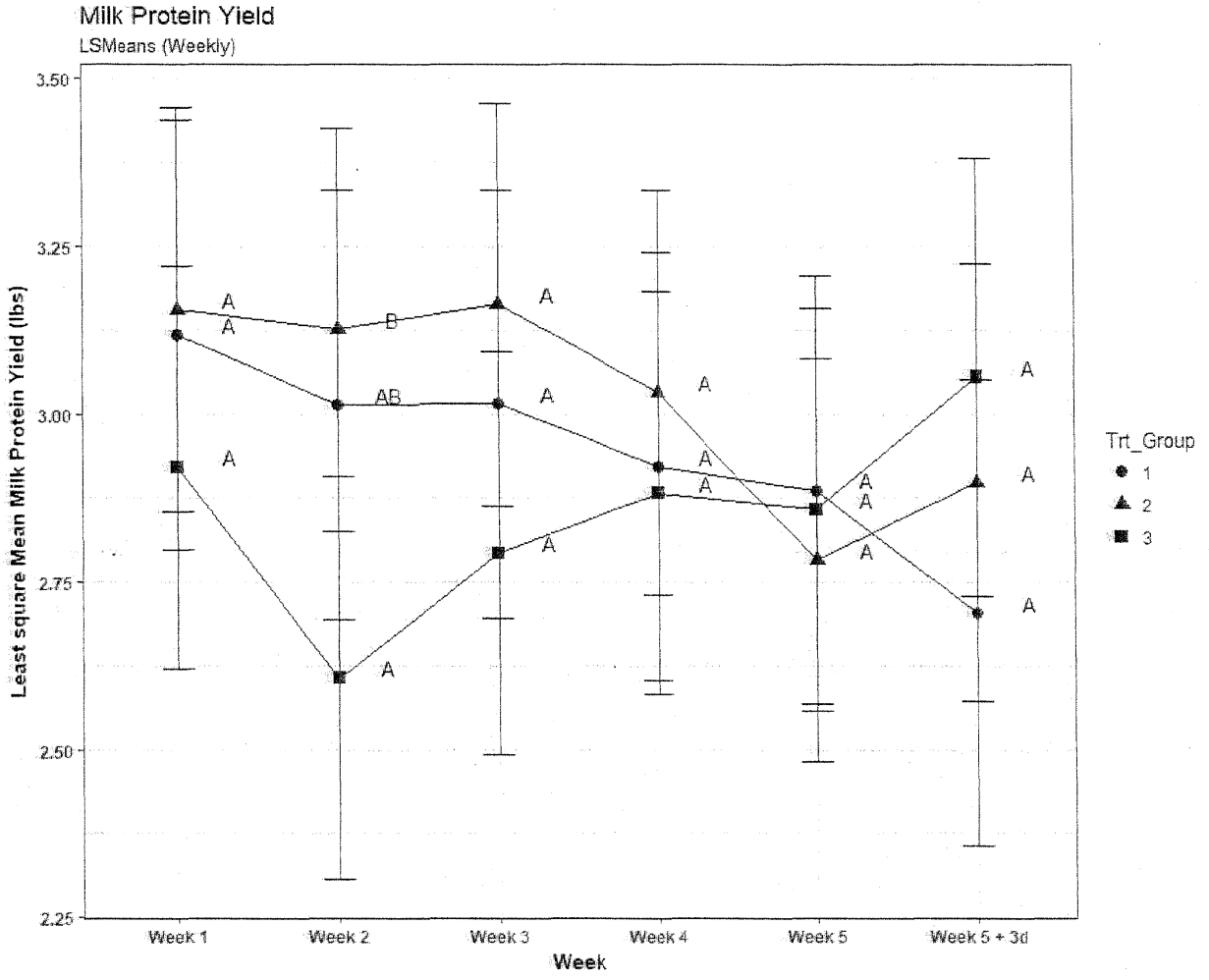
Milk Production (daily total, lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Figure 2: Graph of Weekly Least Square Means for Milk Fat Yield



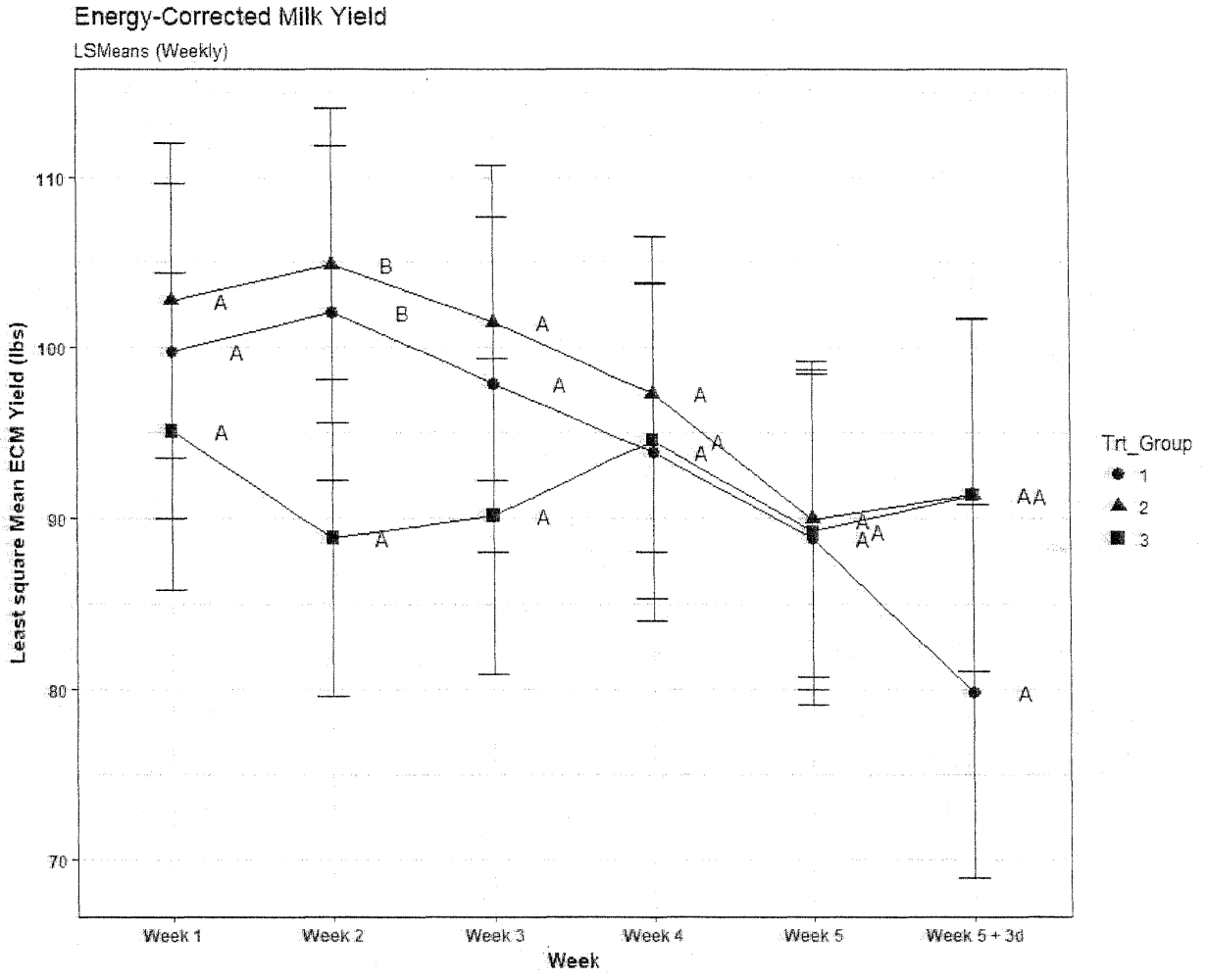
Milk Fat Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Figure 3: Graph of Weekly Least Square Means for Milk Protein Yield



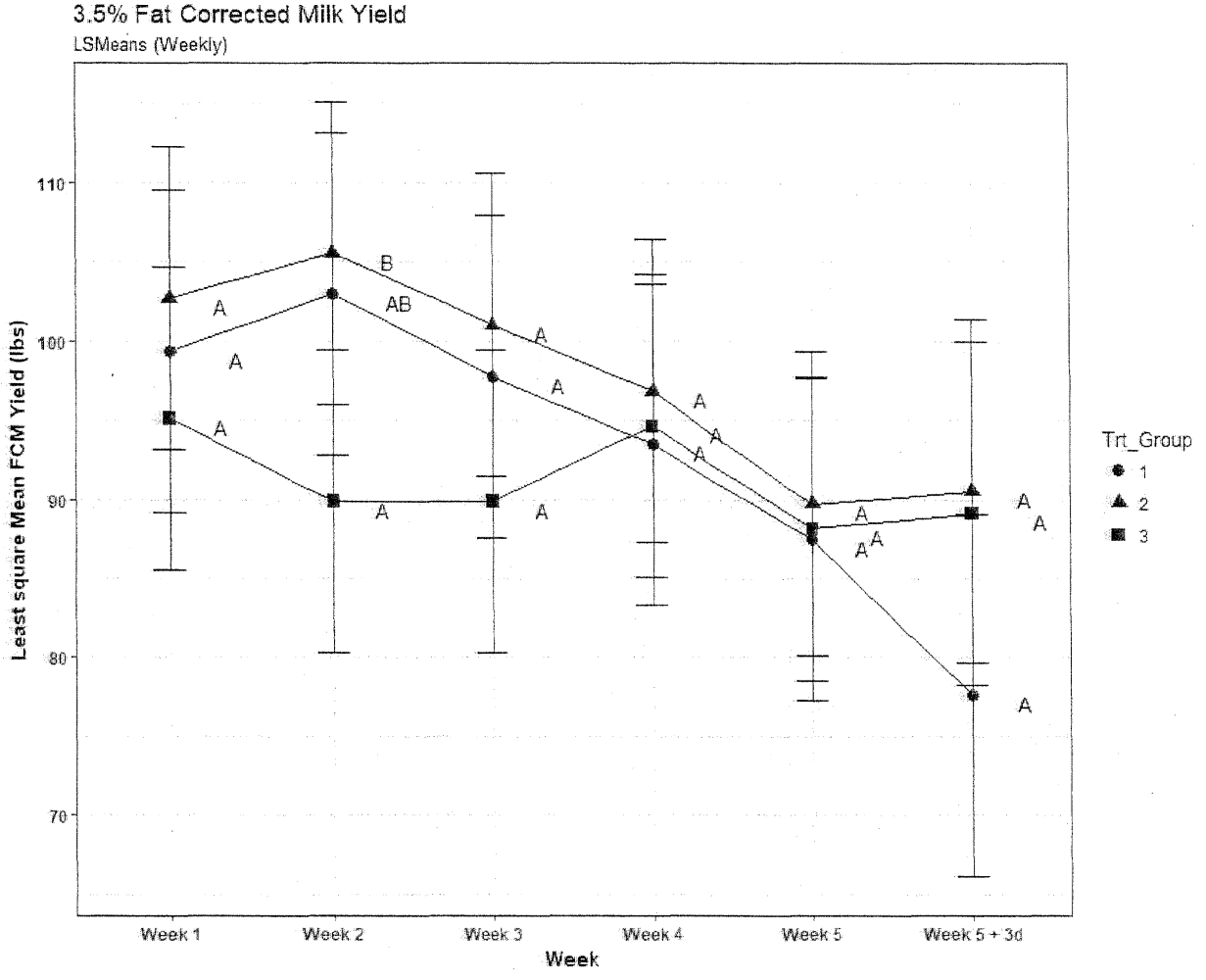
Milk Protein Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Figure 4: Graph of Weekly Least Square Means for Energy-Corrected Milk Yield



Energy-Corrected Milk Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Figure 5: Graph of Weekly Least Square Means for 3.5% Fat Corrected Milk Yield



3.5% Fat Corrected Milk Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Milk Component Data

Milk data (Milk Fat Percentage, Milk Protein Percentage, Milk Lactose Percentage, Milk Solids Percentage, Milk Somatic Cell Count) were measured on Study Days -7 to 38 for AM measurements, and on Study Days 8 to 38 for PM measurements. The milk data from Study Days 1 to 38 were analyzed. AM and PM measurements were averaged per study day per cow for analysis. The data for Study Days 1 through 7 were only AM measurements. Table 5 contains Model Information for Milk Component Data. Table 6 contains Milk Component Data for treatment effects. There were no significant individual Treatment Group LSMean differences within week for Milk Fat %. Figure 6 show the Graph of Weekly Least Square Means for Milk Fat Percentage. For Milk Protein %, Treatment Group 1 had significantly higher values than Treatment Group 2 during Week 5 + 2d, p=0.0001. Treatment Group 1 had significantly higher values than Treatment Group 3 during Week 5 + 2d, p=0.0009. Figure 7 shows the Graph of Weekly Least Square Means for Milk Protein Percentage. For Milk SCC, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 1, p=0.0273. Figure 8 shows the Graph of Weekly Least Square Means for Milk Somatic Cell Count.

Table 5: Model Information for Milk Component Data

Variable	Model Type	P-values		Decision
		Treatment Group	Treatment Group*Week	
Milk Fat %	Mixed	0.8392	0.1733	(1)
Milk Protein %	Mixed	0.7404	<0.0001	(3)
Milk SCC	Mixed	0.1310	0.0218	(3)

Decisions:

(1) There were no significant terms involving Treatment_Group. No further evaluation is needed.

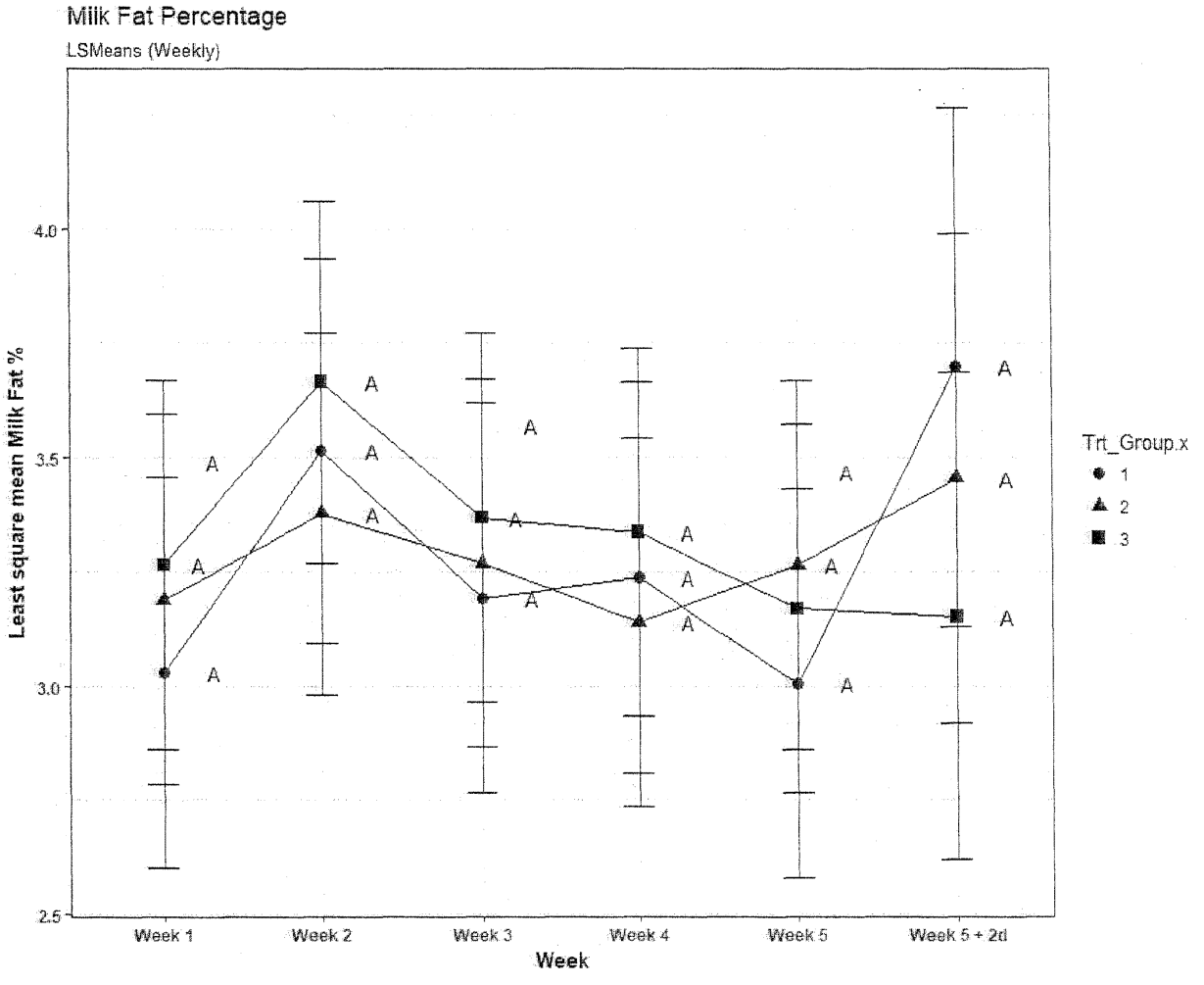
(2) The Treatment_Group main effect is significant at α=0.10, and the 2-way interaction is not significant. Compare treatment means from the main effect of Treatment_Group.

(3) The Treatment_Group by Week interaction is significant at α=0.10. Compare treatment means within each week.

Table 6: Milk Component Data for Treatment Effects

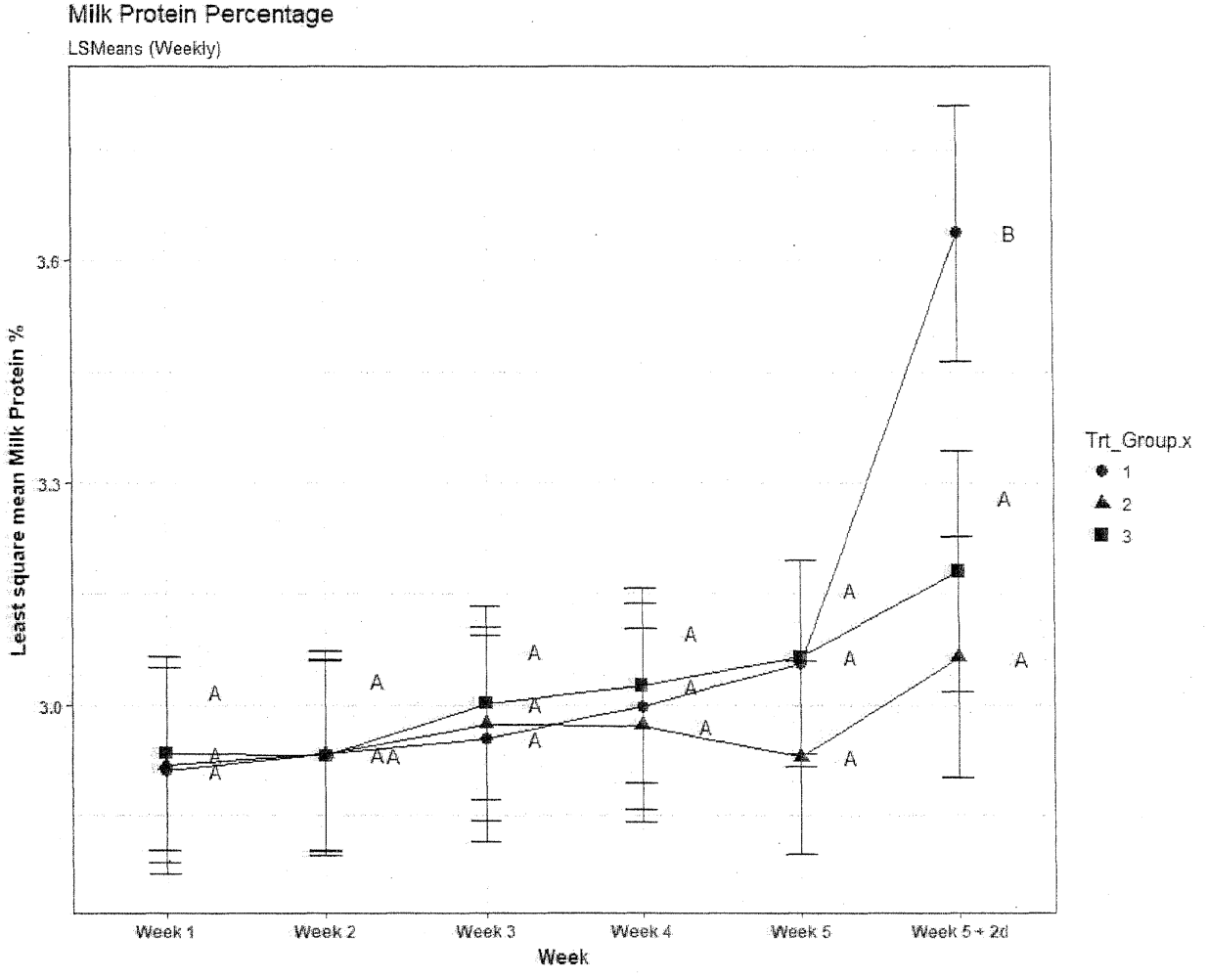
Variable	Week	Compare	Difference	Standard Error	P-value
Milk Protein %	5 + 2d	Treatment Group 1 vs. Treatment Group 2	0.5731	0.1051	0.0001
Milk Protein %	5 + 2d	Treatment Group 1 vs. Treatment Group 3	0.4569	0.1051	0.0009
Milk SCC	1	Treatment Group 2 vs Treatment Group 3	1.494	0.5299	0.0273

Figure 6: Graph of Weekly Least Square Means for Milk Fat Percentage



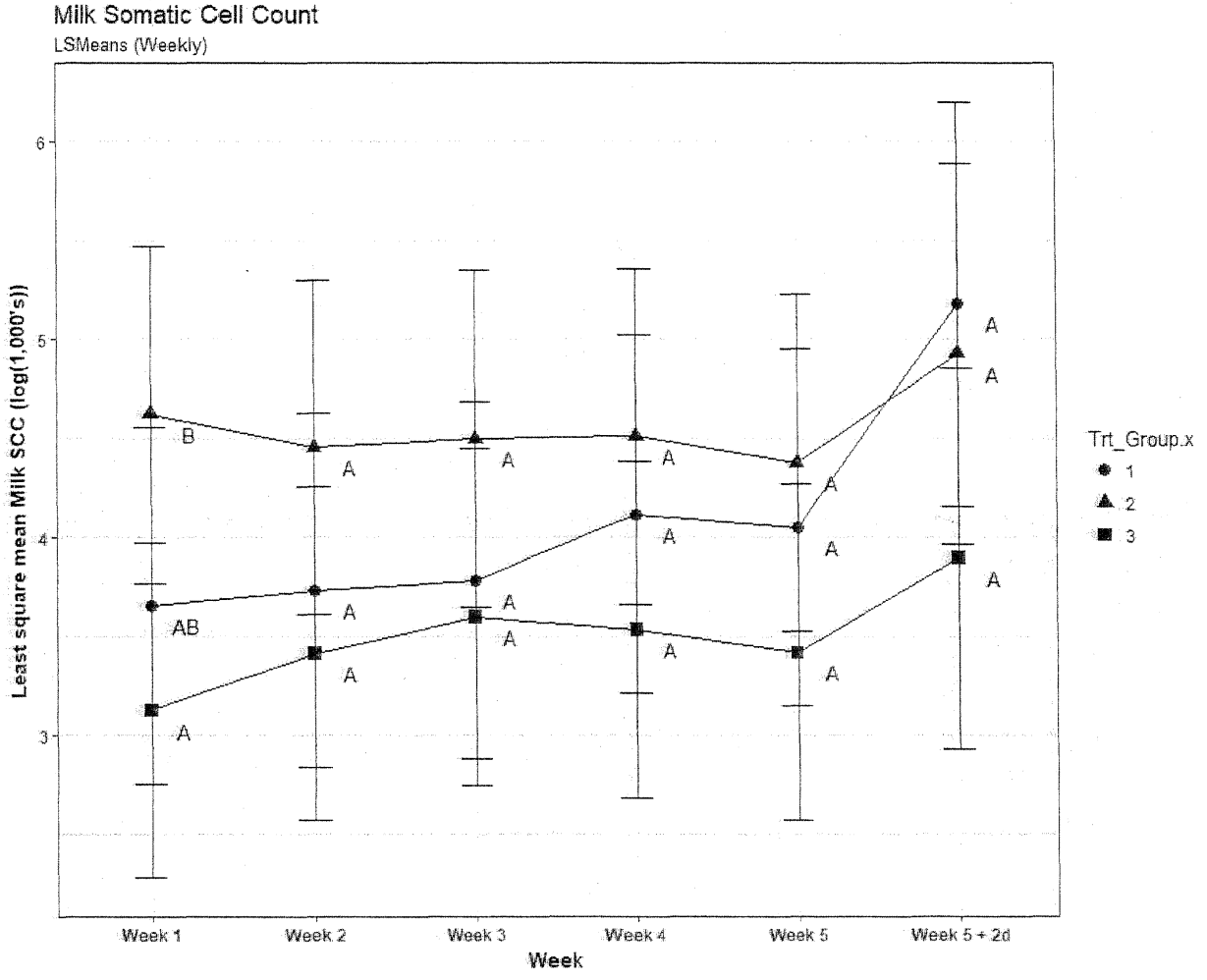
Milk Fat Percentage for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Figure 7: Graph of Weekly Least Square Means for Milk Protein Percentage



Milk Protein Percentage for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Figure 8: Graph of Weekly Least Square Means for Milk Somatic Cell Count



Milk Somatic Cell Count (log(1,000's)) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Feed Data

For feed, Dry Matter Percentage, ADF (% DM), NDF (% DM), and NDFom (% DM) values for samples were measured on Study Days 1, 8, 16, 24, and 28. Table 7 shows the Summary of Feed Data.

Table 7: Summary of Feed Data

	Dry Matter Percentage	ADF (% DM)	NDF (% DM)	NDFom (% DM)
Minimum	0.4654	0.1901	0.2764	0.2587
1st Quartile	0.4755	0.1978	0.2835	0.2636
Median	0.4847	0.1985	0.2879	0.2649
Mean	0.4813	0.1982	0.2861	0.2648
3rd Quartile	0.4855	0.1998	0.2892	0.2667
Maximum	0.4952	0.2046	0.2937	0.2702
Standard Deviation	0.01128	0.005229	0.006547	0.004226
Coefficient of Variation (%)	2.344	2.639	2.288	1.596

Clinical Udder Evaluations

Abnormal clinical udder findings were considered minimal, incidental and not treatment group-related during the study.

Cow #51005, Treatment Group 1, (b) (4) one episode of mastitis from Study Day 1 to 9 (multiple quarters scored 4-2), and did not respond well to Spectromast LC® (Zoetis) intra-mammary antimicrobial treatment. This cow was replaced with cow 54027 on Study Day 10.

Cow #49155, Treatment Group 1, had one episode of mastitis on Study Days 35 to 38 (multiple quarters scored 4-2) and was treated with Spectromast LC® (Zoetis) intra-mammary antimicrobial treatment. This cow did not respond well to intra-mammary treatment, but completed the study.

Cow #47520, Treatment Group 2, had two episodes of mastitis, the first on Study Days 2-4 (one quarter scored 4-2) and was treated with Spectromast LC® (Zoetis) intra-mammary antimicrobial treatment. This cow responded well to intra-mammary treatment and returned to normal. The second episode of mastitis was on Study Days 29 to 38 (one quarter scored 3-2), and was not treated and completed the study.

Cow #49654, Treatment Group 2, had two episodes of mastitis, the first on Study Days 11-14 (one quarter scored 3-2) and was not treated and this cow returned to normal. The second episode of mastitis was on Study Days 22 to 24 (the same one quarter scored 3-2), and was not treated, returned to normal and completed the study.

Cow #53110, Treatment Group 3, had one episode of mastitis on Study Days 2 to 7 (one quarter scored 4-2) and was treated with Spectromast LC® (Zoetis) intra-mammary antimicrobial treatment. This cow responded well to intra-mammary treatment, and completed the study.

Overall Clinical Health Observations

Abnormal clinical health observations, as determined by observing the cows in their pen) were considered minimal, incidental and not treatment group-related during the study.

All animals were clinically normal from Study Day -7 to 35 (except for mastitis cases stated above, which were determined at the time of milking.

On Study Days 36 to 38 two cows, #51562, Treatment Group 2 and #49155, Treatment Group 1, were both observed depressed. Cow #49155 had an episode of mastitis ongoing and was being treated with Spectromast LC® (Zoetis) intra-mammary antimicrobial treatment (described above). Both animals completed the study. Cow #51562 did not have mastitis and depression was the only abnormal clinical observation and considered most likely due to focal local injection site inflammation due to the multiple intra-rumen injections.

Fecal ADF and NDF

Fecal ADF (% DM), NDF(% DM), NDFom (% DM), and Dry Matter Percentage were measured from Study Days 1, 8, 16, 24 and 28. Table 8 contains the model information for Fecal Data. Table 9 contains Fecal Data Dry Matter % Differences for Treatment Effects. For Fecal Data Dry Matter Percentage, Treatment Group 1 had significantly higher values than Treatment Group 3, p=0.0229. Table 10 contains the Fecal Data NDF (%DM) Differences for Treatment Effects. For NDF (% DM), Treatment Group 1 had significantly higher values than Treatment Group 2 on Day 1, p=0.0146. Treatment Group 2 had significantly lower values than Treatment Group 3 on Day 1, p=0.0631.

Table 8: Model Information for Fecal Data

Variable	Model Type	P-values		Decision
		Treatment Group	Treatment Group*Study Day	
ADF (% DM)	Fixed Effects Only	0.2433	0.1497	(1)
NDF (% DM)	Fixed Effects Only	0.2833	0.05478	(3)
NDFom (% DM)	Fixed Effects Only	0.2386	0.1796	(1)
Dry Matter %	Fixed Effects Only	0.03432	0.1777	(2)

Decisions:

(1) There were no significant terms involving Treatment Group. No further evaluation is needed.

(2) The Treatment Group main effect is significant at α=0.10, and the 2-way interaction is not significant. Compare treatment means from the main effect of Treatment Group.

(3) The Treatment Group by Study Day interaction is significant at α=0.10. Compare treatment means within each day.

Table 9 Fecal Data Dry Matter % Differences for Treatment Effects

Variable	Compare	Difference	Standard Error	P-value	Significance
Dry Matter %	Treatment Group 1 vs. Treatment Group 2	0.005060	0.005165	0.5914	
Dry Matter %	Treatment Group 1 vs. Treatment Group 3	0.01416	0.005272	0.0229	*
Dry Matter %	Treatment Group 2 vs. Treatment Group 3	0.009096	0.005201	0.1923	

Table 10 Fecal Data NDF (% DM) Differences for Treatment Effects

Variable	Study Day	Compare	Difference	Standard Error	P-value	Significance
NDF (% DM)	1	Treatment Group 1 vs. Treatment Group 2	0.1081	0.03793	0.0146	*
NDF (% DM)	1	Treatment Group 2 vs. Treatment Group 3	-0.08360	0.03665	0.0631	*

Rumen Samples

Rumen samples were submitted to the Sponsor for evaluation and the results are not reported in this report.

Conclusions:

In the opinion of the Investigator, abnormal clinical udder findings and abnormal clinical health observations were considered minimal, incidental and not treatment group-related during the study.

Statistically significant differences between treatment groups were determined to be as follows:

For Fecal Data Dry Matter Percentage, Treatment Group 1 had significantly higher values than Treatment Group 3, p=0.0229.

For Fecal Data NDF (% DM), Treatment Group 1 had significantly higher values than Treatment Group 2 on Day 1, p=0.0146. Treatment Group 2 had significantly lower values than Treatment Group 3 on Day 1, p=0.0631.

For Milk Protein %, Treatment Group 1 had significantly higher values than Treatment Group 2 during Week 5 + 2d, p=0.0001. Treatment Group 1 had significantly higher values than Treatment Group 3 during Week 5 + 2d, p=0.0009.

For Milk SCC, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 1, p=0.0273.

For Milk Production, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0185. Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 3, p=0.0754.

For Milk Fat Yield, Treatment Group 1 had significantly higher values than Treatment Group 2 during Week 1, p=0.0905.

For Milk Protein Yield, Treatment Group 1 had significantly higher values than Treatment Group 2 during Week 1, p=0.0251. Treatment Group 2 had significantly lower values than Treatment Group 3 during Week 1, p=0.0932.

For Energy-corrected Milk Yield, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0614.

For 3.5% Fat-Corrected Milk Yield, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0405.

Attachment 1 Ration Composition and Calculated Nutrient Analysis

(b) (4)

Ration Outputs (Fresh Cows)

AMTS.Cattle.Professional

Farm: LoneOak
Cattle: Fresh Cows

FBW: 1550 lbs
BCS (1-5): 3.00
ADG: 0.000 lbs/day

DIM: 30
Milk: 84.9 lbs/day
Milk Fat: 3.70%
Milk Prt: 3.10%

Inputted DMI: 52.41 lbs
Predicted DMI: 40.76 lbs

Ration Fed				
Ingredient	$/hd	%DM	DM lbs/day	AF lbs/day
Alfalfa Hay 20 CP 37 NDF 17 [illegible]	1.53	90.0	11.00	12.22
Corn Silage	1.10	35.1	12.46	35.50
Corn	0.41	85.0	3.00	3.53
EnerGII Regular	0.31	98.0	0.60	0.61
High Moisture Corn 30%	0.48	70.3	3.50	4.98
Soy Plus	0.41	89.1	1.37	1.53
Molasses Cane	0.08	71.0	0.64	0.90
Almond Hulls-Alpha Dairy [illegible]	0.10	89.0	1.28	1.44
Canola	0.91	90.0	4.27	4.74
Cottonseed	0.63	91.0	3.20	3.52
LO MC Min 070912	0.23	98.5	1.28	1.30
Wheat Straw 5 CP 79 NDF 16 [illegible]	0.10	92.0	1.71	1.86
Soyhull Pellets	0.46	90.0	4.27	4.74
DDG	0.67	89.0	3.84	4.32
Totals	7.43	64.6	52.41	81.19

Cost/ton As-Fed: $183.04

Output	Min	Value	Max	Status
Cost/hd	2.75	7.43	100.00	OK
DM (%)	20.00	64.56	80.00	OK
Dry Matter Intake (lbs/day)	24.60	52.41	24.70	HIGH
Forage NDF (%NDF)	0.00	57.57	100.00	OK
Forage (%DM)	0.00	48.02	100.00	OK
ADF (%DM)	0.00	25.71	100.00	OK
NDF (%DM)	0.00	37.25	100.00	OK
peNDF (%DM)	22.00	25.28	35.00	OK
ME Allowable Milk (lbs/day)	84.04	66.47	85.74	LOW
MP Allowable Milk (lbs/day)	84.04	78.45	85.74	LOW
ME (%Rqd)	99.00	85.49	101.00	LOW
MP (%Rqd)	99.00	95.12	101.00	LOW
CP (%)	-	16.32	-	-
SP (%CP)	-	31.81	-	-
RDP (%DM)	-	8.53	-	-
NFC (%DM)	0.00	31.75	40.00	OK
Sugar (%DM)	0.00	6.10	12.00	OK
Starch (%DM)	0.00	17.11	30.00	OK
Soluble Fiber (%DM)	0.00	6.79	10.00	OK
EE (%DM)	0.00	5.76	6.50	OK
LCFA (%DM)	0.00	4.74	6.50	OK
Total Unsaturate (%DM)	0.00	3.30	3.00	HIGH
NEl (Mcal/lb)	-	0.67	-	-
DCAD1 (meq/kg)	-200.00	305.89	500.00	OK
MP Supply (g)	500.00	2633.39	3000.00	OK
CHO-C (g)	0.00	3102.24	2200.00	HIGH
Ferm. CHO (%DM)	10.00	36.19	70.00	OK
Fermentable CHO (%CHO)	0.00	53.14	70.00	OK
IOFC	0.00	-7.43	100.00	LOW
Ca (g)	0.00	212.27	200.00	HIGH
Ca (%DM)	0.00	0.89	2.00	OK
Mg (%DM)	0.00	0.34	2.00	OK
P (%DM)	0.00	0.40	2.00	OK
K (%DM)	0.00	1.49	2.00	OK
S (%DM)	0.00	0.26	2.00	OK
Na (%DM)	0.00	0.44	2.00	OK
Cl (%DM)	0.00	0.39	2.00	OK
Vit-A (KIU)	0.00	64.56	110.00	OK
Vit-D (KIU)	0.00	11.39	50.00	OK
Vit-E (IU)	0.00	172.16	3000.00	OK
LYS (%MP)	0.00	6.29	7.60	OK
MET (%MP)	0.00	1.97	2.40	OK
LYS:MET	2.80	3.19	4.00	OK
ME (Mcal/lb)	-	1.04	-	-
NEg (Mcal/lb)	-	0.38	-	-
NEm (Mcal/lb)	-	0.65	-	-
Monensin (mg/day)	0.00	225.25	480.00	OK
IOpurFC	-	-4.52	-	-
Purchased Cost/hd	-	4.52	-	-
Total Manure N (g)	0.00	0.00	100.00	OK
Total Manure P (g)	0.00	0.00	100.00	OK

Attachment 2 Statistical Report

Statistical Report

"Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms in Live Lactating Dairy Holstein Heifers via Direct Injection into the Rumen"

Study Number (b) (4)

Study Sponsor:

Ascus Biosciences, Inc.

6450 Lusk Blvd

Suites E109 / 209

San Diego, CA 92121

In-Life Test Facility:

(b) (4)

Prepared by:

(b) (4) Date: 04 August 17

(b) (4)

Table of Contents

1.0 INTRODUCTION

There were 3 treatment groups in the study. 8 experimental Holstein cows (average ~100 days in milk) received 2 microbes via injection into the rumen (Treatment Group 1). 8 experimental Holstein cows (average ~100 days in milk) received 3 microbes via injection into the rumen (Treatment Group 2). 8 experimental Holstein cows (average ~100 days in milk) received 3 basal suspension medias (no microbes) via injection into the rumen (Treatment Group 3).

The cows were inoculated daily after the morning milking for 28 days. Fecal contents were sampled from each cow on study day 1 (prior to inoculation), and on study day 8, study day 16, study day 24, and study day 28. Samples had NDF and ADF determined. Feed samples were collected on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, and Study Day 28. Samples had NDF and ADF determined. Rumen contents were sampled from each cow on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, Study Day 28, Study Day 35 and Study Day 38. Twice daily milking, milk production measurements and clinical udder evaluations by quarter were performed every day from Study Day -7 to Study Day 38 for every individual animal, except for Cow 54027, which was not enrolled until Study Day 10, and for Cow 51005, which was removed from the study after Study Day 9. Cows were observed daily for overall clinical health from Study Day -7 to Study Day 38. Milk component measurements were taken on Study Days -7 to 38 in the AM and on Study Days 8 to 38 in the PM. Cows 54027 and 51005 were not included in the analysis.

Each individual cow was the experimental unit. The parameters statistically analyzed include the following:

- Fecal ADF, NDF, NDFom, and Dry Matter Percentage
- Feed ADF, NDF, NDFom, and Dry Matter Percentage
- Milk Production (Milk Production, Milk Fat Yield, Milk Protein Yield, Energy-Corrected Milk Yield, 3.5% Fat Corrected Milk Yield
- Milk Component Data: Milk Fat Percentage, Milk Protein Percentage, Milk Somatic Cell Count

2.0 STATISTICAL ANALYSIS METHODS

All statistical comparisons of the treatment main effect and two-way interactions with the treatment main effect were performed at the 0.10 level of significance. Statistical analyses were performed using R statistical software version 3.4.0.

2.1 Fecal ADF and NDF

Fecal ADF (% DM), NDF (% DM), NDFom (% DM), and Dry Matter Percentage values from Study Days 1, 8, 16, 24, 28 were analyzed using the R package "nlme" and the lme function for linear mixed models, with treatment, study day, and treatment by study day interaction as fixed effects and Cow ID as a random effect (where appropriate).

fit <- lme (Response ~ Treatment_Group*Day, random = ~ 1 | ID, data=fecal_data)

Each model was compared to a fixed-effect only model using a Chi-squared test. Least square means were used to compare treatment groups using the unadjusted p-values and Satterthwaite degrees of freedom was used to test for significant differences. Tukey's method was used to adjust the p-values for multiple comparisons.

2.2 Milk Production

The daily total milk production data was transformed into four additional variables: Milk Fat Yield, Milk Protein Yield, Energy-Corrected Milk Yield, and 3.5% Fat Corrected Milk Yield. Milk Fat Yield was obtained using the following formula:

$$Milk\ Fat\ Yield = Milk\ Production\ (lbs) \times Milk\ Fat\ Percentage$$

Daily total milk production measurements were transformed into Milk Fat Yield using the average of the AM and PM Milk Fat Percentages for each cow on the corresponding Study Day. There were no PM Milk Fat Percentage measurements on Study Days 1 through 7, so the AM measurement was used for the calculation of Milk Fat Yield on these Study Days instead of the average.

Milk Protein Yield was obtained using the following formula:

$$Milk\ Protein\ Yield = Milk\ Production\ (lbs) \times Milk\ Protein\ Percentage$$

Daily total milk production measurements were transformed into Milk Protein Yield using the average of the AM and PM Milk Protein Percentages for each cow on the corresponding Study Day. There were no PM Milk Protein Percentage measurements on Study Days 1 through 7, so the AM measurement was used for the calculation of Milk Protein Yield on these Study Days instead of the average.

Energy-Corrected Milk Yield was obtained using the following formula:

$$ECM = 0.327 \times Milk\ Production\ (lbs) + 12.95 \times Milk\ Fat\ Yield + 7.2 \times Milk\ Protein\ Yield$$

Daily total milk production measurements were transformed into Energy-Corrected Milk Yield using the average of the AM and PM Milk Protein Percentages and the average of the AM and PM Milk Fat Percentages for each cow on the corresponding Study Day. There were no PM Milk Protein Percentage or Milk Fat Percentage measurements on Study Days 1 through 7, so the AM measurements were used for the calculation of Energy-Corrected Milk Yield on these Study Days instead of the averages.

3.5% Fat Corrected Milk Yield was obtained using the following formula:

$$FCM = 0.432 \times Milk\ Production\ (lbs) + 16.23 \times Milk\ Fat\ Yield$$

Daily total milk production measurements were transformed into 3.5% Fat Corrected Milk Yield using the average of the AM and PM Milk Protein Percentages and the average of the AM and PM Milk Fat Percentages for each cow on the corresponding Study Day. There were no PM Milk Protein Percentage or Milk Fat Percentage measurements on Study Days 1 through 7, so the AM measurements were used for the calculation of 3.5% Fat Corrected Milk Yield on these Study Days instead of the averages.

Milk Production, Milk Fat Yield, Milk Protein Yield, Energy-Corrected Milk Yield, and 3.5% Fat Corrected Milk Yield measurements from Study Days 1 to 38 were analyzed using the R package "nlme" and the lme function for linear mixed models, with treatment, week (time period), and the treatment by week interaction term as fixed effects and Cow ID as a random effect (where appropriate).

```
fit <- lme (Response ~ Trt_Group*Time_Period + (1 | Cow_ID), data=avgdataset, na.action =
na.omit)
```

Each model was compared to a fixed-effect only model using a Chi-squared test. Least square means were used to compare treatment groups using the unadjusted p-values and Satterthwaite degrees of freedom was used to test for significant differences. Tukey's method was used to adjust the p-values for multiple comparisons.

2.3 Milk Component Data

Milk data (Milk Fat Percentage, Milk Protein Percentage, Milk Somatic Cell Count) measurements from Study Days 1 to 38 AM and 8 to 38 PM were analyzed using the R package "nlme" and the lme function for linear mixed models, with treatment, week (time period), and the treatment by week interaction terms as fixed effects and Cow ID as a random effect (where appropriate). AM and PM measurements were averaged per study day per cow for analysis. The data for Study Days 1 through 7 were only AM measurements.

```
fit <- lme(Response ~ Trt_Group*Time_Period + (1 | Cow_ID), data=milk_data, na.action =
na.omit)
```

Each model was compared to a fixed-effect only model using a Chi-squared test. Least square means were used to compare treatment groups using the unadjusted p-values and Satterthwaite degrees of freedom was used to test for significant differences. Tukey's method was used to adjust the p-values for multiple comparisons. The Milk SCC data were log-transformed.

2.4 Feed Data

The feed data was a set of Dry Matter Percentage, ADF (% DM), NDF (% DM), and NDFom (% DM) values for samples taken on Study Days 1, 8, 16, 24, and 28. A summary table was produced for this data set.

3.0 RESULTS

Variables are grouped by model outcome (where appropriate): non-significant TRT effect, significant TRT effect, significant TRT*Time Period (week) or TRT*Day effect. Statistically significant results for variables follow, when necessary. Only the appropriate differences are listed and significant differences are denoted with an asterisk (*).

3.1 Fecal ADF and NDF

Fecal ADF (% DM), NDF (% DM), NDFom (% DM), and Dry Matter Percentage were measured from Study Days 1, 8, 16, 24, 28. The R output and code is in Appendix A.

Table 3.1.1 Model Information for Fecal Data

Variable	Model Type	P-values		Decision
		Treatment_Group	Treatment_Group*Study_Day	
ADF (% DM)	Fixed Effects Only	0.2433	0.1497	(1)
NDF (% DM)	Fixed Effects Only	0.2833	0.05478	(3)
NDFom (% DM)	Fixed Effects Only	0.2386	0.1796	(1)
Dry Matter %	Fixed Effects Only	0.03432	0.1777	(2)

Decisions:
(1) There were no significant terms involving Treatment_Group. No further evaluation is needed.
(2) The Treatment_Group main effect is significant at α=0.10, and the 2-way interaction is not significant. Compare treatment means from the main effect of Treatment_Group.
(3) The Treatment_Group by Study_Day interaction is significant at α=0.10. Compare treatment means within each day.

Table 3.1.2 Decision (2) Fecal Data Dry Matter %: Differences and Standard Deviations for Treatment Effects

Variable	Compare	Difference	Standard Error	P-value	Significance
Dry Matter %	Treatment Group 1 vs. Treatment Group 2	0.005060	0.005165	0.5914	
Dry Matter %	Treatment Group 1 vs. Treatment Group 3	0.01416	0.005272	0.0229	*
Dry Matter	Treatment Group 2 vs. Treatment Group 3	0.009096	0.005201	0.1923	

%					

For Dry Matter Percentage, Treatment Group 1 had significantly higher values than Treatment Group 3, p=0.0229.

For decision (3) for NDF (% DM), only the significant contrasts between treatments within Study Days are displayed. The R output and code is in Appendix A.

Table 3.1.3 Decision (3) Fecal Data NDF (% DM): Significant Differences and Standard Deviations for Treatment Effects

Variable	Study Day	Compare	Difference	Standard Error	P-value	Significance
NDF (% DM)	1	Treatment Group 1 vs. Treatment Group 2	0.1081	0.03793	0.0146	*
NDF (% DM)	1	Treatment Group 2 vs. Treatment Group 3	-0.08360	0.03665	0.0631	*

For NDF (% DM), Treatment Group 1 had significantly higher values than Treatment Group 2 on Day 1, p=0.0146. Treatment Group 2 had significantly lower values than Treatment Group 3 on Day 1, p=0.0631.

3.2 Milk Production

Daily total milk production (sum of AM and PM) measurements were taken on Study Days -7 to 38, but only the measurements from Study Days 1 to 38 were analyzed. Descriptions of the calculations performed to obtain all variables in this section are given in Section 2.2. The R output and code is in Appendix A.

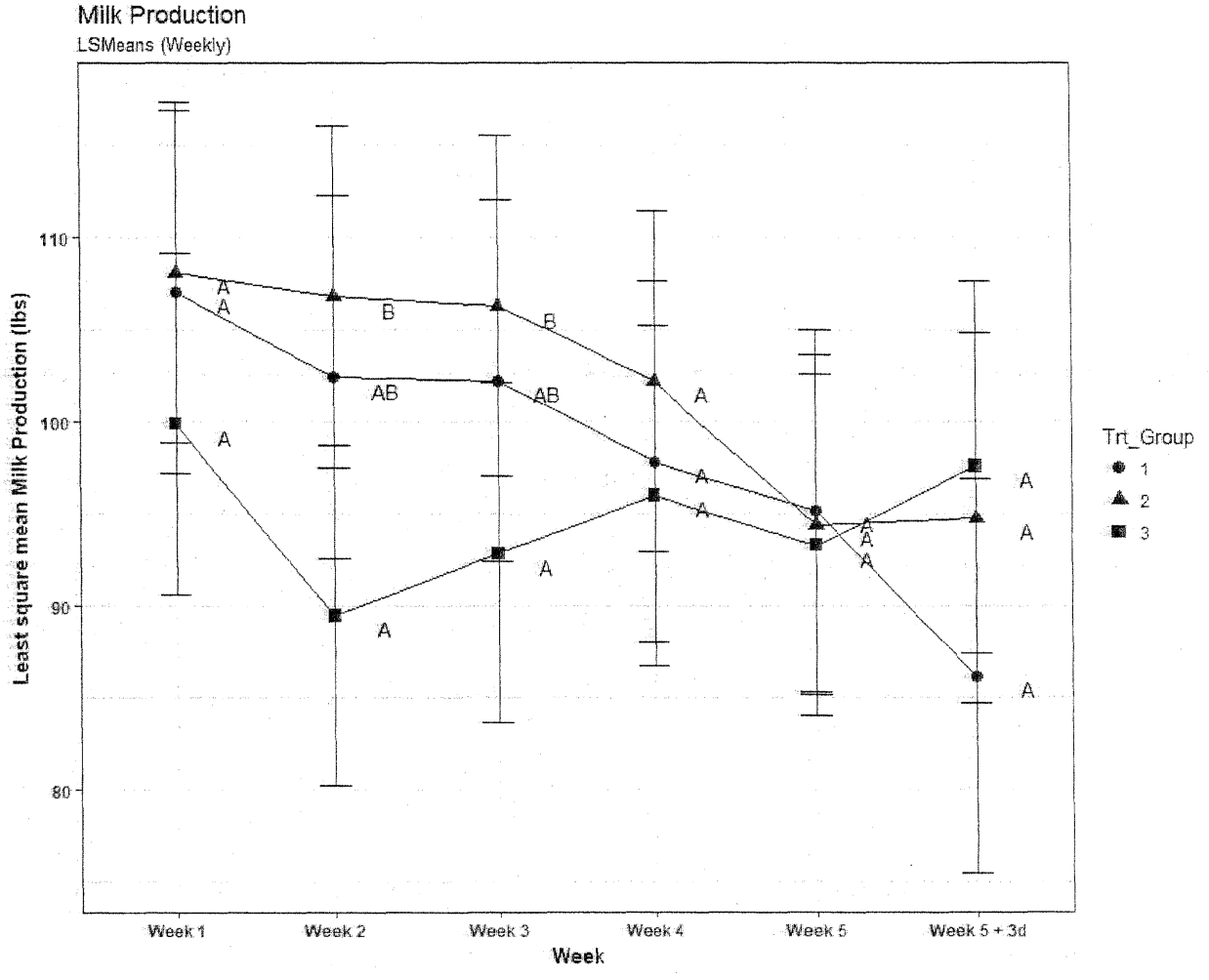
Table 3.2.1: Model Information for Milk Production Data				
Variable	Model Type	P-values		Decision
		Treatment_Group	Treatment_Group*Week	
Milk Production	Mixed	0.3233	<0.0001	(3)
Milk Fat Yield	Mixed	0.637	0.022	(3)
Milk Protein Yield	Mixed	0.5017	<0.0001	(3)
Energy-Corrected Milk Yield	Mixed	0.4284	<0.0001	(3)
3.5% Fat-Corrected Milk Yield	Mixed	0.4348	<0.0001	(3)

Decisions:
(1) There were no significant terms involving Treatment_Group. No further evaluation is needed.
(2) The Treatment_Group main effect is significant at α=0.10, and the 2-way interaction is not significant. Compare treatment means from the main effect of Treatment_Group.
(3) The Treatment_Group by Week interaction is significant at α=0.10. Compare treatment means within each week.

Table 3.2.2: Decision (3) Milk Production Data Differences and Standard Deviations for Treatment Effects					
Variable	Week	Compare	Difference	Standard Error	P-value
Milk Production	2	Treatment Group 2 vs. Treatment Group 3	17.3214	5.7712	0.0185
Milk Production	3	Treatment Group 2 vs. Treatment Group 3	13.4107	5.7712	0.0754
Milk Protein Yield	2	Treatment Group 2 vs Treatment Group 3	0.5191	0.1872	0.0302
Energy-Corrected Milk Yield	2	Treatment Group 1 vs Treatment Group 3	13.1688	5.9642	0.0942
Energy-Corrected Milk Yield	2	Treatment Group 2 vs Treatment Group 3	15.9651	5.7620	0.0303
3.5% Fat-Corrected Milk Yield	2	Treatment Group 2 vs Treatment Group 3	15.7085	5.9674	0.0405

For Milk Production, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0185. Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 3, p=0.0754.

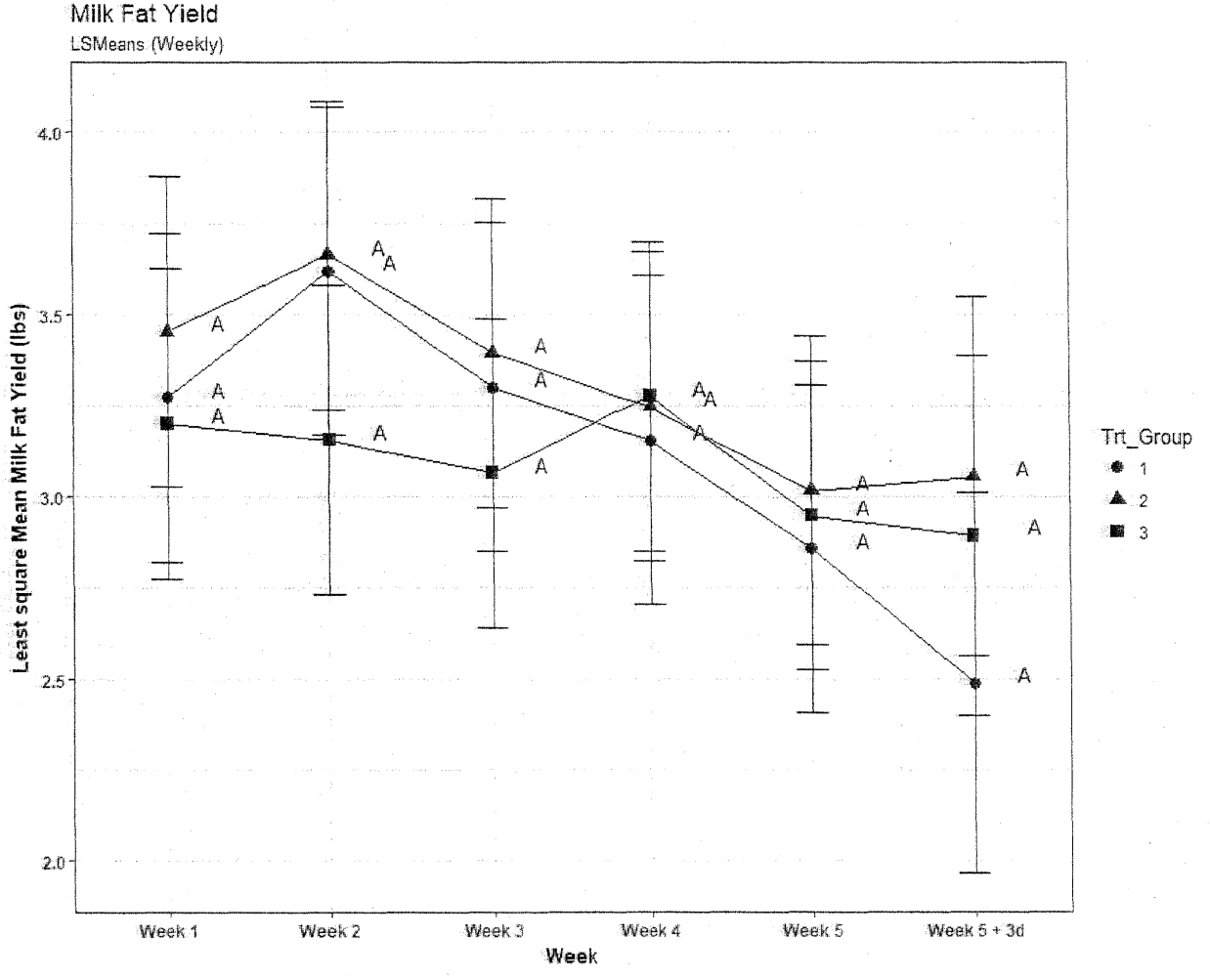
Figure 3.2.1: Graph of Weekly Least Square Means for Milk Production



Milk Production (daily total, lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Although the Treatment Group by Week interaction was significant, there were no significant individual Treatment Group LSMean differences within week for Milk Fat Yield. The adjustment for multiple comparisons created this disparity.

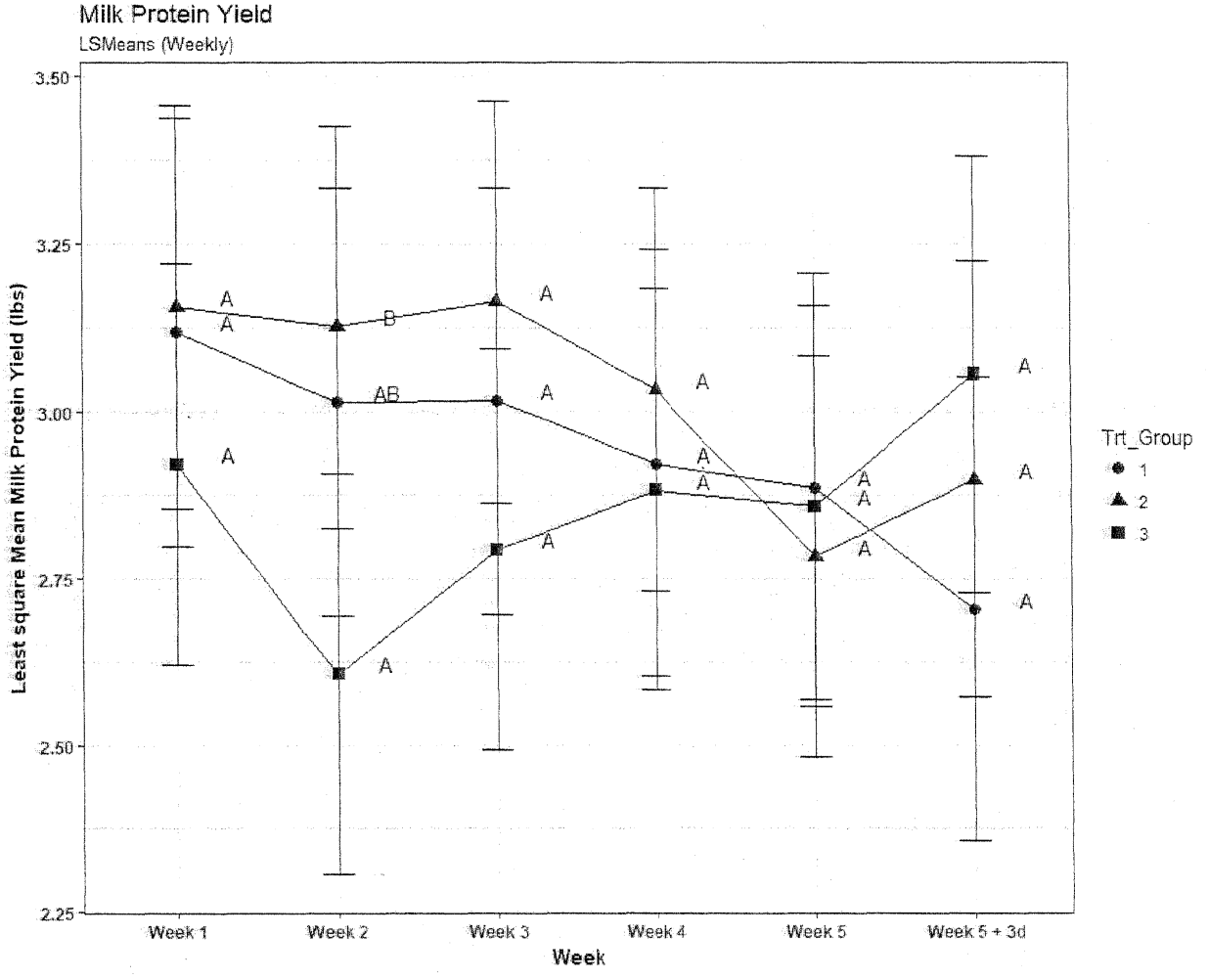
Figure 3.2.2: Graph of Weekly Least Square Means for Milk Fat Yield



Milk Fat Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

For Milk Protein Yield, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0302.

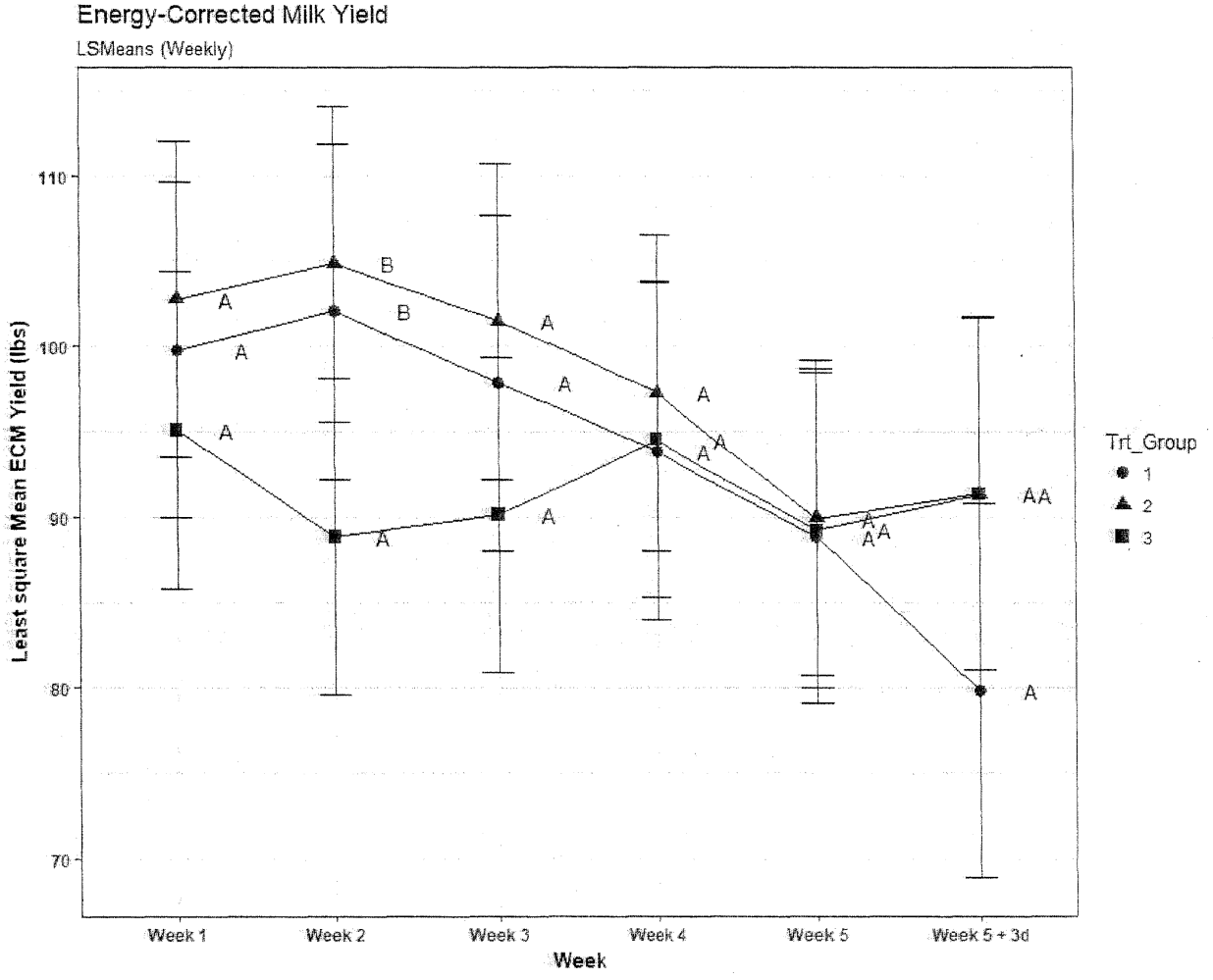
Figure 3.2.3: Graph of Weekly Least Square Means for Milk Protein Yield



Milk Protein Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

For Energy-corrected Milk Yield, Treatment Group 1 had significantly higher values than Treatment Group 3 during Week 2, p=0.0942. Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0303.

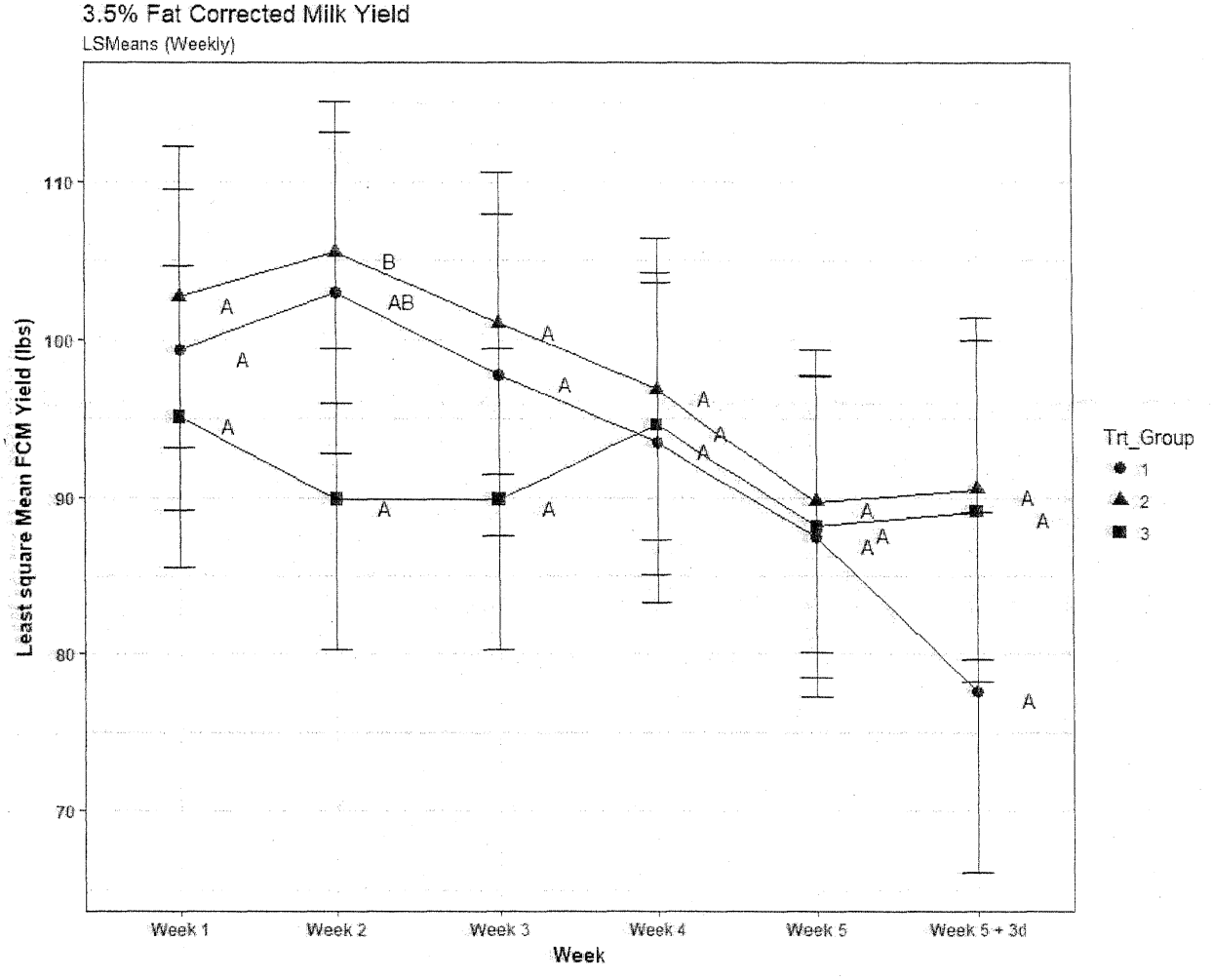
Figure 3.2.4: Graph of Weekly Least Square Means for Energy-Corrected Milk Yield



Energy-Corrected Milk Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

For 3.5% Fat-Corrected Milk Yield, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0405.

Figure 3.2.5: Graph of Weekly Least Square Means for 3.5% Fat Corrected Milk Yield



3.5% Fat Corrected Milk Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

3.3 Milk Component Data

Milk data (Milk Fat Percentage, Milk Protein Percentage, Milk Lactose Percentage, Milk Solids Percentage, Milk Somatic Cell Count) were measured on Study Days -7 to 38 for AM measurements, and on Study Days 8 to 38 for PM measurements. The milk data from Study Days 1 to 38 were analyzed. AM and PM measurements were averaged per study day per cow for analysis. The data for Study Days 1 through 7 were only AM measurements. The R output and code is in Appendix A.

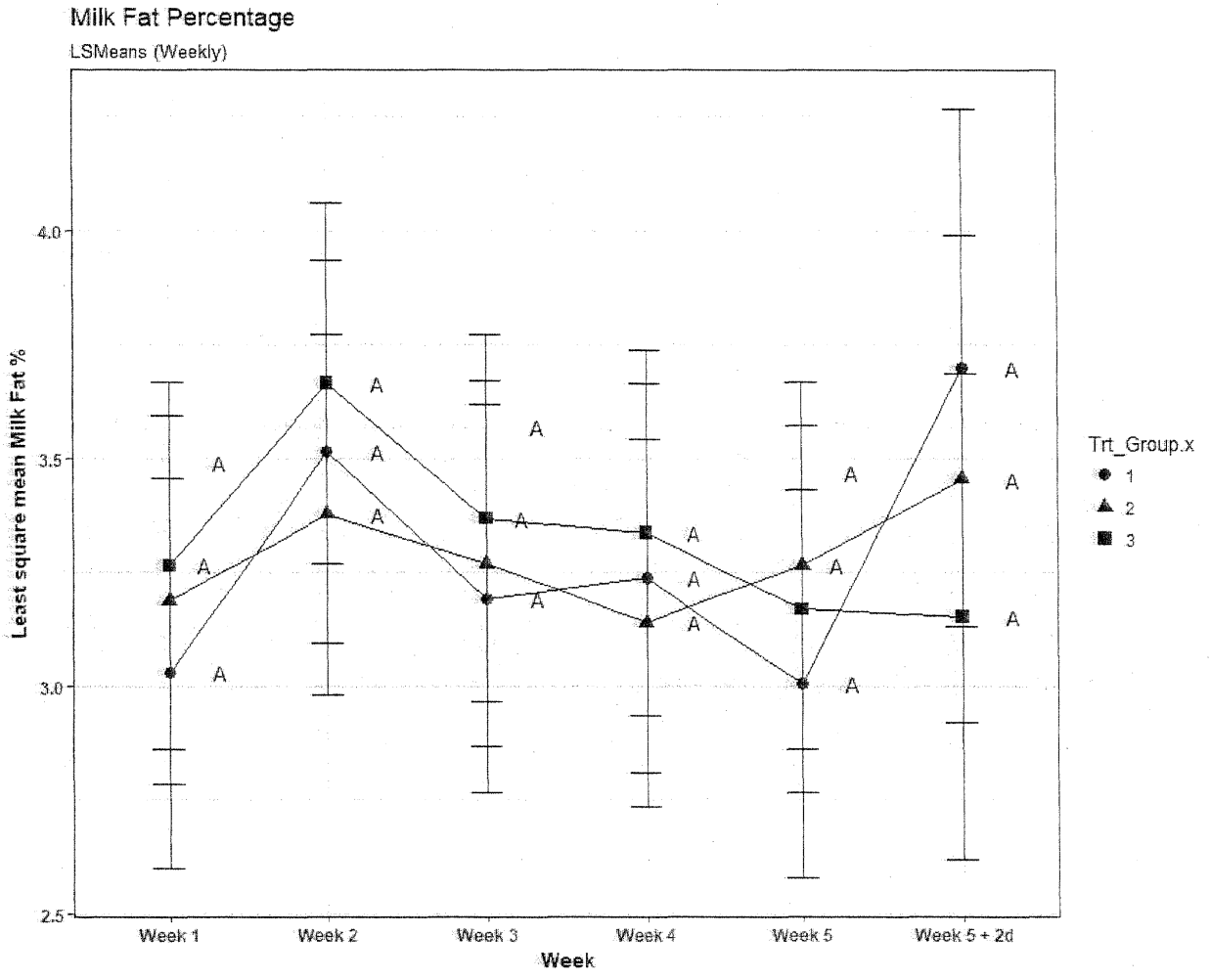
Table 3.3.1: Model Information for Milk Component Data				
Variable	Model Type	P-values		Decision
		Treatment_Group	Treatment_Group*Week	
Milk Fat %	Mixed	0.8392	0.1733	(1)
Milk Protein %	Mixed	0.7404	<0.0001	(3)
Milk SCC	Mixed	0.1310	0.0218	(3)

Decisions:
(1) There were no significant terms involving Treatment_Group. No further evaluation is needed.
(2) The Treatment_Group main effect is significant at α=0.10, and the 2-way interaction is not significant. Compare treatment means from the main effect of Treatment_Group.
(3) The Treatment_Group by Week interaction is significant at α=0.10. Compare treatment means within each week.

Table 3.3.2: Decision (3) Differences and Standard Deviations for Treatment Effects					
Variable	Week	Compare	Difference	Standard Error	P-value
Milk Protein %	5 + 2d	Treatment Group 1 vs. Treatment Group 2	0.5731	0.1051	0.0001
Milk Protein %	5 + 2d	Treatment Group 1 vs. Treatment Group 3	0.4569	0.1051	0.0009
Milk SCC	1	Treatment Group 2 vs Treatment Group 3	1.494	0.5299	0.0273

There were no significant individual Treatment Group LSMean differences within week for Milk Fat %.

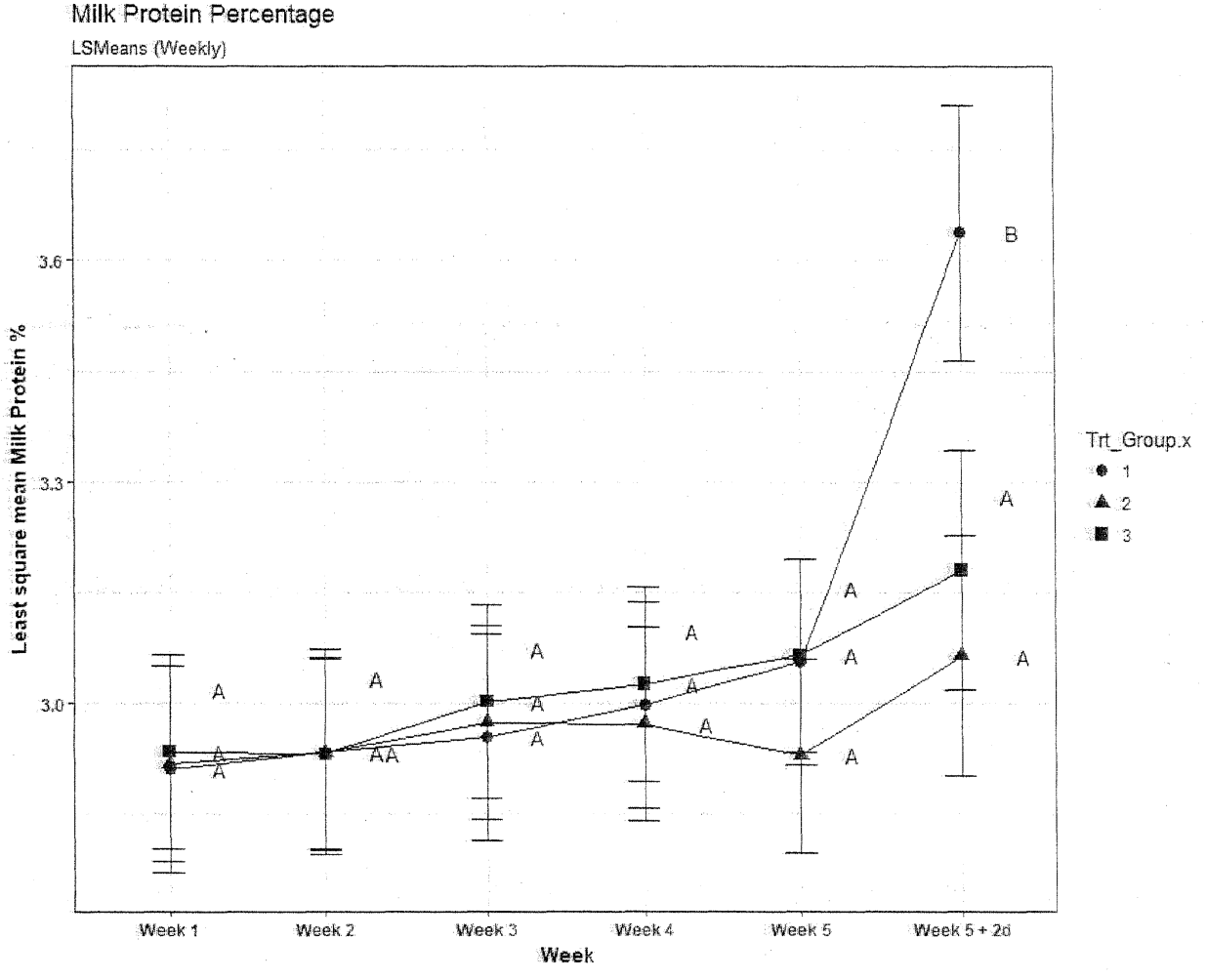
Figure 3.3.1: Graph of Weekly Least Square Means for Milk Fat Percentage



Milk Fat Percentage for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

For Milk Protein %, Treatment Group 1 had significantly higher values than Treatment Group 2 during Week 5 + 2d, p=0.0001. Treatment Group 1 had significantly higher values than Treatment Group 3 during Week 5 + 2d, p=0.0009.

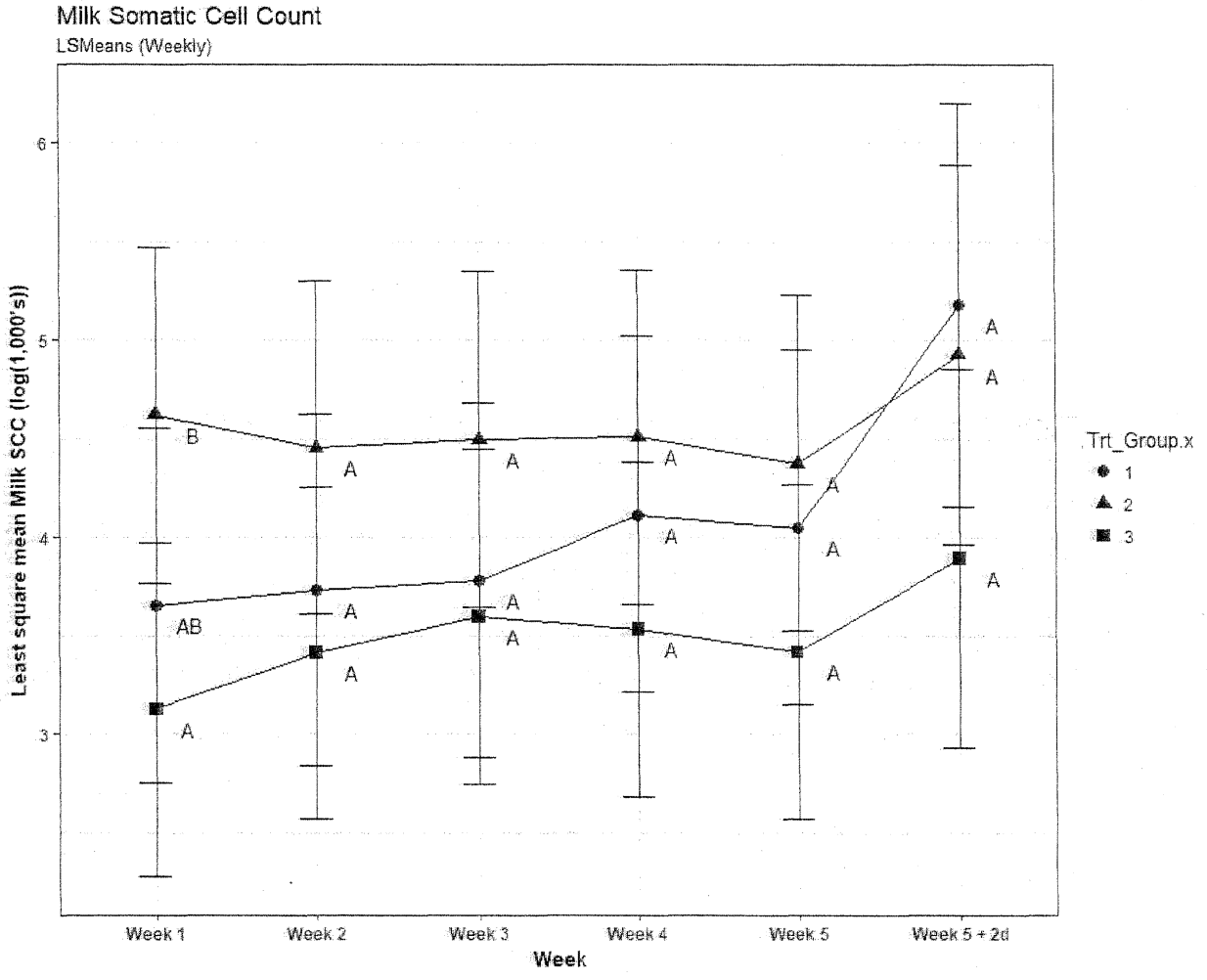
Figure 3.3.2: Graph of Weekly Least Square Means for Milk Protein Percentage



Milk Protein Percentage for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

For Milk SCC, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 1, p=0.0273.

Figure 3.3.3: Graph of Weekly Least Square Means for Milk Somatic Cell Count



Milk Somatic Cell Count (log(1,000's)) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

3.4 Feed Data

For feed, Dry Matter Percentage, ADF (% DM), NDF (% DM), and NDFom (% DM) values for samples were measured on Study Days 1, 8, 16, 24, and 28.

Table 3.4.1 Summary of Feed Data

	Dry Matter Percentage	ADF (% DM)	NDF (% DM)	NDFom (% DM)
Minimum	0.4654	0.1901	0.2764	0.2587
1st Quartile	0.4755	0.1978	0.2835	0.2636
Median	0.4847	0.1985	0.2879	0.2649
Mean	0.4813	0.1982	0.2861	0.2648
3rd Quartile	0.4855	0.1998	0.2892	0.2667
Maximum	0.4952	0.2046	0.2937	0.2702
Standard Deviation	0.01128	0.005229	0.006547	0.004226
Coefficient of Variation (%)	2.344	2.639	2.288	1.596

4.0 CONCLUSIONS

Statistically significant differences between treatment groups were determined to be as follows:

For Fecal Data Dry Matter Percentage, Treatment Group 1 had significantly higher values than Treatment Group 3, p=0.0229.

For Fecal Data NDF (% DM), Treatment Group 1 had significantly higher values than Treatment Group 2 on Day 1, p=0.0146. Treatment Group 2 had significantly lower values than Treatment Group 3 on Day 1, p=0.0631.

For Milk Protein %, Treatment Group 1 had significantly higher values than Treatment Group 2 during Week 5 + 2d, p=0.0001. Treatment Group 1 had significantly higher values than Treatment Group 3 during Week 5 + 2d, p=0.0009.

For Milk SCC, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 1, p=0.0273.

For Milk Production, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0185. Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 3, p=0.0754.

For Milk Fat Yield, Treatment Group 1 had significantly higher values than Treatment Group 2 during Week 1, p=0.0905.

For Milk Protein Yield, Treatment Group 1 had significantly higher values than Treatment Group 2 during Week 1, p=0.0251. Treatment Group 2 had significantly lower values than Treatment Group 3 during Week 1, p=0.0932.

For Energy-corrected Milk Yield, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0614.

For 3.5% Fat-Corrected Milk Yield, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0405.

APPENDIX 1

Certificates of Analysis

(b) (4)

Certificate of Analysis

March 02, 2021

(b) (4)

Order No. 520720
Sample No. 1074364

SAMPLE INFORMATION

Description DY21 Palm Oil Encapsulate
Lot Number 787A-2106-E603
Received February 25, 2021

ANALYTICAL RESULTS

Analysis Heavy Metals - Food
Method ICP-MS
Analysis Date February 25, 2021 to March 02, 2021

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	0.004/0.016	(b) (4)
Cadmium	0.001/0.004	
Mercury	0.001/0.004	
Lead	0.001/0.004	

Reported by
(b) (4)

March 02, 2021

ND = None Detected

If there are any questions with this report, please contact (b) (4)

(b) (4)

Certificate of Analysis

March 11, 2021

(b) (4)

Order No. 520720
Sample No. 1074364

SAMPLE INFORMATION

Description DY21 Palm Oil Encapsulate
Lot Number 787A-2106-E603
Received February 25, 2021

ANALYTICAL RESULTS

Analysis Date February 25, 2021 to March 11, 2021

Findings

Analysis	Results	Method
Coliforms	(b) (4)	FDA BAM - ECC Agar
E. coli		FDA BAM - ECC Agar
Listeria		AOAC 2013.10
Salmonella		AOAC 2013.01

Reported by

(b) (4)

Analyst

If there are any questions with this report, please contact (b) (4)

(b) (4)

Tel: 415 822 1100
Fax: 415 822 6615

(b) (4)

(b) (4)

Product Certificate of Analysis

Product Name	Fat Encapsulated *Pichia kudriavzevii* ASCUSDY21 (DY21 POE)
Batch Number	787A-2106-E603
Date of Manufacture	15Feb2021
Expiration Date	N/A
Retest Date	15Feb2022
Storage Conditions	2 – 10 °C

Analytical Property	**Specification**	**Result**
DY21-POE Microbe Enumeration	>4.0 E07 CFU/g	(b) (4)
Coliform	<10 CFU/g	
E. coli	<10 CFU/g	
Salmonella	Negative/25g	
Listeria	Negative/25g	

Approval (Name, Title, Signature, and Date)

This batch was manufactured according to (b) (4) standards and meets the registered specifications.

(b) (4) 3/15/2021

Quality

(b) (4)

Certificate of Analysis

March 02, 2021

(b) (4)

Order No. 520720
Sample No. 1074365

SAMPLE INFORMATION

Description DY21 Palm Oil Encapsulate
Lot Number 787A-2106-E604
Received February 25, 2021

ANALYTICAL RESULTS

Analysis Heavy Metals - Food
Method ICP-MS
Analysis Date February 25, 2021 to March 02, 2021

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	0.004/0.016	(b) (4)
Cadmium	0.001/0.004	
Mercury	0.001/0.004	
Lead	0.001/0.004	

Reported by
(b) (4)

March 02, 2021

ND = None Detected

If there are any questions with this report, please contact (b) (4).

(b) (4)

Certificate of Analysis

March 11, 2021

(b) (4)

Order No. 520720
Sample No. 1074365

SAMPLE INFORMATION

Description	DY21 Palm Oil Encapsulate
Lot Number	787A-2106-E604
Received	February 25, 2021

ANALYTICAL RESULTS

Analysis Date February 25, 2021 to March 11, 2021

Findings

Analysis	Results	Method
Coliforms	(b) (4)	FDA BAM - ECC Agar
E. coli		FDA BAM - ECC Agar
Listeria		AOAC 2013.10
Salmonella		AOAC 2013.01

Reported by

(b) (4)

Analyst

If there are any questions with this report, please contact (b) (4)

(b) (4)

Tel: 415 822 1100
Fax: 415 822 6615

(b) (4)

(b) (4)

Product Certificate of Analysis

Product Name	Fat Encapsulated *Pichia kudriavzevii* ASCUSDY21 (DY21 POE)
Batch Number	787A-2106-E604
Date of Manufacture	18Feb2021
Expiration Date	N/A
Retest Date	18Feb2022
Storage Conditions	2 – 10 °C

Analytical Property	**Specification**	**Result**
DY21-POE Microbe Enumeration	>4.0 E07 CFU/g	(b) (4)
Coliform	<10 CFU/g	
E. coli	<10 CFU/g	
Salmonella	Negative/25g	
Listeria	Negative/25g	

Approval (Name, Title, Signature, and Date)

This batch was manufactured according to (b) (4) standards and meets the registered specifications.

(b) (4)

3/15/2021

Quality

(b) (4)

Certificate of Analysis

March 11, 2021

(b) (4)

Order No. 521068
Sample No. 1075577

SAMPLE INFORMATION

Description	DY21 POE
Lot Number	787A-2106-E607
Received	March 08, 2021

ANALYTICAL RESULTS

Analysis	Heavy Metals - Food
Method	ICP-MS
Analysis Date	March 08, 2021 to March 11, 2021

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	0.004/0.016	(b) (4)
Cadmium	0.001/0.004	
Mercury	0.001/0.004	
Lead	0.001/0.004	

Reported by

(b) (4)

March 11, 2021

ND = None Detected

If there are any questions with this report, please contact (b) (4)

(b) (4)

Certificate of Analysis

March 16, 2021

(b) (4)

Order No. 521068
Sample No. 1075577

SAMPLE INFORMATION

Description	DY21 POE
Lot Number	787A-2106-E607
Received	March 08, 2021

ANALYTICAL RESULTS

Analysis Date March 08, 2021 to March 16, 2021

Findings

Analysis	Results	Method
Coliforms	(b) (4)	FDA BAM - ECC Agar
E. coli		FDA BAM - ECC Agar
Listeria		AOAC 2013.10
Salmonella		AOAC 2013.01

Reported by

(b) (4)

Microbiologist (b) (4)

If there are any questions with this report, please contact (b) (4)

(b) (4)

Tel: 415 822 1100
Fax: 415 822 6615

(b) (4)

(b) (4)

Product Certificate of Analysis

Product Name	Fat Encapsulated *Pichia kudriavzevii* ASCUSDY21 (DY21 POE)
Batch Number	787A-2106-E607
Date of Manufacture	26Feb2021
Expiration Date	N/A
Retest Date	26Feb2022
Storage Conditions	2 – 10 °C

Analytical Property	**Specification**	**Result**
DY21-POE Microbe Enumeration	>4.0 E07 CFU/g	(b) (4)
Coliform	<10 CFU/g	
E. coli	<10 CFU/g	
Salmonella	Negative/25g	
Listeria	Negative/25g	

Approval (Name, Title, Signature, and Date)

This batch was manufactured according to (b) (4) standards and meets the registered specifications.

(b) (4) 3/18/2021

Quality

(b) (4) **RESPONSE TO GRAS AGRN 38 (*Pichia kudriavzevii* ASCUSDY21) REVIEW BY FDA-CVM**

APPENDIX 2

Aflatoxin Testing

(b) (4)

Certificate of Analysis

March 25, 2021

(b) (4)

Order No. 521761
Sample No. 1077486

SAMPLE INFORMATION

Description	Dairy-21
Lot Number	18-0202-001-P86-1
Received	March 24, 2021

ANALYTICAL RESULTS

Analysis	Aflatoxin (non-Fda)
Instrument	LC-MS/MS
Method	AOAC Official Method 990.33
Analysis Date	March 24, 2021 to March 25, 2021

Analyte	LOQ (ppb)	Findings (ppb)
Aflatoxin B1	2	(b) (4)
Aflatoxin B2	1	
Aflatoxin G1	2	
Aflatoxin G2	1	

ND = None Detected

Reported by

(b) (4)

Senior Analyst

March 25, 2021

If there are any questions with this report, please contact (b) (4)

(b) (4)

Certificate of Analysis

March 25, 2021

(b) (4)

Order No. 521761
Sample No. 1077487

SAMPLE INFORMATION

Description	Dairy-21
Lot Number	18-0202-001-P86-2
Received	March 24, 2021

ANALYTICAL RESULTS

Analysis	Aflatoxin (non-Fda)
Instrument	LC-MS/MS
Method	AOAC Official Method 990.33
Analysis Date	March 24, 2021 to March 25, 2021

Analyte	LOQ (ppb)	Findings (ppb)
Aflatoxin B1	2	(b) (4)
Aflatoxin B2	1	
Aflatoxin G1	2	
Aflatoxin G2	1	

ND = None Detected

Reported by

(b) (4)

Senior Analyst

March 25, 2021

If there are any questions with this report, please contact (b) (4)

(b) (4)

Certificate of Analysis

March 25, 2021

(b) (4)

Order No. 521761
Sample No. 1077488

SAMPLE INFORMATION

Description	Dairy-21
Lot Number	18-0202-001-P87-1
Received	March 24, 2021

ANALYTICAL RESULTS

Analysis	Aflatoxin (non-Fda)
Instrument	LC-MS/MS
Method	AOAC Official Method 990.33
Analysis Date	March 24, 2021 to March 25, 2021

Analyte	LOQ (ppb)	Findings (ppb)
Aflatoxin B1	2	(b) (4)
Aflatoxin B2	1	
Aflatoxin G1	2	
Aflatoxin G2	1	

ND = None Detected

Reported by

(b) (4)

Senior Analyst

March 25, 2021

If there are any questions with this report, please contact (b) (4)

(b) (4)

Certificate of Analysis

March 25, 2021

(b) (4)

Order No. 521761
Sample No. 1077489

SAMPLE INFORMATION

Description	Dairy-21
Lot Number	787A-2105-B024
Received	March 24, 2021

ANALYTICAL RESULTS

Analysis	Aflatoxin (non-Fda)
Instrument	LC-MS/MS
Method	AOAC Official Method 990.33
Analysis Date	March 24, 2021 to March 25, 2021

Analyte	LOQ (ppb)	Findings (ppb)
Aflatoxin B1	2	(b) (4)
Aflatoxin B2	1	
Aflatoxin G1	2	
Aflatoxin G2	1	

ND = None Detected

Reported by

(b) (4)

Senior Analyst

March 25, 2021

If there are any questions with this report, please contact (b) (4)

(b) (4)

Certificate of Analysis

March 25, 2021

(b) (4)

Order No. 521761
Sample No. 1077490

SAMPLE INFORMATION

Description	Dairy-21
Lot Number	787A-2105-B029
Received	March 24, 2021

ANALYTICAL RESULTS

Analysis	Aflatoxin (non-Fda)
Instrument	LC-MS/MS
Method	AOAC Official Method 990.33
Analysis Date	March 24, 2021 to March 25, 2021

Analyte	LOQ (ppb)	Findings (ppb)
Aflatoxin B1	2	(b) (4)
Aflatoxin B2	1	
Aflatoxin G1	2	
Aflatoxin G2	1	

ND = None Detected

Reported by

(b) (4)

Senior Analyst

March 25, 2021

If there are any questions with this report, please contact (b) (4)

(b) (4)

Certificate of Analysis

March 25, 2021

(b) (4)

Order No. 521761
Sample No. 1077491

SAMPLE INFORMATION

Description	Dairy-21
Lot Number	787A-2105-B031
Received	March 24, 2021

ANALYTICAL RESULTS

Analysis	Aflatoxin (non-Fda)
Instrument	LC-MS/MS
Method	AOAC Official Method 990.33
Analysis Date	March 24, 2021 to March 25, 2021

Analyte	LOQ (ppb)	Findings (ppb)
Aflatoxin B1	2	(b) (4)
Aflatoxin B2	1	
Aflatoxin G1	2	
Aflatoxin G2	1	

ND = None Detected

Reported by

(b) (4)

Senior Analyst

March 25, 2021

If there are any questions with this report, please contact (b) (4)