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(54) **Titre : SOUCHE DE BACILLUS PUMILUS ET PROCEDES POUR AMELIORER LA SANTE ET LES PERFORMANCES DE RUMINANTS**
(54) **Title: A BACILLUS PUMILUS STRAIN AND METHODS FOR IMPROVING RUMINANT HEALTH AND PERFORMANCE**

(57) **Abrégé/Abstract:**
Described are strains including Enterococcus faecium strain 8G-1 (NRRL B- 50173), Enterococcus faecium strain 8G-73 (NRRL B-50172), Bacillus pumilus strain 8G-134 (NRRL B-50174) and strains having all of the identifying characteristics of each of these strains. One or more of the strains can be used to treat or prevent acidosis. They can also be used to improve other measures of ruminant health and/or performance including but not limited to increased milk production, increased milk fat content, decreased somatic cell count (SCC), improved immunological response and health as evidenced by decreased SCC, and increased efficiency of milk production. Methods of using the strains, alone and in combination, are described. Methods of making the strains are also provided.

ABSTRACT

Described are strains including *Enterococcus faecium* strain 8G-1 (NRRL B-50173), *Enterococcus faecium* strain 8G-73 (NRRL B-50172), *Bacillus pumilus* strain 8G-134 (NRRL B-50174) and strains having all of the identifying characteristics of each of these strains. One or more of the strains can be used to treat or prevent acidosis. They can also be used to improve other measures of ruminant health and/or performance including but not limited to increased milk production, increased milk fat content, decreased somatic cell count (SCC), improved immunological response and health as evidenced by decreased SCC, and increased efficiency of milk production. Methods of using the strains, alone and in combination, are described. Methods of making the strains are also provided.

A *BACILLUS PUMILUS* STRAIN AND METHODS FOR IMPROVING RUMINANT HEALTH AND PERFORMANCE

BIBLIOGRAPHY

Complete bibliographic citations of the references referred to herein by the first author's last name and year of publication can be found in the Bibliography section, immediately preceding the claims.

FIELD OF THE INVENTION

The invention relates to strains and methods for controlling acidosis. More particularly, the invention relates to bacterial strains useful for improving ruminant health and/or performance and methods of making and using the strains.

DESCRIPTION OF THE RELATED ART

The feeding of high concentrations of fermentable carbohydrate to ruminants has become a common practice in the beef and dairy cattle industry over the last 50 years. The need for improving the production efficiency and quality of meat has led to this trend. Improvements in production have not occurred without certain difficulties. Increasing the ruminant consumption of fermentable carbohydrate by feeding higher levels of cereal grains has resulted in increased incidence of metabolic disorders such as acidosis. The relationship between high concentrate consumption and ruminal acidosis has been well documented in reviews (Dunlop, 1972; Slyter, 1976). Many researchers have shown a decline in ruminal pH following the feeding of high levels of readily fermentable carbohydrate (RFC) to cattle and the subsequent disruption of ruminal microbiota and physiological changes occurring in the animal (Allison, et.al., 1975, Hungate et.al., 1952; Elam, 1976). Most have attributed this decline to an over production of organic acids by ruminal bacteria such as *Streptococcus bovis*. However, the effect of excessive carbohydrate on the ruminal microbiota that initiates this response has not been well documented.

In the past, intensive management of feeding has been the only method to combat acidosis. More specifically, grains are diluted with roughage and the increase in dietary concentrate percentage is carefully controlled in a step-wise method to ensure smooth transition to high levels of concentrate over a 14-21 day period. Most commercial feedlots formulate and deliver several "adaptation" diets that contain different ratios of grain to forage.

Although intensive feeding management is usually quite effective in controlling acidosis, it is very costly to the producer due to the high cost of producing, transporting, chopping forage, disposing of increased animal waste, and lower production efficiencies. Producers and feedlot managers need to implement strategies that will allow for efficient production of livestock fed high concentrate rations.

Other strategies have been to combine the use of adaptation diets with feeding antimicrobial components such as ionophores. Ionophores inhibit intake and reduce the production of lactic acid in the rumen by reducing the ruminal populations of gram-positive, lactic acid-producing organisms such as *Streptococcus bovis* and *Lactobacillus* spp. (Muir et al. 1981).

Although the usage of ionophores have reduced the incidence of acute acidosis in feedlots, consumer concern about the use of antibiotics in meat production and the need for feedlot managers to continually find ways to reduce costs while improving animal performance and carcass composition has lead to the examination of alternative methods to reduce acidosis and improve feedlot cattle performance.

The use of direct-fed microbials as a method to modulate ruminal function and improve cattle performance has been gaining increased acceptance over the past 10 years. There are two basic direct-fed microbial technologies that are currently available to the beef industry for the control of ruminal acidosis: (1) using lactic acid producing DFM technology and (2) adding specific bacterial species capable of utilizing ruminal lactic acid. While the reported mode of action of each of these technologies is different, they both attempt to address the accumulation of ruminal lactic acid.

The first approach, i.e., using lactic acid producing DFM technology, attempts to increase the rate of ruminal lactic acid utilization by stimulating the native ruminal microbiota. As reported, the addition of relatively slow growing lactic acid producing bacteria, such as species of *Enterococcus*, produces a slightly elevated concentration of ruminal lactic acid. The gradual increase forces the adaptation of the ruminal

microflora to a higher portion of acid tolerant lactic acid utilizers. However, these *Enterococcus* strains failed to adequately control and prevent acidosis.

The second approach, i.e., adding specific bacterial species capable of utilizing ruminal lactic acid, is based on the finding that species of *Propionibacterium* significantly minimize the accumulation of ruminal lactic acid during an acidosis challenge with a large amount of Readily Fermentable Carbohydrate (RFC). *Propionibacterium* are natural inhabitants of the rumen in both dairy and beef cattle and function in the rumen by using lactic acid to produce important volatile fatty acids like acetate and propionate.

Current DFM technologies developed to date have been developed based upon an antiquated microbiological understanding of the incidence of acidosis in the rumen. Until recently, methods of studying the microbial ecology of the rumen have relied on cultivation techniques. These techniques have been limited due to unknown growth requirements and unsuitable anaerobic conditions for many of the rumen microorganisms. Thus, ecological studies relying on these cultivation techniques have been based on a limited understanding of the rumen microbiota.

Current DFMs when used alone or with yeast to minimize the risk of ruminal acidosis and to improve utilization of a feedlot cattle diet containing high concentrate provide mixed results. However, a study of DFM strains *Propionibacterium* P15, and *Enterococcus faecium* EF212, and *E. faecium* EF212, fed alone or fed combined with a yeast, *Saccharomyces cerevisiae*, indicated that addition of DFM combined with or without yeast had no effect on preventing ruminal acidosis (Yang, W., 2004).

In view of the foregoing, it would be desirable to provide one or more strains to prevent and/or treat acidosis. It would be advantageous if the one or more strains also improved other measures of ruminant health and/or performance. It would also be desirable to provide methods of making and using the strains.

SUMMARY OF THE INVENTION

The invention, which is defined by the claims set out at the end of this disclosure, is intended to solve at least some of the problems noted above. Isolated strains are provided, including *Enterococcus faecium* strain 8G-1 (NRRL B-50173), a strain having all of the identifying characteristics of *Enterococcus faecium* strain 8G-1 (NRRL B-50173), *Enterococcus faecium* strain 8G-73 (NRRL B-50172), a strain having all of the identifying characteristics of *Enterococcus faecium* strain 8G-73

(NRRL B-50172), *Bacillus pumilus* strain 8G-134 (NRRL B-50174), a strain having all of the identifying characteristics of *Bacillus pumilus* strain 8G-134 (NRRL B-50174), and combinations thereof.

Additionally provided is a combination including one or more of the strains listed above and monensin.

Also provided is a method of administering an effective amount of one or more of the strains listed above to an animal and a method of administering a combination including an effective amount of one or more of the strains listed above and monensin to an animal.

In at least some embodiments, the administration of the one or more strain to the animal provides at least one of the following benefits in or to the animal when compared to an animal not administered the strain: (a) reduces acidosis, (b) stabilizes ruminal metabolism as indicated by delayed lactic acid accumulation and prolonged production of volatile fatty acids, (c) recovers more quickly from acidosis challenge as measured by pH recovery and lactic acid decline, (d) reduces exhibition of clinical signs associated with acidosis (e) increased milk production in lactating dairy cows, (f) increased milk fat content in lactating dairy cows, (g) decreased somatic cell count (SCC) in lactating dairy cows, (h) improved immunological response and health as evidenced by decreased SCC and (i) increased efficiency of milk production in lactating dairy cows.

Also provided is a method of making a direct-fed microbial. In the method, a strain selected from the group consisting of *Enterococcus faecium* strain 8G-1 (NRRL B-50173), *Enterococcus faecium* strain 8G-73 (NRRL B-50172), and *Bacillus pumilus* strain 8G-134 (NRRL B-50174) is grown in a liquid nutrient broth. The strain is separated from the liquid nutrient broth to make the direct-fed microbial. In at least some embodiments of the method, the strain is freeze dried.

Additionally provided is a method of making a direct-fed microbial. In the method, a strain selected from the group consisting of *Enterococcus faecium* strain 8G-1 (NRRL B-50173), *Enterococcus faecium* strain 8G-73 (NRRL B-50172), and *Bacillus pumilus* strain 8G-134 (NRRL B-50174) is grown in a liquid nutrient broth. The strain is separated from the liquid nutrient broth to make the direct-fed microbial. Monensin is added to the direct-fed microbial.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred exemplary embodiments described herein are illustrated in the accompanying drawings, in which like reference numerals represent like parts throughout and in which:

Figure 1 is a graph showing pH differences between tester (non-acidotic; Cluster 2) and driver (acidotic; Cluster 1) populations.

Figure 2 is a graph showing lactic acid accumulation differences between tester (non-acidotic; Cluster 2) and driver (acidotic; Cluster 1) populations.

Figure 3 is a graph showing in vitro glucose by treatment over time.

Figure 4 is a graph showing in vitro lactic acid accumulation by treatment over time.

Figure 5 is a graph showing total VFA (acetate+propionate+butyrate) accumulation over time.

Figure 6 is a graph showing mean ruminal pH over time in control and candidate DFM cattle.

Figure 7 is a graph showing mean ruminal lactate over time in control and candidate DFM cattle.

Figure 8 is a graph showing ruminal VFA concentrations over time treatment. (Total VFA = acetate + propionate + butyrate).

Before explaining embodiments described herein in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

DETAILED DESCRIPTION

Provided herein are strains. Methods of making and using the strains are also provided.

In at least some embodiments, a direct-fed microbial (DFM) made with one or more of the strains provided herein allows beef and dairy producers to continue managing feeding regimens to optimize growth and performance without sacrificing health due to digestive upset associated with ruminal acidosis. At least some

embodiments of the DFMs were selected on the basis of managing ruminal lactate concentrations via lactate utilization or priming the rumen to maintain lactate utilizing microflora. At least some embodiments of the DFMs were developed to manage ruminal energy concentrations. Unlike the current DFMs marketed to cattle producers to alleviate acidosis, at least some of the embodiments of the invention were not developed to manage a problem after it occurs, but rather to alleviate the problem before it happens.

Strains:

The strains provided herein include *Enterococcus faecium* strain 8G-1, *Enterococcus faecium* strain 8G-73, and *Bacillus pumilus* strain 8G-134, which are also referred to herein as 8G-1, 8G-73, and 8G-134, respectively.

Strains *Enterococcus faecium* strain 8G-1, *Enterococcus faecium* strain 8G-73, and *Bacillus pumilus* strain 8G-134 were deposited on August 29, 2008 at the Agricultural Research Service Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois, 61604 and given accession numbers B-50173, B-50172 , and B-50174, respectively. The deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. One or more strain provided herein can be used as a direct-fed microbial (DFM).

For purposes of this disclosure, a "biologically pure strain" means a strain containing no other bacterial strains in quantities sufficient to interfere with replication of the strain or to be detectable by normal bacteriological techniques. "Isolated" when used in connection with the organisms and cultures described herein includes not only a biologically pure strain, but also any culture of organisms which is grown or maintained other than as it is found in nature. In some embodiments, the strains are mutants, variants, or derivatives of strains 8G-1, 8G-73, or 8G-134 that also provide benefits comparable to that provided by 8G-1, 8G-73, and 8G-134. In some embodiments, the strains are strains having all of the identifying characteristics of strains 8G-1, 8G-73, or 8G-134. Further, each individual strain (8G-1, 8G-73, or 8G-134) or any combination of these strains can also provide one or more of the benefits described herein. It will also be clear that addition of other microbial strains, carriers, additives, enzymes, yeast, or the like will also provide control of acidosis and will not constitute a substantially different DFM.

Bacillus strains have many qualities that make them useful as DFMs. For example, several *Bacillus* species also have GRAS status, i.e., they are generally recognized as safe by the US Food and Drug Administration and are also approved for use in animal feed by the Association of American Feed Control Officials (AAFCO). The *Bacillus* strains described herein are aerobic and facultative sporeformers and thus, are stable. *Bacillus* species are the only sporeformers that are considered GRAS. A *Bacillus* strain found to prevent or treat acidosis is *Bacillus pumilus* strain 8G-134 .

Enterococcus strains also have many qualities that make them useful as DFMs.

Enterococcus strains are known to inhabit the gastrointestinal tract of monogastrics and ruminants and would be suited to survive in this environment. *Enterococcus* have been shown to be facultatively anaerobic organisms, making them stable and active under both aerobic and anoxic conditions. *Enterococcus faecium* strain 8G-1 and *Enterococcus faecium* strain 8G-73 were identified by the inventors as being useful for these purposes.

Preparation of the Strains:

In at least one embodiment, each one of the strains described herein is cultured individually using conventional liquid or solid fermentation techniques. In at least one embodiment, the *Bacillus* strain and *Enterococcus* strains are grown in a liquid nutrient broth, in the case of the *Bacillus,* to a level at which the highest number of spores are formed. The *Bacillus* strain is produced by fermenting the bacterial strain, which can be started by scaling-up a seed culture. This involves repeatedly and aseptically transferring the culture to a larger and larger volume to serve as the inoculum for the fermentation, which can be carried out in large stainless steel fermentors in medium containing proteins, carbohydrates, and minerals necessary for optimal growth. Non-limiting exemplary media are MRS or TSB. However, other media can also be used. After the inoculum is added to the fermentation vessel, the temperature and agitation are controlled to allow maximum growth. In one embodiment, the strains are grown at 32° to 37° under agitation. Once the culture reaches a maximum population density, the culture is harvested by separating the cells from the fermentation medium. This is commonly done by centrifugation.

In one embodiment, to prepare the *Bacillus* strain, the *Bacillus* strain is fermented to a 5×10^8 CFU/ml to about 4×10^9 CFU/ml level. In at least one

embodiment, a level of 2 x 10^9 CFU/ml is used. The bacteria are harvested by centrifugation, and the supernatant is removed. The pelleted bacteria can then be used to produce a DFM. In at least come embodiments, the pelleted bacteria are freeze-dried and then used to form a DFM. However, it is not necessary to freeze-dry the *Bacillus* before using them. The strains can also be used with or without preservatives, and in concentrated, unconcentrated, or diluted form.

The count of the culture can then be determined. CFU or colony forming unit is the viable cell count of a sample resulting from standard microbiological plating methods. The term is derived from the fact that a single cell when plated on appropriate medium will grow and become a viable colony in the agar medium. Since multiple cells may give rise to one visible colony, the term colony forming unit is a more useful unit measurement than cell number.

Using the Strains:

In at least some embodiments, one or more strain is used to form a DFM. One or more carriers, including, but not limited to, sucrose, maltodextrin, limestone, and rice hulls, can be added to the strain.

To mix the strain(s) and carriers (where used), they can be added to a ribbon or paddle mixer and mixed preferably for about 15 minutes, although the timing can be increased or decreased. The components are blended such that a uniform mixture of the cultures and carriers result. The final product is preferably a dry, flowable powder, and may be formulated based upon the desired final DFM concentration in the end product.

In at least one embodiment of a method of making a DFM, a strain described herein is grown in a medium, such as a liquid nutrient broth. The strain is separated from the liquid nutrient broth to make the direct-fed microbial. The strain can be freeze dried after it is separated from the broth.

One or more of *Enterococcus faecium* strain 8G-1, *Enterococcus faecium* strain 8G-73, and *Bacillus pumilus* strain 8G-134 can be fed to animals to reduce or even eliminate the occurrence of acidosis. For this, an effective amount of one or more of these strains is administered to the animals. Upon administration to the animals, the strain(s) provides at least one of the following benefits in or to the animals: (a) reduces acidosis in the animals, (b) stabilizes ruminal metabolism as indicated by delayed lactic acid accumulation and prolonged production of volatile

fatty acids, (c) recovers more quickly from acidosis challenge as measured by pH recovery and lactic acid decline, and (d) does not exhibit clinical signs associated with acidosis.

The animals can be cattle, including both beef cattle and dairy cattle, that is, one or more bull, steer, heifer, calf, or cow; goats; sheep; llamas; alpacas; other four-compartment stomached, and ruminant animals that may encounter ruminal imbalance when fed readily fermentable carbohydrate (RFC).

In at least one embodiment, when *Enterococcus faecium* strain 8G-1 or *Enterococcus faecium* strain 8G-73 is fed, the strain is administered to the animals at a level such that the animals are dosed daily with about 5 x 10^8 CFU/animal/day to about 5 x 10^{10} CFU/animal/day. In at least one embodiment, when *Bacillus pumilus* strain 8G-134 is fed, the strain is administered to the animals at a level such that the animals are dosed daily with about 5 x 10^8 CFU/animal/day to about 5 x 10^{10} CFU/animal/day. In at least one embodiment, two or more strains of *Enterococcus faecium* strain 8G-1, *Enterococcus faecium* strain 8G-73 and *Bacillus pumilus* strain 8G-134 are fed, and the strains are administered to the animals at a level such that the animals are dosed daily with about 5 x 10^8 CFU/animal/day to about 5 x 10^{10} CFU/animal/day as the total dose of the combined strains. Other levels of one or more strains can be fed to the animals. The strain can be administered to the animals from about 30 days of age through the remainder of the adult ruminant productive life or for other time periods.

In at least one embodiment, the strain is fed as a direct-fed microbial (DFM), and the DFM is used as a top dressing on a daily ration. In addition, the strain can be fed in a total mixed ration, pelleted feedstuff, mixed in with liquid feed, mixed in a protein premix, delivered via a vitamin and mineral premix.

In at least one embodiment, the strain is fed as a DFM, and the DFM is fed in combination with Type A Medicated Article monensin (Rumensin®), with a daily dose about 50mg to 660 mg per head. Monensin is fed to increase feed efficiency. Monensin, as an ionophore, creates permeability in bacterial cell membrane creating an ion imbalance between the intracellular and extracellular spaces. This response affects ruminal microbiota populations and influence feedstuff fermentation to improve livestock feed efficiency.

In at least one embodiment, the strain is fed as a DFM, and the DFM is fed in combination with Type A Medicated Article tylosin phosphate (Tylan®), with a daily

dose of about 60 to 90 mg/head. Tylosin phosphate is fed to beef cattle to reduce liver abscesses caused by *Fusobacterium necrophorum* and *Actinomyces pyogenes*.

EXAMPLES

The following Examples are provided for illustrative purposes only. The Examples are included herein solely to aid in a more complete understanding of the presently described invention. The Examples do not limit the scope described herein described or claimed herein in any fashion.

Example 1

Acidosis Model Experimental Design:

Ten crossbred steers were blocked by weight and assigned to two pens. The daily feed ration for all treatment groups prior to challenge consisted of 45% roughage and 55% concentrate on a dry matter basis. Cattle were fed 15 lbs/head/day of the ration once in the morning and had remaining feed pushed closer to the feeding stanchion late in the afternoon. Both pens were fasted for 24 hours before challenge with the concentrate diet treatments. Concentrate diet treatments consisted of highly fermentable carbohydrate sources of steam flaked corn on a 90% as fed basis. After fasting for 24 hours, the concentrate diet was fed ad libitum at 100 lbs/pen to all pens (0h). Challenge diet consumption was visually monitored and additional feed added on an as needed basis.

Rumen fluid samples were obtained from individual animals via oral intubation using a collection tube attached to a vacuum flask. Different flasks and collection tubes were used for each pen to minimize cross contamination of microbiota between treatments. Ruminal fluid collected in the vacuum flasks was decanted into sterile 50 ml Falcon tubes labeled with sample time and animal identification number (ear tag number). Ruminal samples were collected from all pens at -36h, -24h, and -12h. Time -36h and -24h samples represented the physiological baseline for each animal. Time -12h samples represented rumen fluid in the fasted state for each animal. Time 0h was designated as the beginning of the feeding challenge. Ruminal samples were collected from all animals every 4 hours from +6 to +22 hours. All pens were sampled at +28, +36, and +48 hours. The pH from individual ruminal samples were analyzed immediately after acquisition. All

samples were frozen and prepared for shipment to Agtech Products, Inc. (Waukesha, WI) for further analysis.

Volatile fatty acids and carbohydrate concentrations were measured in individual ruminal samples. Samples were prepared for HPLC analysis by aseptically removing duplicate 1.0 ml samples from the rumen fluid collected from each animal at each time period. Samples were placed in a 1.5 ml microcentrifuge tube and the debris was pelleted by centrifugation (10 minutes, at 12,500 rpm). The supernatant fluid (750 µl) was transferred to a clean tube and acidified with an equal volume of 5 mM H_2SO_4. The acidified fluid was thoroughly mixed and filtered through 0.2 µm filter directly into a 2 ml HPLC autosampler vial and capped. Samples were analyzed using a Waters 2690 HPLC system (Waters Inc., Milford, MA). The sample were injected into 5 mM H_2SO_4 mobile phase heated to 65˚C and separated using a BioRad HPX-87H Column (Bio-Rad Laboratories, Inc., Hercules, CA). The HPLC was standardized using a set of concentrations for each compound of interest. Compounds used as standards were include dextrose (glucose), lactate, methylglyoxal, butyrate, propionate, and acetate.

Discovery of Bacterial Genes in Non-Acidotic Cattle Ruminal Microflora:

Suppressive Subtractive Hybridization:

The Genome Subtraction Kit (Clontech, Palo Alto, CA) was utilized to determine microbial population differences between two sets of pooled ruminal samples. Hierarchal clustering analysis was performed to determine similarities and differences between animals based on pH and lactic acid profiles over time. Cluster analysis positioned cattle 2069, 2071, 2078, 2113, and 2127 in Cluster 1 and cattle 2107, 2115, 2088, 2133, and 2124 in Cluster 2. Repeated measures analysis was performed to compare pH and lactic acid from Cluster 1 to Cluster 2. All variables were analyzed separately. Cluster 1 had a significantly higher mean lactic acid profile than Cluster 2 (P=0.0004) accompanied with lower mean pH (P=0.0075) throughout the course of the challenge diet period (Figures 1 and 2). The rumen fluid from individual animal was pooled within cluster for suppressive subtractive hybridization (SSH) procedures.

Suppressive subtraction hybridization (SSH) strategies were developed to compare pooled ruminal DNA samples from cattle in Cluster 1 to those in Cluster 2 at sample times +6h, +10h, +14h, and +18h. Suppressive subtraction hybridization was

performed utilizing Cluster 2 as the tester (non-acidotic cattle) and Cluster 1 as the driver (acidotic cattle). The SSH was hypothesized to result in unique DNA fragments from organisms that resulted in lower levels of lactic acid and a higher pH (ruminal energy modulating organism). By performing subtractions using samples from time +10h, the DNA fragments (genes) found, were from organisms that were able to modulate the utilization of excess energy in the ruminal environment in the form of RFC and alleviate potential effects of acidosis.

Cloning and screening of unique tester sequence library:

Strain specific DNA sequences that are recovered after subtraction were cloned for further analysis. DNA sequences were inserted into the pCR2.1 vector (Invitrogen) and transformed into *E. coli* chemically competent TOP10 cells. The transformation mixture was plated onto 22 x 22 cm LB agar plates containing 50 µg/ml kanamycin and overlaid with 40 mg/ml X-gal in DMF. Plates were incubated at 37˚C for 24h. Recombinant colonies (white colonies) were picked into sterile microtitre plates containing LB medium and kanamycin at 50 µg/ml. All wells containing recombinant PCR products were separated into 1 ml aliquots. One aliquot was purified using the Qiaquick PCR Purification Kit (Qiagen), with the second aliquot pelleted via centrifugation, resuspended in LB+Kan + 10% glycerol and stored at -80˚ C.

Southern Hybridization:

Slot-blot hybridizations were conducted using standard protocols. To confirm the specificity of the cloned DNA inserts, positively charged Zeta-Probe® Blotting Membranes (Bio-Rad Laboratories; Hercules, CA) were hybridized with probes made from the original tester and driver DNA digested with *Alu* I and labeled with the DIG High Prime DNA labeling kit (Roche Diagnostics Corporation, Indianapolis, IN). Recombinant inserts showing sequence homology to the tester DNA but not the driver DNA was selected for sequence analysis. Hybridizations were conducted on cloned inserts. At each time period, subtraction was performed, SSH 6, 10, 14, and 18. From SSH 6, 10, 14, and 18, there were 12, 29, 105, and 29 cloned inserts, respectively, that were tester specific.

The DNA sequence from each tester positive insert was determined (Lark Technologies; Houston, TX). Sequence from each insert was compared with

sequences from the NCBI database using the blastX function. Nucleotide sequences were translated and gene function was deduced by comparing sequences to those found in the NCBI database using the blastX function. Gene function was placed in a gene category using the Clusters of Orthologous Groups (COG) web site. Specific COG genes identified were used to construct oligonucleotide probes for colony hybridization and slot-blot hybridization experiments. Four genes of the twenty-nine were selected from SSH 10 to be utilized for colony hybridization based upon functional attributes based on selection from non-acidotic cattle. The genes were selected from clones 79, 84, 94, and 110 were identified via using the NCBI blastX function with assigned functions: beta-xylosidase, glucose/galactose transporter, 4-alpha-glucanotransferase, and 4-alpha-glucanotransferase, respectively. All genes selected for colony hybridization had assigned properties as identified by COG as Carbohydrate and Transport Metabolism function, which would have provided bacteria containing these genes an advantage at metabolizing excess energy such as that found in the rumen when challenged with RFC.

Colony Hybridization:

Rumen fluid collected during the acidosis trial from cattle at times +10h, +14h, and +18h was utilized. Cattle 2107, 2124, 2115, 2088, and 2133 were selected from each of these time periods. These cattle are representative of animals that were previously selected for the "tester population" or non-acidotic group. Individual rumen samples were taken from -20° C and allowed to thaw at room temperature. Thawed rumen samples were individually plated on three separate mediums in duplicate. Media utilized consisted of sodium lactate agar (NLA), Lactate Propionibacterium Selective Agar (LPSA), and modified reinforced Clostridial media (RCS). The RCS was prepared similar to commercially available reinforced Clostridial media sans glucose. Thus, the major carbohydrate source in RCS is starch. Table 1 below indicates the incubation conditions and dilutions of rumen fluid plated on each media.

Table 1. Incubation conditions and dilutions plated on each media.

	Incubation Conditions			
Media	**O2 Conditions**	**Incubation Time**	**Incubation Temperature**	**Dilutions Plated**
LPSA	Anaerobic	7 Days	32° C	10-1, 10-2
NLA	Anaerobic	5 Days	37° C	10-2, 10-3
RCS	Anaerobic	48 Hours	37° C	10-1, 10-2

After incubation, individual colonies were picked off of each plate and inoculated into 10 ml broth tubes consisting of the respective media, except LPSA, which was inoculated into NLB. Colonies were selected from each time period and each animal (five cattle x three time periods). For the RCS media, five colonies were picked for each animal-time period. The LPSA exhibited less colonies and diversity on the plates and number of colonies selected per animal-time period was variable. Two colonies per animal-time period were selected from the NLA media, except animal 2107 at time period 18. Six colonies were picked from this animal-time period due to increased visible diversity. Not all inoculated tubes exhibited growth after incubation.

Tubes showing growth were separated into two separate aliquots of 9 ml and 1 ml. The 1 ml aliquot was utilized for DNA isolation procedures utilizing the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals; Mannheim, Germany). The 9 ml aliquot was transferred to a sterile 15 ml Falcon Tube and centrifuged until a solid pellet was formed. The pellet was then reconstituted in NLB or RCS broths containing 10% glycerol. The reconstituted sample was placed in the -80°C for future use. The extracted DNA was then used for RAPD-PCR analysis of individual isolates to determine phylogenic relationships. Analysis was performed using Bio-Numerics (Applied Maths Inc., Austin, TX) on the RAPD DNA banding patterns to determine the relatedness of the isolates. The similarity coefficient of isolates was determined using the Dice coefficient and an un-weighted pair group method (UPGMA). A similarity of 80% or greater was used to group the 109 isolates into 65 separate clusters. Of the 65 clusters, 23 grew only on RCS, 11 grew only on LPSA, 14 grew only on NLA, 4 clusters grew both on RCS and LPSA, 6 grew on both RCS and NLA, 3 grew on both LPSA and NLA, and 4 clusters were found to be present on all three media.

Slot blot hybridizations were prepared utilizing the Bio-Dot™ SF Microfiltration Apparatus (BIO-RAD; Hercules, CA). The genomic DNA of a single isolate within a cluster was selected to represent the cluster and blotted onto membranes. Probes were prepared for hybridization using the PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals; Mannheim, Germany). Probes selected were derived from the cloned insert analysis described above and consisted of four clone inserts (Clones 79, 84, 94, and 110) from SSH10. Labeled probes were pooled prior to hybridizations. Hybridizations were conducted at 45°C for 5 hours. Colorimetric reactions were

allowed to develop overnight on the membranes. Thirty of the 37 isolates (clusters) on the RCS membrane exhibited hybridization as identified by colorimetric reaction and 25 of the 28 isolates on the LPSA/NLA membrane

Isolates exhibiting hybridization were then prepared for 16s rRNA sequencing. Briefly, the 16s rRNA of each of the 55 isolates was amplified via PCR using the primers 8F (SEQ ID NO: 1, AGAGTTTGATYMTGGCTCAG) and 1406R (SEQ ID NO: 2, ACGGGCGGTGTGTRC). The PCR product was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Purified product was analyzed by gel electrophoresis. When sufficient product was available, the purified sample was sent overnight on ice for single pass sequencing (Lark Technologies, Houston, TX). The 16s sequences from each cluster were compared with sequences from the NCBI database using the blastn function. Organisms of interest brought forward from this comparison consisted of *Enterococcus faecium* strain 8G-1, *Enterococcus faecium* strain 8G-73, and *Bacillus pumilus* strain 8G-134.

Example 2

In vitro Strain Testing:

Rumen fluid was collected for in vitro trials from two yearling Hereford heifers. Heifers were identified by identification tags and were referred to as 101 and 133. Heifers were fed 6 lbs/head/day of dried distillers grain (DDGS) and had access to free choice haylage.

The in vitro protocol was followed as closely as possible to decrease experimental error between each trial. Briefly, rumen fluid was collected from each heifer and placed into marked, pre-warmed thermoses. Thermoses were transported to Agtech Products, Inc. for processing. Rumen fluid was added in duplicate to bottles containing McDougall's Buffer and 3.0% glucose (final concentration after McDougall's Buffer and rumen fluid have been mixed to a volume of 180 ml), which had been tempered to 39 °C. Candidate DFM strains, *Enterococcus faecium* strain 8G-1, *Enterococcus faecium* strain 8G-73, and *Bacillus pumilus* strain 8G-134, were added to designated bottles at 1.0×10^7 CFU/ml (final concentration). The unit of observation was the bottle, and treatments were performed in quadruplicate. Treatments consisted of Control (glucose added but no DFM), *Enterococcus faecium* strain 8G-1, *Enterococcus faecium* strain 8G-73, and *Bacillus pumilus* strain 8G-134. Bottles were then purged with of CO_2 and capped. Bottles were maintained in a

shaking water bath at 39°C and 140 rpm. Approximately 10 minutes prior to sampling, bottles were briefly vented to release gases produced as a byproduct of fermentation. Rumen fluid was withdrawn from each bottle initially and every 6 hours until the 36 hour mark. Rumen pH and volatile fatty acids were measured and recorded. Statistical analysis was performed using repeated measures analysis to determine DFM effects over time or one-way ANOVA to determine treatment affects at a specific points in time.

The focus of the ruminal in vitro experiments was to determine if candidate DFM strains, *Enterococcus faecium* strain 8G-1, *Enterococcus faecium* strain 8G-73, and *B. pumilus* strain 8G-134, could positively influence ruminal fermentation in an energy excess environment. Excess glucose was added to each ruminal in vitro, to replicate cattle engorgement with a readily fermentable carbohydrate. As shown in Figure 3, the addition of each of the candidate strains significantly increased the utilization of glucose over time (P=0.0001). In comparison to the control (Figure 4), the influence on lactic acid production over time was also significantly impacted by the addition of the candidate DFM to the challenged in vitro model (P=0.0025). By time point 36 hours, there was 17% less lactic acid production in the *B. pumilus* and 32% less lactic acid accumulation in both the *Enterococcus* candidates.

Volatile fatty acid analysis was performed via HPLC. Total VFA (acetate+propionate+butyrate) were significantly affected by addition of the *Enterococcus* candidates (P=0.0279) (Figure 5). The *Enterococcus* candidates, 8G-1 and 8G-73, appeared to increase the amount of total VFA produced over time. There was no significant affect on total VFA production when comparing the *B. pumilus* candidate to that of the control.

The in vitro results indicated that the candidate DFMs 8G-1, 8G-73, and 8G-134 positively affected ruminal fermentation by increasing glucose utilization without a corresponding increase in lactic acid production in comparison to that of the control treatment. Excess glucose in the rumen is typically fermented rapidly with the production lactic acid. It is the accumulation of lactic acid which drives an acute acidotic response. By utilizing glucose without the concomitant production of lactic acid, the candidate DFMs have demonstrated the potential to ameliorate the affects of acidosis. The ruminal in vitro model suggested that these strains may be able to successfully modulate excess ruminal energy in cattle fed high amounts of readily fermentable carbohydrates.

Example 3

Candidate DFM Testing in Cattle Fed a Readily Fermentable Carbohydrate—an Acute Acidosis Challenge:

Materials and Methods:

Cattle and Pens Assignments:

Twenty cross-bred beef steers were purchased at local sale barns. Cattle were housed at the research facility for a period of two weeks prior to trial initiation for observation of morbidity or mortality. Cattle were randomly blocked across treatment by weight. Five head of cattle were assigned to a pen and pens designated to one of four treatments. Treatments consisted of 3 pens each receiving a different DFM as is detailed below with the fourth pen receiving no DFM (control). Treatment assignments can be seen in Table 2 below.

Table 2. Treatment assignments by pen.

Pen ID	Candidate DFM (TX)	Minimum Dose (CFU/Head/Day)	16s rRNA Identification
1	None	0	None (Control)
2	8G-1	5×10^{10}	*Enterococcus spp.*
3	8G-73	5×10^{10}	*Enterococcus spp.*
4	8G-134	5×10^{9}	*Bacillus pumilus*

The daily feed ration for all treatment groups prior to challenge, consisted of 62.5% roughage and 30% cracked corn and 7.5% protein supplement (Table 3) below. The protein supplement contained monensin (Rumensin®) fed at 375 mg/head/day. The protein supplement also contained tylosin phosphate (Tylan®).

Table 3. Challenge Ration Composition

	Ingredient	% of Diet (DM)
Pre-challenge diet	Ground Hay	62.5
	Cracked Corn	30
	Steakmaker® K+ 45-25 R500 T180*	7.5
Challenge Diet	Steam Flaked Corn	87.4
	Alfalfa Pellets	5.1
	Steakmaker® K+ 45-25 R500 T180*	7.5

*Both rations contain Rumensin and Tylan.

Cattle were fed 15 lbs/head/day of the ration once in the morning and had any remaining feed pushed closer to the feeding stanchion late in the afternoon. Fourteen days prior to fasting, treatment groups were fed candidate DFMs at the dose designated in Table 2 above as a top dressing on the daily ration.

Bacillus pumilus strain 8G-134 was fed at a minimum of 5×10^9 CFU/Head/Day. The *Enterococcus* candidates 8G-1 and 8G-73 were fed at 5×10^{10} CFU/Head/Day.

Candidate DFM Preparation:

Candidate DFM strains, previously selected for the challenge trial, were *Enterococcus spp*. 8G-1 and 8G-73; and *Bacillus pumilus* strain 8G-134. Strains were stored at -80°C. Each culture was inoculated into 10 ml broth tubes containing MRS (Man, Rogosa and Sharp) or TSB (tryptic soy broth). Broth tubes were incubated for 24 hours at 32° and 37°C for the *Bacillus* and *Enterococcus* candidates, respectively. Cultures were struck for isolation on respective agar medium and incubated. An isolated colony was picked into 10 ml of broth and allowed to grow to mid log phase (18 to 24 h) and transferred into fresh broth (10% vol/vol transfer). *Enterococcus* candidates were grown at 37°C in MRS broth. *Bacillus* was grown at in a shaking incubator at 130 rpm at 32°C in horizontal TSB tubes. For the growth of *Enterococcus*, 2 ml were transferred into a 250 ml bottle containing 198 ml of broth and incubated for 18hrs.

The 200 ml of culture was inoculated into a 2 L bottle containing 1.8 L of broth and allowed to incubate for 18hr. For the *Bacillus* candidates, 5 ml were transferred into a 250 flask containing 50 ml of TSB and then was incubated at 32°C in a shaking incubator at 130 rpm for 24hr. The 50 ml was used to inoculate a 1L flask containing 600 ml and allowed to incubate for another 24 hours.

The optical density (OD) of the 18hr culture of *Enterococcus* candidates was taken before harvesting the cells. The OD was compared to previous growth curves to determine the cfu/ml of culture. Samples were plated for enumeration and genetic fingerprinting. Quality control was ensured between each fermentation batch via RAPD-PCR analysis. With a target minimum of 5.0e10 cfu/head/day for *Enterococcus* candidates, the calculated amount of culture was dispensed into 250 ml Nalgene™ centrifuge bottle and spun at 4°C for 10min at 4500 rpm. Target minimum for *Bacillus* candidate was 5.0e9 cfu/head/day, and a total of 100 ml of the *Bacillus* culture was spun down similar to the *Enterococcus*. Supernatant was discarded. The pellet was resuspended in 30 ml of growth media containing 10% glycerol. This amount was transferred to a 50 ml conical tube. The centrifuge bottles were then rinsed with 10 ml of broth and transferred to the same conical tube. Samples were

labeled with strain, date the candidate was harvested, and fermentation batch number. Plate counts were used to determine the total cfu in each tube. Tubes were combined to deliver counts of a minimum of 5.0e10 cfu/head/day for *Enterococcus* candidates and 5.0e9 cfu/head/day for *Bacillus* candidates. All conical tubes were frozen at -20°C

Challenge Diet and Rumen Fluid Collection Phase:

Rumen fluid samples were obtained from individual animals via ruminal intubation using a collection tube fitted with a strainer and attached to a vacuum source through a vacuum flask. The pH was immediately measured after rumen fluid acquisition and samples were frozen to be transported to Agtech Products, Inc. for VFA analysis. Samples were collected from all cattle at sample times -12h, +6h, +10h, +14h, +18h, +22h, +30h, +36h, and +48h, with time 0h representing the initiation of the challenge. All feed was removed from the cattle at time -24h to initiate the fast and encourage cattle to engorge the challenge ration at time 0.

All pens were fasted for 24 hours before challenge with the concentrate diet (Time 0). The concentrate diet consisted of 28 lb flake weight steam flaked corn (Table 3 above). The challenge ration was fed to deliver 20 lbs/head. Challenge ration consumption was visually monitored and additional feed added on an as needed basis through the remainder of the trial.

Ruminal samples were collected every 4 hours from all cattle from +6 to +22 hours. Each rumen sample pH was analyzed immediately after acquisition. Rumen fluid was then frozen and transported to Agtech Products, Inc. for VFA analysis via HPLC. Repeated measures analysis was performed on rumen pH, VFAs, and glucose levels using individual animal as the unit of observation. Pairwise comparisons were performed over time between each candidate DFM treatment pens and the control pen to determine the candidates' effectiveness to alter ruminal fermentation patterns.

Results and Discussion:

Twenty head of crossbred beef cattle weighing on average 731.95 lbs were randomly blocked by weight across treatments such that there were no significant differences by weight between treatment groups (Table 4). There were 3 treatment pens and one control pen with five head/pen. Treatment assignment per pen can be seen in Table 2 above.

Table 4. Treatment assignments by pen.				
Pen	Treatment	N	Ave. Pen Weight in lbs (SD)	Average Feed Consumption/Steer[1] (% of Body Weight)
1	Control	5	731.6 (79.94)	4.8
2	8G-1	5	741.2 (94.03)	3.9
3	8G-73	5	731.6 (70.12)	3.7
4	8G-134	5	727.6 (72.71)	3.6
[1]Average feed consumption/steer was calculated as a percentage of the average steer weight for that pen				

Cattle were fed challenge ration at time 0 and rumen fluid was collected at designated time points to measure ruminal fermentation values. Feed consumption per pen was monitored and recorded after 24 hours. Average feed consumption/steer was calculated as a percentage of the average steer weight for that pen (Table 4 above). The control pen (pen 1) appeared to have the highest consumption of feed in comparison to the other treatment groups with the average steer consuming 4.8% of its body weight. The lowest challenge ration consumption/pen was in pen 4 with the average steer eating approximately 3.6% of its body weight. Cattle in all pens on average would have been consuming approximately 5.625 lbs of concentrate/day as part of the pre-challenge ration, which on average would have constituted 0.8% of the average steers' body weight. Despite the pen variation of challenge ration consumption, the difference was not greater than the increase in concentrate consumption from the pre-challenge ration and would not be causative in fermentation differences between pens.

After 24 hours, feed was removed from the cattle and ground prairie hay was fed ad libitum. Cattle were given free choice hay as a precaution against the continually decreasing rumen pHs. The addition of hay would stimulate additional cud chewing and help to buffer the rumen. Despite the addition of hay, the ruminal pH still continued to decline.

Immediately after rumen fluid collection, sample pH was analyzed. All treatment groups exhibited a decline in ruminal pH as can be observed in Figure 6. The pH for the control group achieved nadir at time 30 hours and began to gradually climb thereafter. By the last rumen sample collection the mean pH for the control pen was still acutely acidotic with a pH of 4.94. Acute acidosis is associated with pH that remains below 5.2 and chronic or subacute acidosis characterized by a pH below 5.6 (Owens, et. al., 1998). Mean numerically higher trends appeared for strains 8G-1, 8G-73, and 8G-134 in pens 2, 3, and 4 when compared to that of the control from time +22 to +48. This suggests that cattle treated with the candidate DFMs in these pens

recovered more quickly from the acidotic challenge. Mean pH for the for cattle treated with 8G-1, 8G-73, and 8G-134 at time +48 was 5.96, 6.02, and 6.14, respectively, which is greater than 1.0 pH unit above the control pen. Repeated measures analysis of these three strain over time (+6 to +48) did not exhibit significant differences when compared to the control pen. However when pH comparisons of pens treated with 8G-1, 8G-73, and 8G-134 to the control pen from time +22h to +48h were performed, differences were or approached significance (P = 0.1562, 0.0965, and 0.0466 for 8G-1, 8G-73, and 8G-134, respectively).

Average lactic acid profiles for all treatment groups are shown in Figure 7. Mean ruminal lactic acid accumulation peaks at 105 mM for the control pen 30 hours after receiving challenge ration. Candidate DFM strains 8G-1, 8G-73, and 8G-134 again exhibited visible mean numeric differences in lactate accumulation in comparison to that of the control pen. Mean lactic acid accumulation was similar between the control cattle and the 8G-1 treated cattle through the first 14 hours of the challenge. Subsequent accumulation levels for the remainder of the trial were much less in the 8G-1 treated cattle although not significant (P= 0.1892). Treatment pens 8G-73 demonstrated decreased levels of lactic acid accumulation at times 30 and remained lower than the control pen for the remainder of the trial. Candidate strain 8G-134 also showed decreased levels of lactic acid starting at +22h and remained consistently lower than the control pen through +48h.

Individual VFAs were measured and analyzed. Volatile fatty acid (VFA) concentrations increased in the control pen and treatments 8G-73 and 8G-134 and peaked at six hours (Figure 8). After six hours each of these treatment pens showed declining levels of total VFA (acetate, propionate and butyrate). There were no significant differences between these treatments and the control. Treatment 8G-1, however, exhibited a delay in VFA decline which did not occur until +14h. Over the course of the trial there were no significant differences in total VFA concentration or the individual VFA (consisting of acetate, propionate, or butyrate) levels.

In addition to monitoring and measuring ruminal fermentation characteristics over the course of the acidotic trial, cattle were observed throughout the trial for visible clinical effects associated with acidosis. Early in the acidotic challenge (+0h to +14h), the effects of the challenge diet were minimal. Cattle did not show signs of depression and continued to feed on the challenge ration. By +22 hours post receiving the challenge ration all cattle except those in receiving Treatment 8G-1)

were showing signs of soreness, depression, and had loose, liquid fecal excretion. Cattle in pen 2 were no longer consuming feed, but did not exhibit clinical symptoms, despite similar having similarly declining pH levels.

Acute ruminal acidosis by definition is the decline in ruminal pH to levels deleterious not only to rumen function but also livestock health. Acute acidosis is marked by the accumulation of lactic acid and the decline in VFA production. Proper rumen function is a combination of managing the available energy and nitrogen components available in feedstuffs. When imbalances in ruminal metabolism occur, digestive upset typically follows and can manifest in the form acidosis. In this trial, strains 8G-1, 8G-73, and 8G-134 enhanced the recovery of rumen function as indicated by ruminal fermentation parameters.

Cattle fed 8G-1, *Enterococcus faecium,* on average recovered more quickly from the acidosis challenge as measured by pH recovery and lactic acid decline. In addition to the measured ruminal fermentation patterns, cattle fed candidate DFM 8G-1 did not exhibit clinical signs associated with acidosis.

Candidate strain 8G-73, *Enterococcus faecium*, improved ruminal fermentation through the course of the trial. Mean lactic acid levels were the lowest for all candidate strains tested at +48h at 12.54 mM. A corresponding increase in pH was also associated with the recovery with a final pH of 6.02, which was 1.08 pH units higher than that of the control.

Candidate strain 8G-134, *Bacillus pumilus*, also enhanced ruminal recovery during the acidotic challenge. Mean lactic acid levels, in cattle fed 8G-134, peaked at 89mM at time +22h, while the control pen continued to increase and peaked at 105mM at time +30 hours. Mean lactic acid levels had dropped to 57mM by +30 hours. As with lactic acid accumulation, ruminal pH in cattle fed 8G-134 recovered more quickly than that of the control and was found to be significantly different from +22 to +48h (P=0.0466).

Example 4

Summary:

Thirty prima and multiparous Holstein cows were blocked by previous lactation and predicted producing ability (PPA) and assigned to one of three treatments. Ten cows were assigned per treatment and treatments consisted of a control group (Treatment 1) which received a basal total mixed ration (TMR),

Treatment 2 and Treatment 3 which received basal total mixed ration TMR and were fed *Bacillus pumilus* 8G-134 at 5 x 10^9 and 1 x 10^{10} CFU/head/day, respectively, from 3 weeks prepartum to 22 week after parturition. The primary objective was to determine the effects of *B. pumilus* 8G-134 on dairy cow milk production and performance above control cattle during this time period. The secondary objective was to determine if there was a dose response associated with feeding *B. pumilus* 8G-134. The *B. pumilus* 8G-134 regimens significantly increased milk production, milk fat, and decreased somatic cell count. These significant *B. pumilus* 8G-134 production effects did not come at the expense of cow body condition score, body weight, increases in dry matter intake or significantly change blood metabolite profiles, and would indicate *B. pumilus* 8G-134 also provided dairy cow efficiency benefits.

Materials and Methods:

Livestock:

Thirty Holstein cows were randomly assigned to one of three dietary treatments in a continuous lactation trial from 3 weeks prior to parturition through 22 weeks postpartum. There were no significant differences for previous milk yield for second and older cows or for predicted producing ability (PPA) for first lactation cows for the different treatment groups. The numbers of first and second lactation animals deviated between the groups, but did not influence overall mean production, as lactation was adjusted in the statistical model. Animals were on study from approximately three weeks prepartum through 22 weeks postpartum.

Nutrition:

Dietary ingredients and formulated composition of total mixed rations (TMRs) are presented in Table 5 below for dry and lactating cows, respectively. The base TMR was the same for each group and differed by top dress treatment. Each group received a top dress of 8 ounces of finely ground corn to which was added 1 ounce of maltodextrin (Treatment 1, control), *Bacillus spp* at 5 x 10^9 CFU/head/day (Treatment 2), and *Bacillus spp* at 1 x 10^{10} CFU/head/day (Treatment 3).

Table 5. Formulated composition of the TMR offered to dry and lactating cows.

	Dry Period	Lactating Period
Ingredients, % DM basis		

Corn Silage	52.57	39.14
Ryelage	18.54	
Alfalfa haylage	.	16.52
Grass hay	14.45	1.77
SBM48	5.74	10.77
Blood Meal	4.41	
AminoPlus	.	6.01
Corn	2.41	21.07
Fat	0.65	1.42
Limestone	.	1.26
Sodium bicarbonate	.	0.84
MagOx	0.65	0.42
Salt	0.32	0.53
TMin Vit	0.27	0.26
Composition, % DM		
CP	16.50	16.64
SP, % CP	32.29	28.58
NDF	41.06	30.65
Starch	19.56	29.82
Sugar	3.19	2.68
NFC	33.47	41.87
Fat	3.82	4.15
Ca	0.31	0.87
P	0.29	0.34
Mg	0.54	0.42
K	1.72	1.33
NeL, mcal/kg	1.60	1.74

Sample and Data Collection:

Daily TMR samples and refusals were collected and composited weekly, weekly composites combined monthly, and monthly samples were analyzed for dry matter (DM), crude protein (CP), acid detergent fiber bound protein (ADF-CP), neutral detergent fiber bound protein (NDF-CP), soluble protein (SP), acid detergent fiber (ADF), neutral detergent fiber (NDF), lignin, fat, starch, sugar, ash, calcium (Ca), phosporus (P), magnesium (Mg), potassium (K), sulfur (S), sodium (Na), chlorine (Cl), iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) by Cumberland Valley Analytical Services, Maugansville, MD.

Cows were milked twice a day, and milk volume was recorded electronically at each milking and am-pm amounts summed for daily total. Once a week milk samples from am and pm milkings were composited for analysis of content of fat, protein, somatic cells, solids not fat, and milk urea nitrogen (MUN) by Dairy One

milk laboratory in State College, PA using a Fossamatic 4000 (FOSS; Eden Prairie, MN).

Animals were on study from approximately three weeks prepartum through 22 weeks postpartum. Animal weight was estimated by heart girth circumference on weeks 1, 3, 7, 11, 15 and 18 postpartum. Body condition was assessed by two independent observers at the same time as body weight was collected.

Blood samples were collected from the coccygeal vein, serum harvested, frozen and analyzed for glucose, beta-hydroxy butyrate (BHB), and non-esterified fatty acids (NEFA) at weeks 2 and 8 postpartum. Glucose and BHB were analyzed using an Abbott Precision Xtra™ meter (Abbott Diabetes Care Inc., Alameda, CA). A Randox™ assay kit (Cat. HN 1530, Randox Laboratories, Northern Ireland) was used to measure non-esterified fatty acid (NEFA) concentration in serum adopted to an enzyme linked immunosorbant assay (ELISA) plate reader at a wave length of 550 nm for multiple samples. The Randox kit uses Acyl CoA synthetase and oxidase to convert NEFA to 2,3-trans-Enoyl-CoA plus peroxide; peroxide plus N-ethyl-N-(2 hydroxy-3-sulphopropyl) m-toluene leads to a purple product, which is the indicator of NEFA concentration in serum.

Statistical Models.

Milk production and content, body weight, and body condition score were analyzed using the mixed procedure in SAS statistical software. Cow was the repeated subject with the covariance matrix set to type 1 correlation structure. Daily milk observations were aggregated by week postpartum. The statistical model was as follows:

$$Y_i = u_i + TRT_j + Lact_k + Week_l + TRT_j*Lact_k + TRT_j*Week_l + TRT_j*Lact_k*Week_l + e_{jklm}$$

Where

Y_i = least square mean of the production dependent variables;

u_i = overall mean of the various production variables;

TRT_j = jth treatment effect, 1, 2, 3;

$Lact_k$ = kth lactation, 1, 2+;

$Week_l$ = lth week, 1..22;

interaction terms (TRTj*Lactk + TRTj*Weekl + TRTj*Lactk*Weekl)

e_{jklm} = error

Monthly samples of TMR and feed refusals for each treatment were tested for difference using means procedure in SAS.

Blood concentrations of glucose, BHB, and NEFA, were analyzed using the general linear models in SAS statistical software. Class variables were cow, week and treatment. Treatment was nested in cow and was the error term for testing treatment significance. Treatment by week interaction was tested for statistical significance using the residual error.

Results:

Mean TMR composition for dry and lactating TMRs is presented in Table 6 over the course of the study. Composition was not different between the treatment groups.

Table 6. Analyzed composition of TMR for dry cows and lactating cows

	Treatment, % DM basis			
Item	1	2	3	SEM
Dry TMR				
N	3	3	3	
CP	13.64	13.17	13.11	0.14
ADF	29.55	28.37	27.88	0.53
NDF	48.54	48.04	47.14	0.93
Lignin	3.64	3.52	3.58	0.09
Starch	18.74	18.19	17.97	0.87
Sugar	6.71	6.15	6.57	0.23
Ash	8.44	7.56	7.79	0.10
NFC	39.49	36.93	38.20	0.86
Ca	0.49	0.46	0.47	0.01
P	0.37	0.35	0.35	0.004
Lactating TMR				
N	9	9	9	
CP, %	14.54	14.81	14.37	0.10
ADF, %	21.54	21.38	21.64	0.25
NDF, %	33.51	33.21	33.30	0.32
Lignin, %	3.27	3.27	3.26	0.06
Starch, %	25.36	26.52	25.48	0.38
Sugar, %	6.25	6.15	6.25	0.15
Fat, %	3.69	3.65	3.62	0.04
Ash, %	8.73	8.67	8.60	0.09
NFC, %	41.22	42.02	41.44	0.34
Ca, %	0.95	0.97	0.96	0.005
P, %	0.36	0.36	0.36	0.003

standard error means (SEM),
NFC, nonfiber carbohydrate
NDF, neutral detergent fiber
ADF, acid detergent fiber
Treatment 1 control; Treatment 2, *Bacillus pumilus* at 8G-134 5 x 10^9; Treatment 3, *Bacillus pumilus* 8G-134 at 1 x 10^{10}.

One control (Treatment 1) cow exhibited abnormal milk production and her data was removed from the data analysis. The analysis of milk production was repeated on 29 cows. Milk production was significantly influenced by treatment (Table 7 below). Cows fed the *Bacillus pumilus* 8G-134 at 5 x 10^9 CFU/head/day (Treatment 2), and *Bacillus pumilus* 8G-134 at 1 x 10^{10} CFU/head/day (Treatment 3)

produced significantly more milk than the cows fed the placebo control. Cows on Treatment 2 and 3 produced approximately 2 kg more milk than treatments 1 (Table 7). There was a significant interaction with parity. Production increases were significant in second parity cows by 5.2 kg, but no significant differences in milk production in first parity cows.

Table 7. Least square mean milk production in Holstein cows from calving through 22 weeks postpartum fed a microbial additive.

Effect	Treatment	Lactation	Milk, kg/d kg/d	sem	Change relative to control, kg/d	sem
Treatment	1		33.12[a]	0.65	0.00	0.66
Treatment	2		35.38[b]	0.60	2.30*	0.60
Treatment	3		35.08[b]	0.61	1.99*	0.61
Lactation		1	31.59[a]	0.47	-0.83	0.47
Lactation		2	36.84[b]	0.39	3.09*	0.39
Interaction						
	1	1	32.44[a]	1.07	0.00	1.07
	2	1	31.70[a]	0.93	-0.72	0.93
	3	1	31.36[a]	0.93	-1.07	0.93
	1	2	33.80[b]	0.74	0.00	0.74
	2	2	39.06[c]	0.76	5.24*	0.76
	3	2	38.79[c]	0.79	5.17*	0.79

Means within group with different superscript differ, $P<0.05$
Mean change with * differ significantly from 0
Treatment 1 control; Treatment 2, *Bacillus pumilus* at 8G-134 5 x 10^9; Treatment 3, *Bacillus pumilus* 8G-134 at 1 x 10^{10}.

Milk fat and yield are presented in Table 8 below. Milk fat was significantly increased by Treatments 2 and 3 above the control. Yield responses followed milk yield with fat yield increased in the *Bacillus pumilus* 8G-134 fed treatment groups. *Bacillus pumilus* 8G-134 cattle for treatment 2 and 3 yielded significantly higher fat percentage above that of the control with 0.24% and 0.31% higher levels, respectively (Table 8). Coupled with the significant increase in milk production, daily fat yield for both treatment 2 and 3 provided significant increases in daily fat production above that of the control (Table 8).

Table 8. Milk fat content by treatment groups.

Effect	Treatment	Lactation	Fat, %	sem	Fat Yield , kg/d	sem
Treatment	1		3.57[a]	0.09	1.206[a]	0.042
	2		3.81[b]	0.08	1.351[b]	0.039
	3		3.88[b]	0.08	1.351[b]	0.039
Lactation		1	3.76	0.06	1.159[a]	0.030
		2	3.78	0.05	1.395[b]	0.025

Means within column with different superscript differ by $P<0.05$
Treatment 1 control; Treatment 2, *Bacillus pumilus* at 8G-134 5 x 10^9; Treatment 3, *Bacillus pumilus* 8G-134 at 1 x 10^{10}.

Log of the linear Somatic cell count (LogSCC) scores were different by treatment and lactation. The *Bacillus pumilus* 8G-134 treatments (Treatments 2 and 3) had significantly lower log linear score than the control cows (Table 9 below). Treatments 2 and 3 cows had LogSCC of 4.97 and 4.96, respectively compared to those of the control cows at 5.92. Parity two cows had significantly higher log linear score than first lactation cows. Somatic cell counts are associated with infection as well as immunological status and health of the lactating dairy cow. Additionally increased SCC is indicative of inflammatory responses to infection. Decreases in SCC demonstrated here may indicate that cows fed the *Bacillus pumilus* 8G-134 are better immunologically to handle infectious challenge during lactation and maintain udder and cow health.

Table 9. Treatment effects on Somatic Cell Count (SCC).

Effect	Treatment	Lactation	LogSCC	sem
Treatment	1		5.92[a]	0.32
	2		4.97[b]	0.30
	3		4.96[b]	0.30
Lactation		1	5.27[a]	0.23
		2	5.86[b]	0.19

LogScc = log of somatic cell count
Treatment 1 control; Treatment 2, *Bacillus pumilus* at 8G-134 5 x 10^9; Treatment 3, *Bacillus pumilus* 8G-134 at 1 x 10^{10}.

Data for mean DMI for groups for dry and lactating periods in Table 10 below. Dry matter intake for dry cows ranged from 10.79 to 11.64 kg/d across the groups. Lactating groups consumed 22.02, 21.31 and 21.48 kg/d for treatment groups 1, 2, and 3, respectively (Table 10). Predicted DMI based on the NRC equation for cows by week postpartum is presented in Table 10. Intake for treatment 1 was 0.83 kg higher than predicted; intake for treatment 2 was -1.37 kg lower than predicted; intake for treatment 3 was -1.04 kg lower than predicted. The increase in milk production on the Treatments 2 and 3 was accomplished with no increase in DMI. In fact the predicted or expected DMI based on NRC predictions compared to the group mean intake suggests these cows consumed 1.0 to 1.5 kg/d less DMI. Thus, the efficiency of DM utilization was increased on Treatments 2 and 3.

Table 10. Least square means for group feed intake, serum glucose, beta-hydroxy butyrate, non-esterified fatty acids, by treatment group.

	Treatment group					
Item	1	sem	2	sem	3	sem
Dry Matter Intake, kg/d						
Dry cows	10.79	0.27	11.64	0.25	11.12	0.25
Lactating cows	22.02	0.11	21.31	0.11	21.48	0.11
Predicted DMI, kg/d	21.19	0.58	22.68	0.57	22.52	0.58
Serum values						
Glucose, mg/dl	53.95	1.98	51.5	1.98	53.5	1.98
Beta-OH butyrate, mg/dl	1.05	0.15	0.88	0.15	1.15	0.15
NEFA, ueq/ml	0.23	0.04	0.16	0.04	0.17	0.04
Serum values by week, 2, 8;						
Glucose, mg/dl,	52.90	2.80	47.00	2.80	48.90	2.80
	55.00	2.80	56.00	2.80	58.10	2.80
BHB, mg/dl	0.92	0.21	0.86	0.21	1.39	0.21
	1.17	0.21	0.89	0.21	0.91	0.21
NEFA, ueq/ml	0.41	0.05	0.23	0.05	0.28	0.05
	0.07	0.05	0.10	0.05	0.07	0.05

BHB = beta-hydroxy butyrate
Treatment 1 control; Treatment 2, *Bacillus pumilus* at 8G-134 5 x 10^9; Treatment 3, *Bacillus pumilus* 8G-134 at 1 x 10^{10}.
Predicted DMI = (.372*FCM + 0.0968*BWT(kg)^.75)*(1-exp(-0.192*(Week+3.67)))

The DMI differences and production values could suggest that cows on Treatments 2 and 3 could have mobilized more body tissue than the control cows to produce more milk and eat less than expected. However, serum NEFA, glucose, and

BHB suggest these cows were in similar energy status as control cows (Table 10 above). Additionally, body weight and BCS were similar for the *Bacillus* groups relative to the control group (Table 11 below). This suggests they did not mobilize more body tissue to produce the additional milk volume, indicating feed conversion efficiency in cows feed Treatments 2 and 3 regardless of dose.

Table 11. Least square mean body weight (lb) and body condition score by treatment groups. Body condition score is the average score of two observers.

Effect	Treatment	Lactation	Wt, (lb)	sem	BCS	sem
Treatment	1		1343.10	28.49	2.92	0.03
	2		1324.60	27.54	3.13	0.03
	3		1388.02	27.73	3.06	0.03
Lactation		1	1223.31	21.87	3.04	0.02
Lactation		2	1476.62	17.94	3.01	0.02
Interaction Treatment x lactation						
	1	1	1170.78	27.72	3.06	0.05
	2	1	1242.64	25.26	3.17	0.05
	3	1	1208.50	24.00	2.94	0.05
	1	2	1486.02	19.33	2.78	0.04
	2	2	1406.42	19.96	3.10	0.04
	3	2	1547.85	20.79	3.19	0.04

Wt = weight, lbs
BCS = body condition score, scale 1 to 5 by 0.25 points; 1=emaciated, 2=thin, 3= average, 4= fat, 5=obese
Treatment 1 control; Treatment 2, *Bacillus pumilus* at 8G-134 5 x 10^9; Treatment 3, *Bacillus pumilus* 8G-134 at 1 x 10^{10}.

It is understood that the various preferred embodiments are shown and described above to illustrate different possible features described herein and the varying ways in which these features may be combined. Apart from combining the different features of the above embodiments in varying ways, other modifications are also considered to be within the scope described herein. The invention is not intended to be limited to the preferred embodiments described above.

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CLAIMS

1. An isolated *Bacillus pumilus* strain 8G-134 (NRRL B-50174).

2. A combination comprising:

an isolated *Bacillus pumilus* strain 8G-134 (NRRL B-50174); and

monensin.

3. The use of *Bacillus pumilus* strain 8G-134 (NRRL B-50174) for the treatment of a ruminant animal to provide at least one of the following benefits as compared to an untreated ruminant animal: (a) reduced acidosis; (b) quicker recovery from acidosis challenge as measured by pH recovery and lactic acid decline; (c) increased milk production; (d) increased milk fat content; (e) decreased somatic cell count (SCC); (f) improved immunological response and health as evidenced by decreased SCC; and (g) increased efficiency of milk production.

4. The use of claim 3, wherein the strain is used in an amount of at least 5×10^9 CFU / animal / day.

5. The use of claim 3 or claim 4, wherein the ruminant animal is at least 30 days of age.

6. The use of any one of claims 3 to 5, wherein the ruminant animal is a beef animal.

7. The use of claim 6, wherein the ruminant animal is a bull, steer, heifer, cow, or calf.

8. The use of any one of claims 3 to 7, wherein the strain is used in combination with monensin.

9. The use of a *Bacillus pumilus* strain 8G-134 (NRRL B-50174) for the treatment of a dairy cow to provide at least one of the following benefits as compared to an untreated dairy cow: (a) increased daily fat yield and milk fat percent; (b)

lowered log somatic cell count (LogSCC) scores; (c) improved immunological response and health as evidenced by decreased LogSCC; (d) increased efficiency of milk production; and (e) when the dairy cow is a second parity or greater cow, increased milk production.

10. The use of claim 9, wherein the increase in milk production in the treated dairy cow is achieved with substantially no increase in dry matter intake in the dairy cow administered the strain.

11. The use of claims 9 or 10, wherein the strain is used in combination with monensin.

12. A method of making a direct-fed microbial, the method comprising:

(a) growing in a liquid nutrient broth *Bacillus pumilus* strain 8G-134 (NRRL B-50174); and

(b) separating the strain from the liquid nutrient broth to make the direct-fed microbial.

13. The method of claim 12, further comprising freeze drying the strain.

14. The method of any one of claims 11 to 13, further comprising adding monensin to the direct-fed microbial.

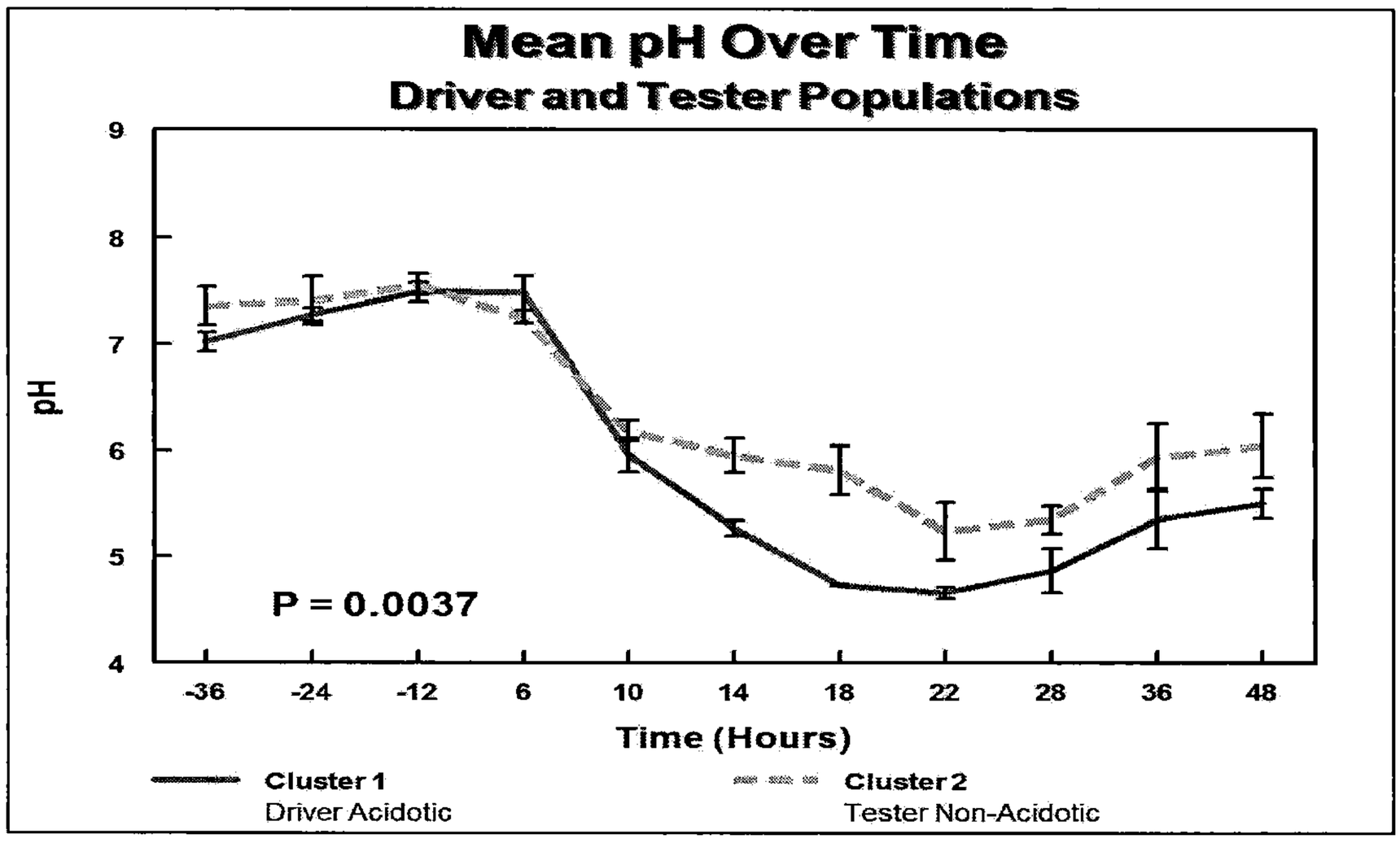

Mean pH Over Time
Driver and Tester Populations
pH
9
8
7
6
5
4
P = 0.0037
-36
-24
-12
6
10
14
18
22
28
36
48
Time (Hours)
Cluster 1
Driver Acidotic
Cluster 2
Tester Non-Acidotic

Fig. 1

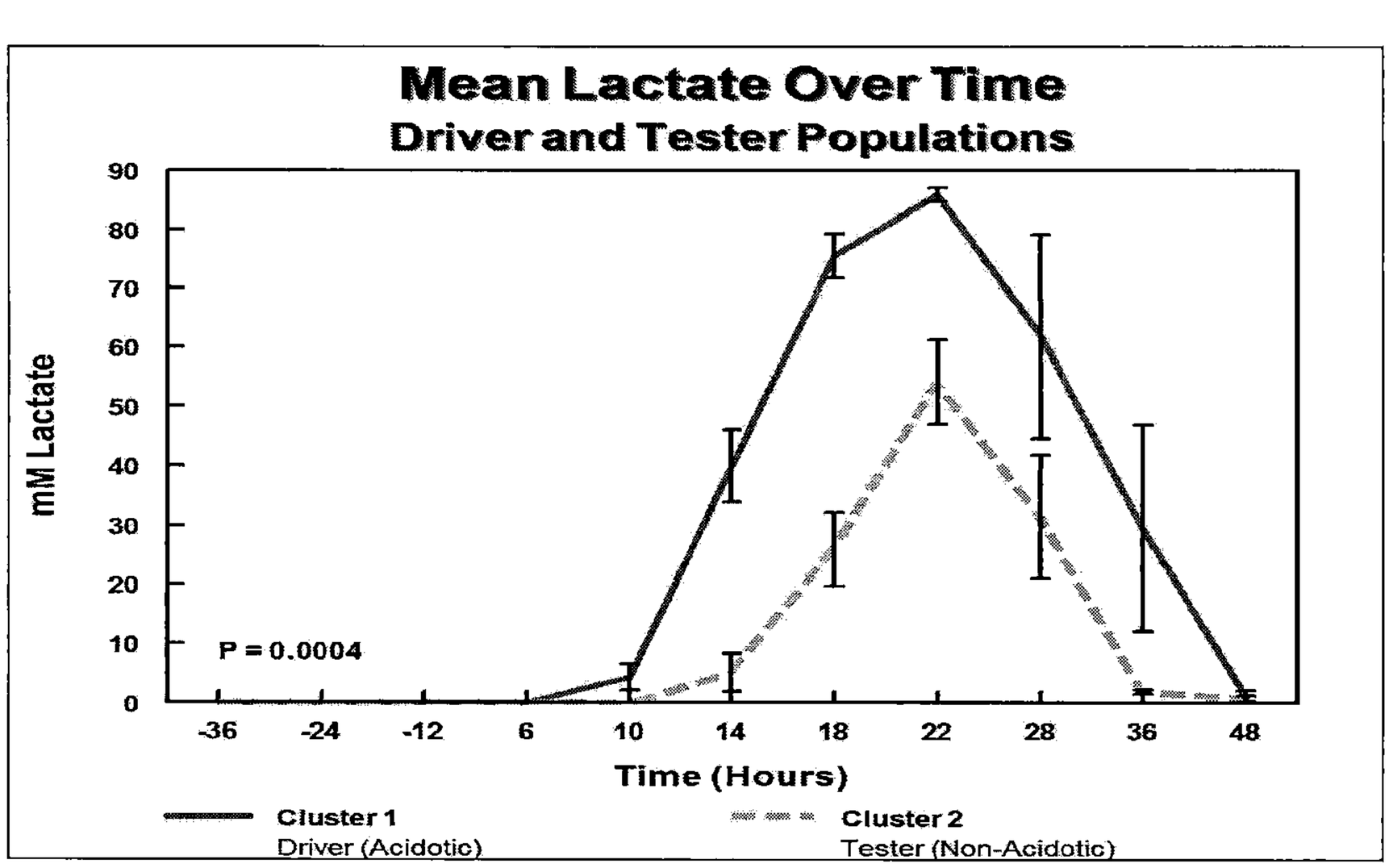

Mean Lactate Over Time
Driver and Tester Populations
mM Lactate
90
80
70
60
50
40
30
20
10
0
P = 0.0004
-36
-24
-12
6
10
14
18
22
28
36
48
Time (Hours)
Cluster 1
Driver (Acidotic)
Cluster 2
Tester (Non-Acidotic)

Fig. 2

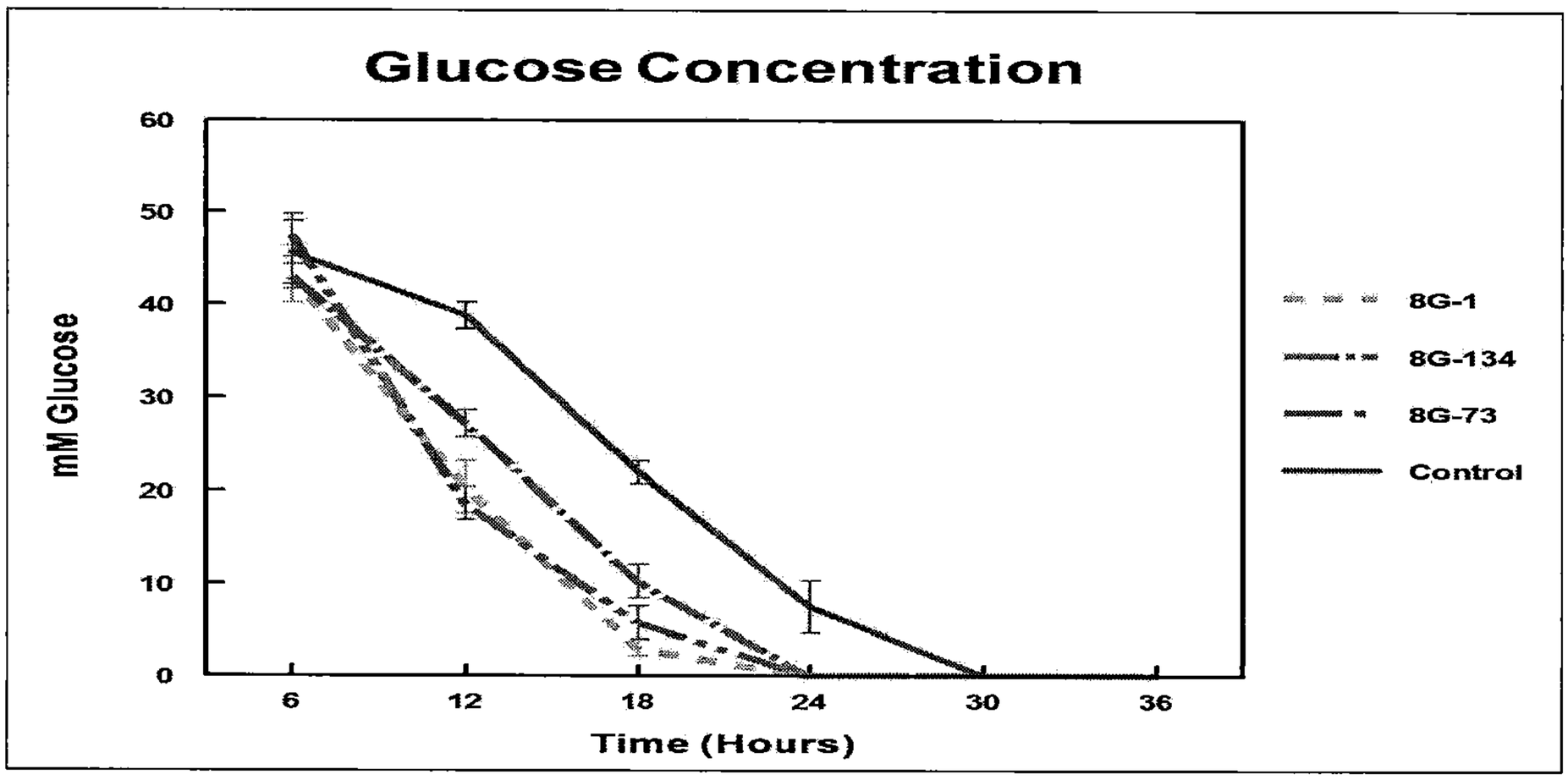

Glucose Concentration
mM Glucose
60
50
40
30
20
10
0
6
12
18
24
30
36
Time (Hours)
8G-1
8G-134
8G-73
Control

Fig. 3

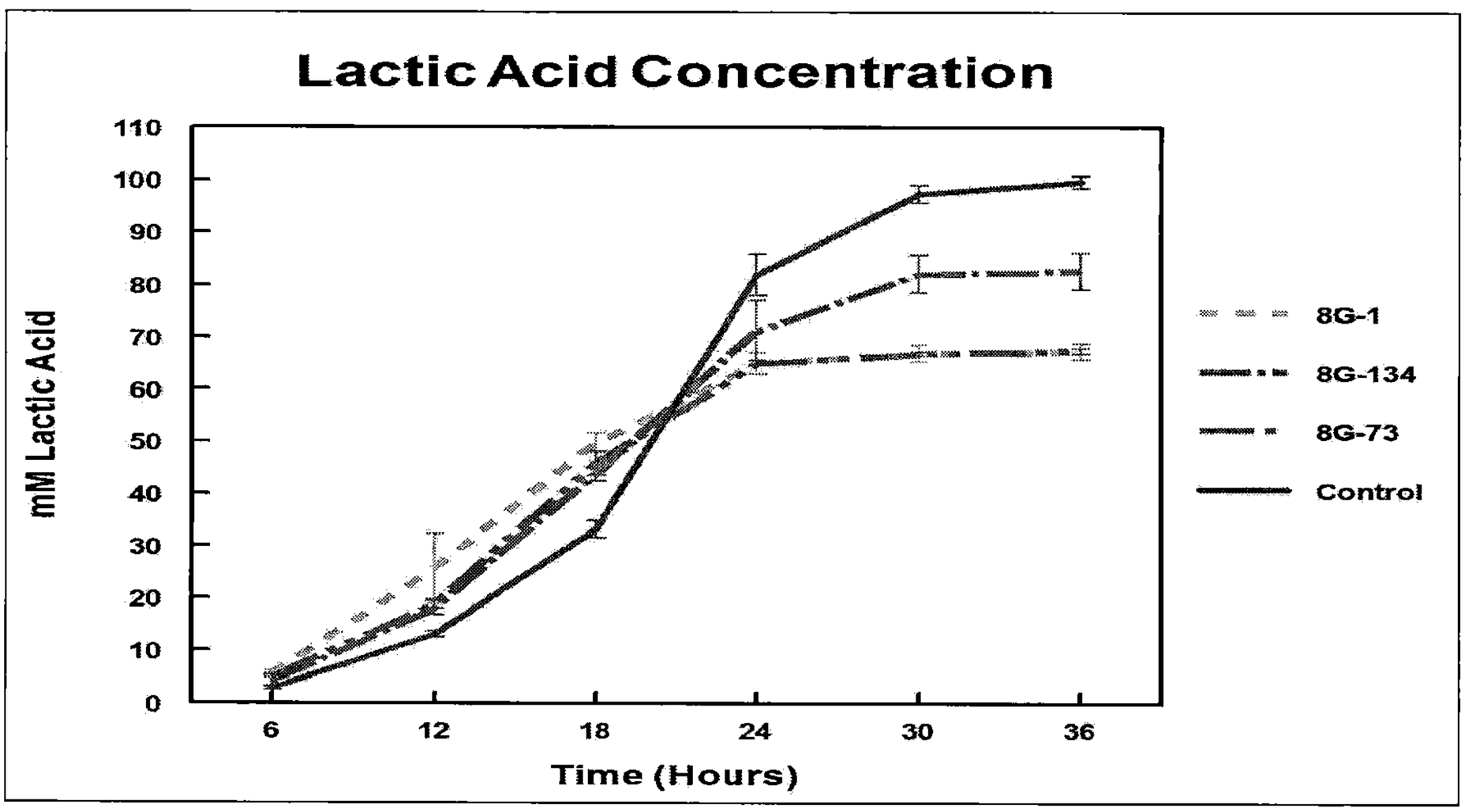

Lactic Acid Concentration
mM Lactic Acid
110
100
90
80
70
60
50
40
30
20
10
0
6
12
18
24
30
36
Time (Hours)
8G-1
8G-134
8G-73
Control

Fig. 4

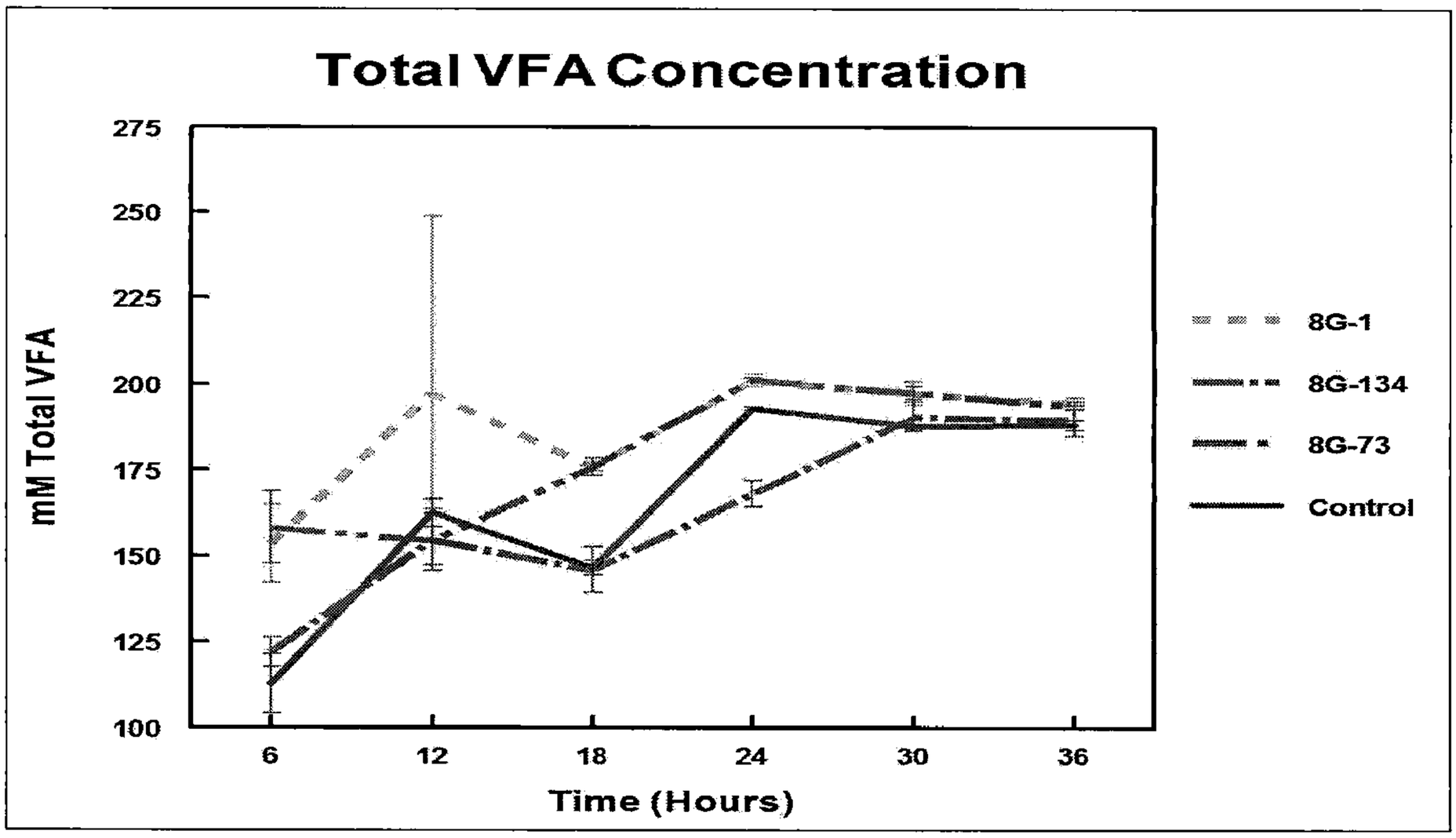

Total VFA Concentration
mM Total VFA
275
250
225
200
175
150
125
100
6
12
18
24
30
36
Time (Hours)
8G-1
8G-134
8G-73
Control

Fig. 5

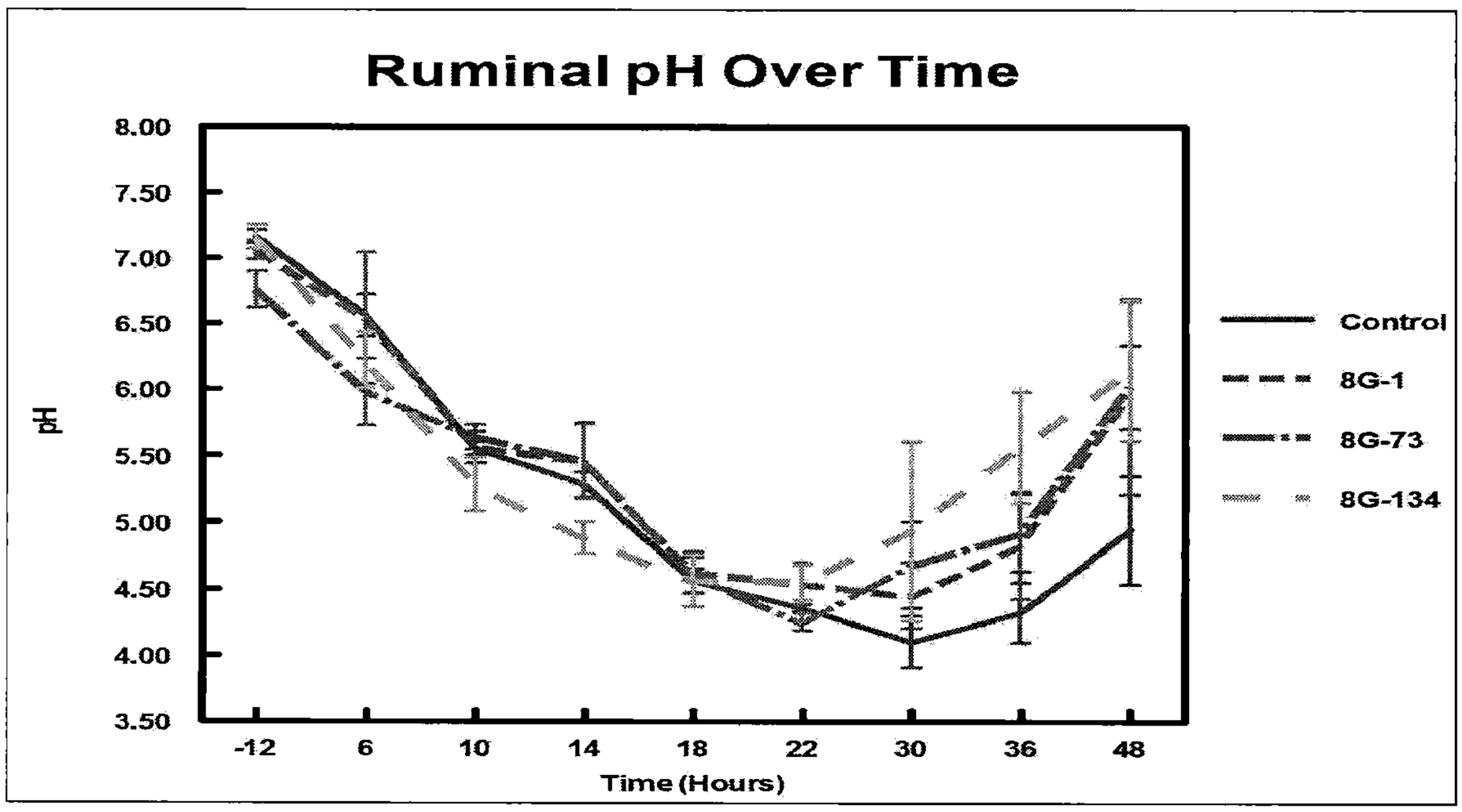

Ruminal pH Over Time
pH
8.00
7.50
7.00
6.50
6.00
5.50
5.00
4.50
4.00
3.50
-12
6
10
14
18
22
30
36
48
Time (Hours)
Control
8G-1
8G-73
8G-134

Fig. 6

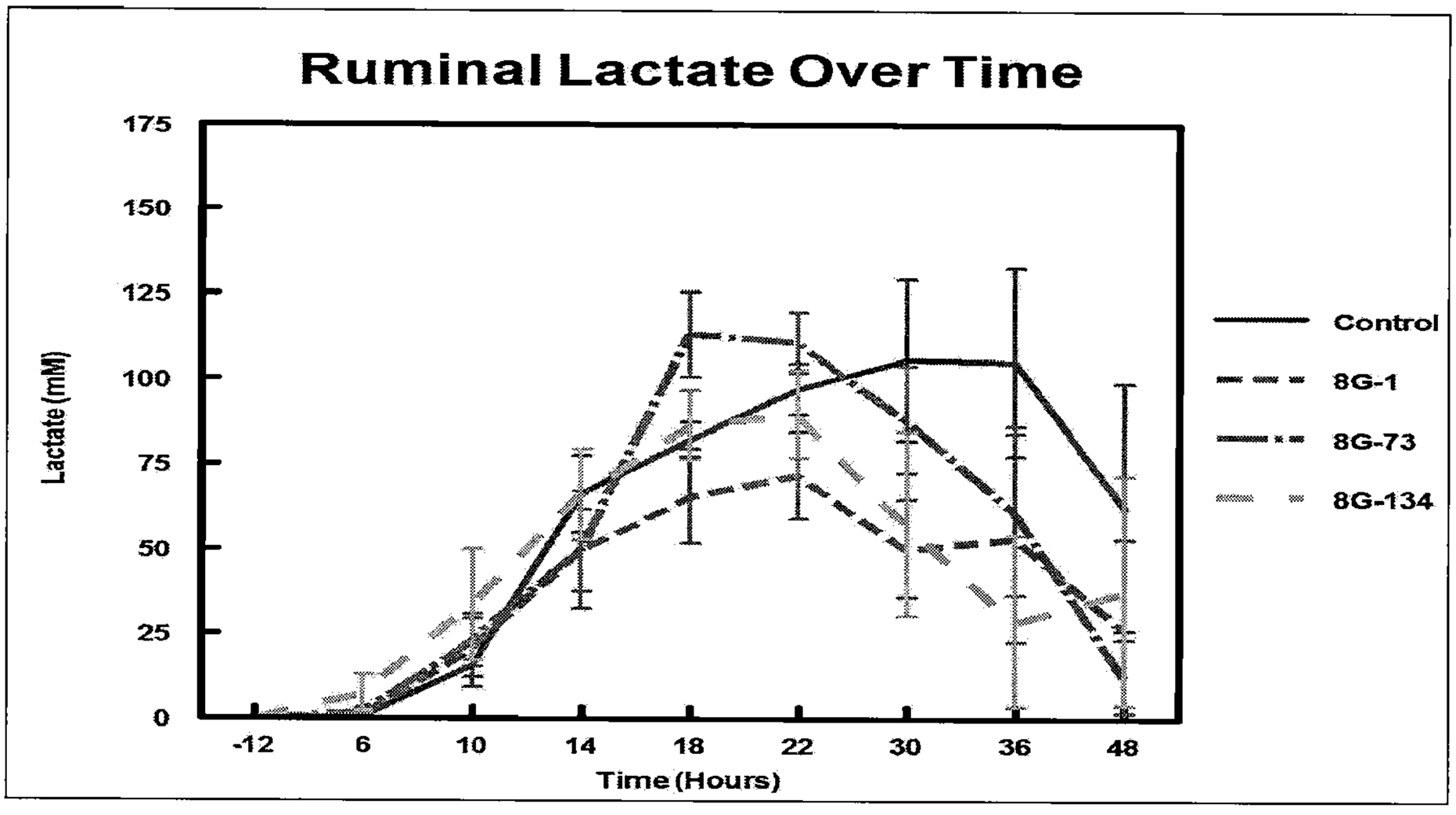

Ruminal Lactate Over Time
Lactate (mM)
175
150
125
100
75
50
25
0
-12
6
10
14
18
22
30
36
48
Time (Hours)
Control
8G-1
8G-73
8G-134

Fig. 7

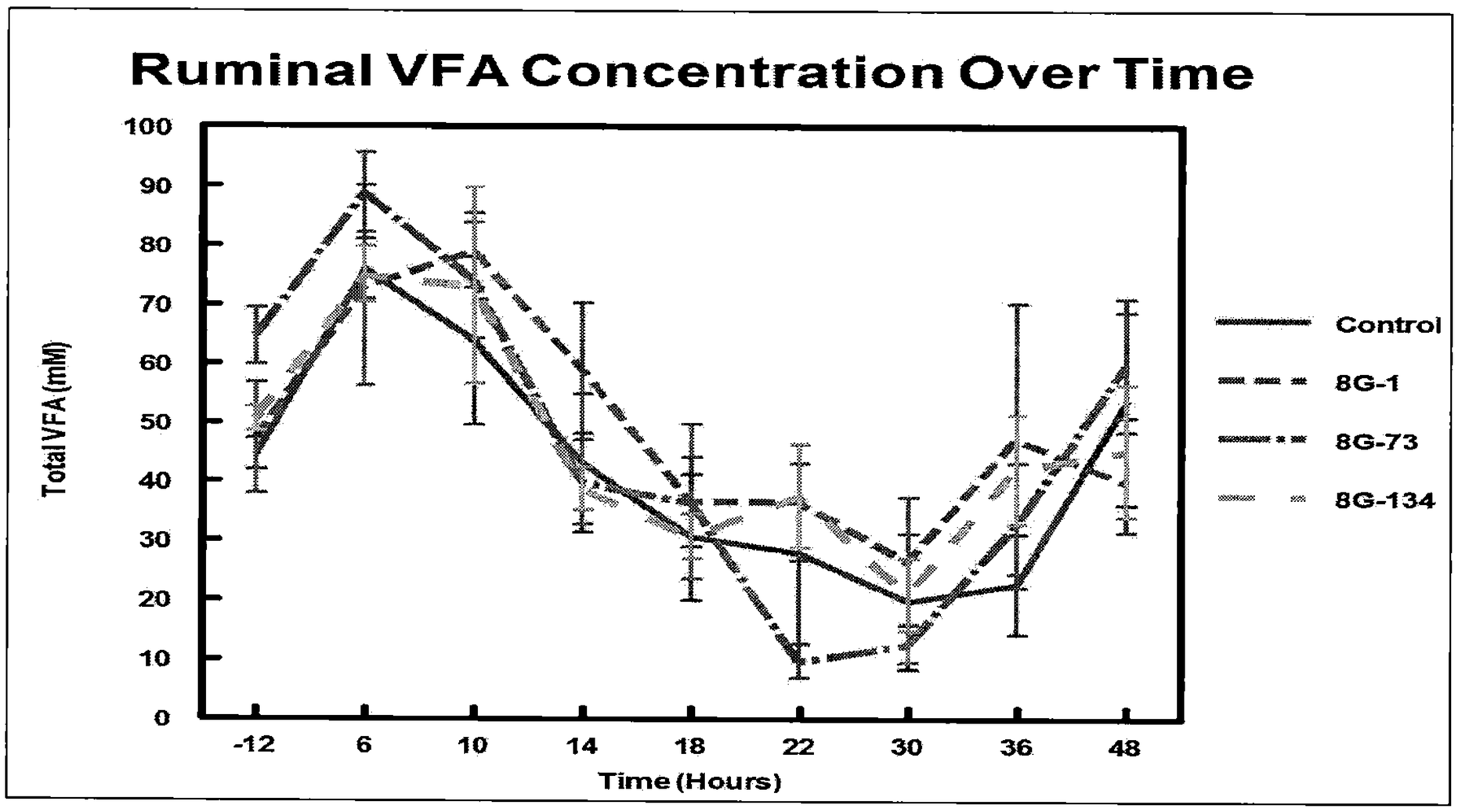

Ruminal VFA Concentration Over Time
Total VFA (mM)
100
90
80
70
60
50
40
30
20
10
0
-12
6
10
14
18
22
30
36
48
Time (Hours)
Control
8G-1
8G-73
8G-134

Fig. 8