McGill
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Department of Animal Science Research Reports

Faculty of Agricultural and Environmental Sciences

2007-08

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Macdonald Campus of McGill University

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Introduction

It is with great pleasure that we invite you to read our 2007-2008 Research Report. Under the watchful eye of our Editor (Professor Arif Mustafa) and with the able help of our Administrative Assistant (Ms. Barbara Stewart), this publication will hopefully provide you with an overview of our department in terms of research, major activities, and future directions.

Since our last report, there have been some changes in the department that may be of interest. After serving on an interim basis, Kevin Wade was appointed Chair of the department for a five-year term, commencing June 1, 2008. We thank Professor Zhao for his previous leadership and look forward to his return to the department in 2008-09 after a well-earned sabbatical leave at the National Taiwan University. Professor Humberto Monardes was also on leave at the Universidade Federal Rural de Pernambuco (Brazil). We hope that their time away will have reignited their ideas on research topics, and look forward to their renewed contributions to the department! Professor Urs Kühnlein retired from the department (December 31, 2007) after a long and distinguished career, which began with his being named to the Shaver-NSERC Industry Chair in Poultry Biotechnology Science in 1985. Over the next twenty years he made significant contributions to the identification of quantitative-trait loci in poultry and was, in 2000, the principal investigator of a major CFI award for the construction of The Donald McQueen Shaver Poultry Complex at the Macdonald Campus Farm. We were delighted that the University saw fit to accept our recommendation that he be named a Professor Emeritus, and he was so recognized at the 2008 Spring Convocation.

Professor Martin Chénier joined us a new Assistant Professor in the areas of Food Safety (this is a joint position between the departments of Animal Science and Food Science). We are extremely excited about Martin's arrival (February 1, 2008) and have great expectations for this joint initiative, especially at a time when food safety is at the forefront of many consumer issues. At the time of writing, a second new Assistant Professor – Raj Duggavathi – has just joined us in the area of female reproductive physiology, using both swine and mice as research models. Our continued efforts to fill positions in areas that are at the front of both consumer concerns and cutting-edge technology should provide the department with a solid footing for the future.

One of the department's major news events came in December 2007 with the "delivery" of Canada's first cloned piglets by Professor Vilceu Bordignon's research group. This event was the focus of local, national and international media. Apart from the amazing technological breakthrough, the expected fallout (in terms of animal models and human medical applications) is almost infinite. This represents a major initiative for our department in terms of research collaboration that extends far beyond the boundaries of the Macdonald Campus. We congratulate Bordignon and his team for their accomplishments to date and wish them much future success in this area.

The following research reports represent, not only a window into the work being done by our staff and their students: they also represent the thrust of future areas in our department and the subsequent training of the next generation of researchers, many of whom have written the actual reports you are about to read. Additional copies of this report are available upon request or on our website (www.mcgill.ca/animal) where additional information may also be found. We would be pleased to have the press and others make use of the material contained herein, although acknowledgements of the source would be appreciated.

Kevin M. Wade
Associate Professor and Chair

September 2008

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Changes in body condition scores before and after calving in Holstein cows

J. Moro-Méndez, R.I. Cue and H. Monardes

ABSTRACT

The objectives of this study were to characterize body condition score (BCS) on Holstein cows under commercial conditions before and after dry off, and during the transition period, and to determine the relative importance of some environmental factors on BCS during those periods. BCS records recorded by farmers in Quebec were used to analyze BCS around dry off and around the transition period. Two models were fitted with the following fixed effects: Herd-Year-Season of calving, calendar month of BCS recording, age (either at dry off or at calving of the lactation being transitioned into), days (either, dry days, or transition days), and level of milk production. Days dry and transition days were highly significant in all parities under study. Body condition score increased after dry off, and decreased after calving. Cows with lower and higher level of production in the previous lactation showed lower BCS.

Keywords: Body condition score, Holstein cows

INTRODUCTION

Body condition score (BCS) of dairy cows is an important trait extensively studied during the lactation (Coffey et al. 2002; Pryce et al. 2000), however, BCS during the dry period has been given less attention. The daily output of milk at the beginning of the lactation exerts additional pressure to mobilize body reserves thus creating negative energy balance (NEB) and a concomitant loss of BCS. Studies in experimental herds have determined the dynamics of BCS during the transition period, and suggest that losses of BCS can be up to 1 BCS from 2 weeks before calving to 3 weeks after calving (Contreras et al. 2004). Thus, the transition period seems to be more critical for the future welfare of the cow than the dry period. Negative energy balance (NEB) is a factor that affect profitability of dairy cows (Collard et al. 2000). Because BCS empirically appraises NEB, the late has an important role in signaling future performance of cows. Body condition scores tend to decrease from the calving day towards the first 4-12 weeks postpartum then increase again for the rest of the lactation as NEB is diminishing; by the end of the lactation, when there is positive energy balance, BCS get its highest level. The dry period is characterized by cessation of milk yield, and renewal, repair and replacement of the mammary epithelial cells (Capuco et al. 1997) in preparation for the following calving and lactation. The objectives of this study were to characterize BCS on Holstein cows under commercial conditions before and after dry off, and during the transition period, and to determine the relative importance of some environmental factors on BCS during those periods.

MATERIALS AND METHODS

The data was provided by the Québec milk recording (Valacta), and consisted of BCS recorded by farmers and supervisors (using a 5-point scale, 1=thin, 5=obese) (Edmonson et al., 1989). There were available 354,958 records of Holstein cows (from first to fifth parities). Body condition scores

were recorded throughout the lactation. Every record had information regarding the identity of the cow, parity, herd, dates of calving, BCS recording, and dry off. For the analysis of BCS before and after dry off, datasets for parities 1 to 3 were obtained from the original database by keeping only records belonging to cows with date of drying-off; for each record the number of days from dry-off date was calculated as the difference in days from the date of BCS recording to the dry-off date. Only records from 60 days before dry to 60 days after dry, inclusive were kept. Approximately seventy-three percent of the animals (in each parity) had only one record of BCS; for the rest of the cows (with two or more BCS per lactation) one record of BCS was kept at random. There were 11784, 10778 and 7840 records of first, second, and third parity, respectively. For the analysis of BCS during the transition period datasets of cows with consecutive pairs of lactations were obtained for each transition period (i.e. from first to second, from second to third and from third to fourth lactations). Only records within the range of 90 days before and 21 days after calving were kept. Only one record of BCS per animal per lactation was kept; approximately seventy percent of the animals (in each parity) had only one record of BCS, thus from the rest one record of BCS was kept at random. There were 8321, 6503, and 4313 records of first to second, second to third, and third to fourth parities, respectively. The transition period from fourth to fifth parity was not analyzed due to the low number of records available after edits. After the edits, a classification variable (level of production) was created in order to adjust for the potential of the cow to mobilize energy that directly affects BCS. Level of production for each cow was defined by classifying total milk yield of the completed lactation in one of eight categories class 1 being the 5% of animals with lower production, and class 8 the 5% of animals with higher production. This classification was defined for both datasets (dry period and transition period). Preliminary descriptive analyses of BCS profiles between 150 days and 300 after second, third, and fourth parities were performed with the objective of obtaining an overview of the changes on BCS occurring during the dry and transition periods. The number of records and average BCS were obtained by each day and plotted in Figure 1 (second lactation shown only). The model applied for the analyses of the dry and transition periods was as follows:

$$Y=M+HYS+MONTH+AGE+DAYS+LEVEL+E$$

Where:

μ = mean

HYS = Herd-Year-Season of calving (Season 1 = November to April, season 2 = May to October)

MONTH = Calendar month of BCS recording

AGE = either age at dry off (in the case of analyses of dry period), or age at calving of the lactation being transitioned into (in the case of transition period analyses)

DAYS = either dry days (in the case of the analysis of the dry period), or transition days (in the case of the analysis of the transition period)

LEVEL = level of production

e = residual, ~ N (0, σ^2e).

The analysis of variance were carried out with Proc Mixed of SAS (SAS 2004).

RESULTS AND DISCUSSION

Phenotypic Distribution of BCS

Figure 1 shows the distribution of average BCS (and number of records) by day within the period of 150 days before second calving and 300 days after. It is evident the decrease in BCS just few days before calving and he recovery of BCS from the nadir at 45 days. Similar pattern was observed in parity third and fourth (not shown).

Fig. 1 shows that the frequency of BCS recording drops after the date of dry off, on average, approximately 75 days before calving; in turn, the average BCS increases from the dry off onwards. The frequency of BCS recording suggests that producers are less interested in BCS during the dry period; this period comprises the pre-fresh transition period (21 days before calving). The number of scores during the last 90 days before calving was lower than during the 21 days after calving (the post-calving transition period). This may reflect the interest of producers in monitoring BCS immediately after calving, but little attention to BCS during the last part of the dry period. Average BCS was higher during the pre-fresh transition period than during the post-calving transition period; this is

expected given that body condition tends to rapidly fall as the high demands for milk yield increase during the first days of the lactation (Banos et al. 2005, Berry et al. 2006). After calving, BCS rapidly fell from around 3.3 to 2.6 at day 45 post partum. This is different from other studies that found increased BCS during the prepartum period (Contreras et al. 2004, Gulay et al. 2003). The decrease of BCS during the first weeks postpartum in our study is similar to report from other dairy populations (Berry et al., 2006, Contreras et al., 2004, Gulay et al. 2003, Kadarmideen and Wegmann 2003).

Table 1 shows the results from the analysis of variance of BCS 60 days before and after dry off date, and Table 2 shows the results from the analyses of the period between 90 days before and 30 days after calving.

Fig. 2 shows the pattern of BCS in the dry period. In a previous analysis (Moro-Méndez et al. 2008) we have reported the curve of BCS along the lactation for Holstein and Ayrshire cows (from first to fifth lactation). Here the focus is on the changes of BCS during the far- and close-dry periods. *There is a trend to increase BCS from -60 days to dry off; and the increase continues after dry off. The high variation on BCS observed around after 30 days from dry off may be result of the low number of records available to obtain the least squares means estimates.* Cows tend to gain weight during the dry period. Aenas et al (2003) found a 72 (+/- 20) kg body weight loss between the week before calving and the week after it.

Figure 3 shows the pattern of BCS during the transition period. In all parities under study, the transition period is characterized by a decline in average BCS. In this study, days in transition affected significantly BCS; in general, cows had higher BCS in the 21 days before parturition; after parturition, it is observed a decline in the average BCS. It is expected that cows that start a lactation exhibit a reduced BCS because increasing daily output of milk requires large amounts of metabolizable energy, which is traduced in loss of weight and in particular deterioration of BCS. Douglas et al (2004) reported a trend to increase BW between 60 days before calving and calving, however BCS tended to maintain a stable level (3.0 to 3.2 BCS between 60 and 45 days before calving), and falling from 3.2 to 2.5 BCS (from 2 weeks to the date of calving). In our study BCS tended to be stable from 60 days before calving to calving date; after calving, BCS declines sharply although the final days of the dry period are characterized by larger variation in BCS, which is mainly caused by the lower number of records in that particular stage of the productive cycle of the cows under study..

CONCLUSIONS

The main environmental effecting BCS during the dry and transition periods were HYS, level of production and days (dry or in transition, respectively). Body condition score showed changes during the dry period. BCS showed an increase after dry off. During the transition period, BCS was stable before calving and declined abruptly after calving.

Table 1. Effects of Herd-Year-Season of calving (HYS), month of BCS recording (MONTH), Age at calving (AGE), level of production (LEVEL), and days from dry off (DAYS DRY)

	Effects				
Parity	HYS	MONTH	AGE	LEVEL	DAYS DRY
First	2.28 ***	0.93	9.64***	9.81***	2.58***
Second	1.99***	1.94*	3.15***	23.70***	2.50***
Third	1.80***	2.63*	2.90*	19.89***	2.04***

Table 2. Effects of Herd-Year-Season of calving (HYS), month of BCS recording (MONTH), Age at calving (AGE), level of production in the previous lactation (LEVEL), and days in transition (DAYS TRANSITION) on BCS

Transition period between parities	Effects				
	HYS	MONTH	AGE	LEVEL	DAYS TRANSITION
First-second	2.34***	1.97*	3.44***	10.63***	5.50***
Second-third	1.96***	2.13*	1.87*	6.21***	6.72***
Third-fourth	1.80***	0.44	1.03	2.50*	4.18***

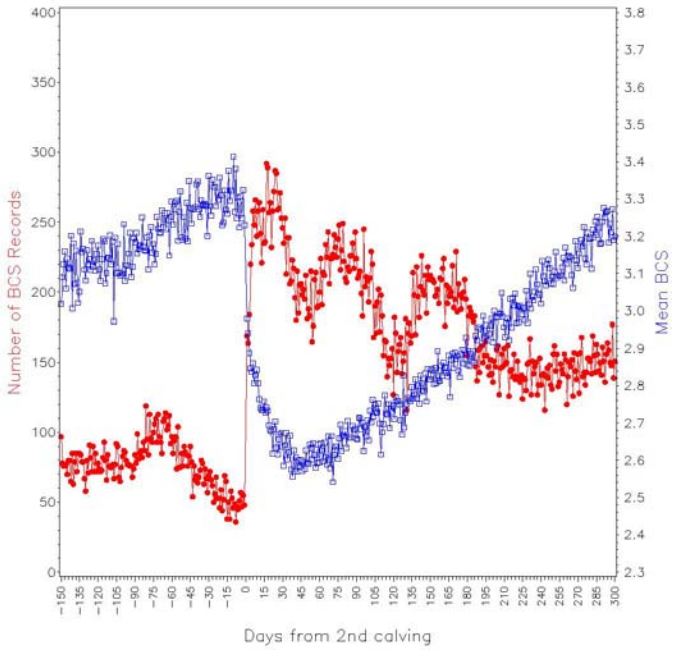

Figure 1. Distribution of BCS 150 days before and 300 days after second calving in Holstein cows.
■= average BCS, • = Number of records.

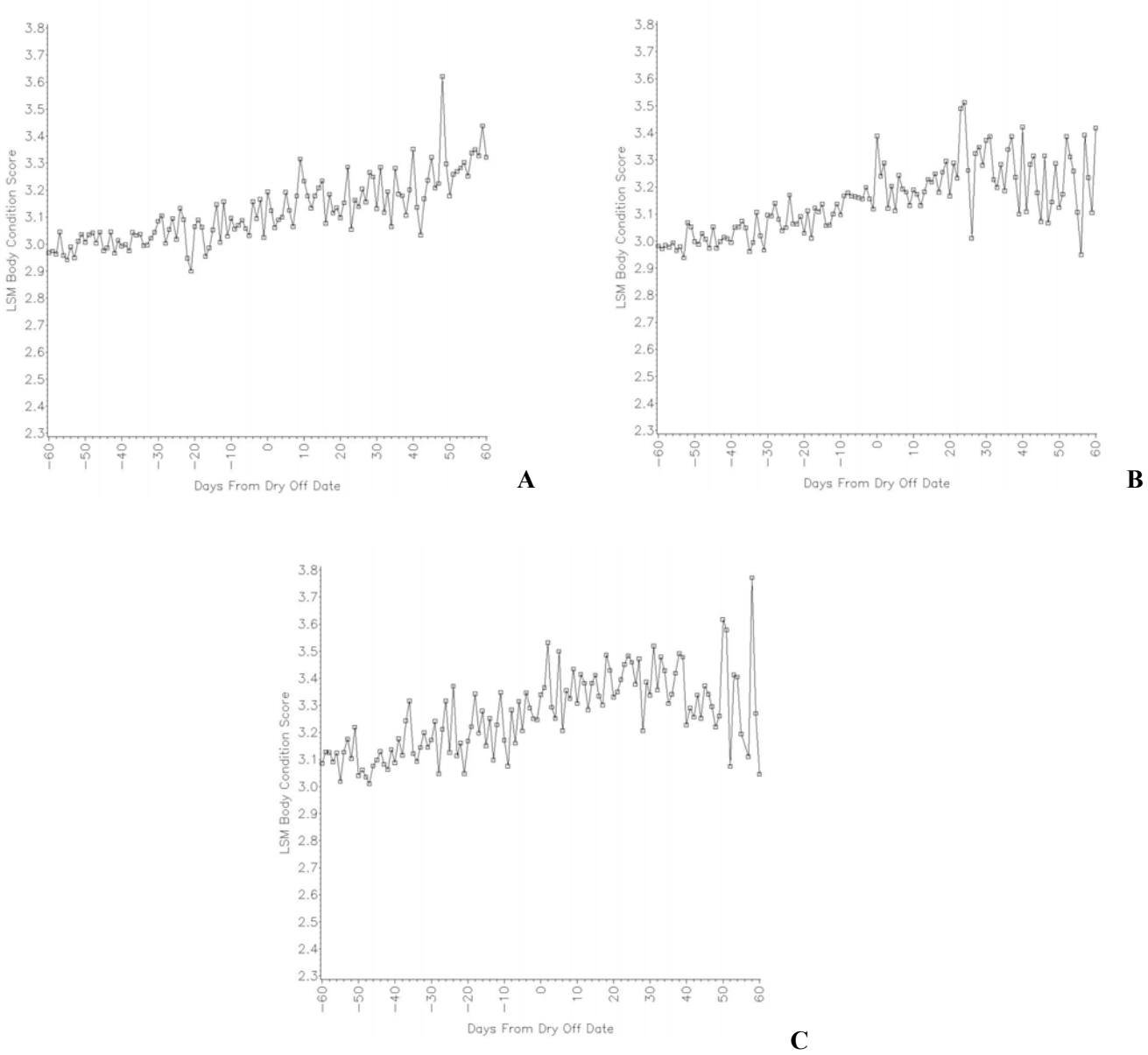

Figure 2. LSM for BCS 60 days before and after dry off: first (A), second (B) and third (C) lactation Holstein cows.

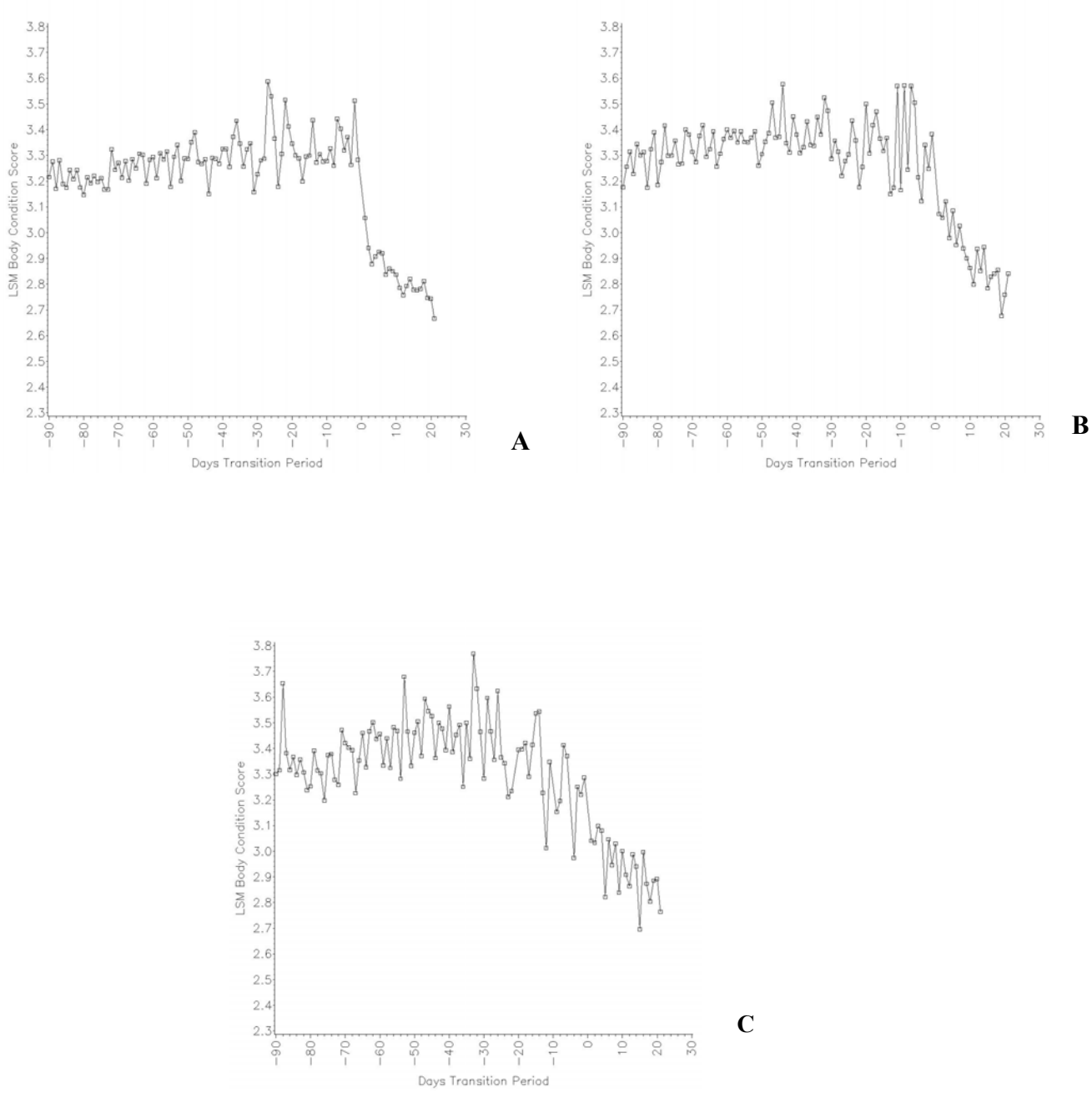

Figure 3. LSM for BCS 90 days before and 30 days after calving: first (A), second (B) and third (C) lactation Holstein cows.

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Combining Veterinary and Milk Recording Data in Québec:

Genetic Variation of Health Traits

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ABSTRACT

The objectives of this study were to investigate the feasibility of combining veterinary health data and milk recording and pedigree information, and to estimate the magnitude of genetic variability of some of the more important health traits in lactating Holstein and Ayrshire cattle. Health data covered the period from 1998 to 2005. Approximately 60% of the health records were successfully matched with milk recording and pedigree information, via matching by herd, cow identification and date of calving. There were 208,552 health events on 73,533 cows. Heritability estimates for the seven traits examined (digestive problems, retained placenta, cystic ovaries, displaced abomasum, mastitis, locomotive problems, and metabolic problems) were low to moderate. This study shows that it is possible to combine health event data and milk recording data, but that correct permanent animal identification and correct recording of date of calving is critical.

INTRODUCTION

The management of dairy cattle has reached such a degree of intensity that many aspects, such as nutrition, genetics, and welfare, must smoothly interplay for producers to obtain sustained profits. In dairy cattle, high milk yield is one of the multiple risk factors for the appearance of diseases (Fleischer et al. 2001). Milk-recording agencies provide valuable support for dairy farmers; however, health events collected by veterinary professionals are not routinely included within such schemes. Here we describe an exploratory study of the genetic variation of diseases affecting cattle, which might provide a basis for a more profitable dairy industry. In Quebec, Valacta provides milk-recording services to approximately 80% of the dairy producers. From the information collected, it has been found that, in 2005, reproductive problems and mastitis were the two main involuntary reasons of culling (31 and 28%, respectively), followed by feet and leg problems (14%) (Valacta 2006). Deaths due to feet and leg problems were almost 11% of mortality cases, followed by 7% due to milk fever, 6% due to mastitis, and 19% due to other diseases. Controlling diseases gives opportunity to increase lifetime production by avoiding involuntary culling, and increasing fertility. Appropriate monitoring of diseases requires access to accurate diagnoses of individual clinical cases. In Québec, DS@HR (Dossier Sante Animal/Animal Health Record) provides veterinary services to a proportion of dairy farmers and maintains a database with health events. The objectives of this study were to evaluate the feasibility of merging veterinary health data and lactation records, estimate incidence of diseases affecting dairy cows in Québec, and study the genetic variation of health traits in Holstein cattle under commercial conditions in Québec.

MATERIAL AND METHODS

Merging Records from DS@HR and Valacta Databases

A file (DS@HR file) containing 296,170 cow-calving dates from 123,867 cows enrolled in herds that received veterinary services was provided by DS@HR; cows were included in this file because they were enrolled with the milk recording service provided by Valacta (formerly Programme d'analyse des troupeaux laitiers du Québec). The information provided in this file was: identity of the cow and herd number on Valacta's milk recording system, identity of the cow and herd number within DS@HR system, and calving date. The initial step was to merge the records from the DS@HR file with records from milk recording. Two data merges were performed, one for Holstein records and one for Ayrshire records. In this initial step, the pedigree information related to the records from DS@HR were retrieved from the CDN pedigree file. The merge processes for Holsteins and Ayrshires produced 155,740 and 8,130 herd-cow-date of calving records from 70,168 and 3,365 cows, respectively, common between DS@HR and Valacta/CDN files. After the merge, a file with matched cows (including Holstein and Ayrshire) was sent to and used by DS@HR in order to retrieve from its database the health events corresponding to any parity belonging to these matched cows. The file contained 10,797 Ayrshire records and 197,755 Holstein records.

Binary Traits for Health Events

The health events file was used to create binary traits for each health event-parity combination as follows: a binary trait was defined as 1 when the cow had the disease during the lactation, otherwise the binary trait was defined as 0. Binary traits were created for milk fever, digestive problems, retained placenta, cystic ovaries, displaced abomasum, reproductive problems, mastitis (first case), mastitis (second case), locomotive problems, and metabolic problems. Lactational incidence rates (LIR) were calculated from the clinical incidence and the number of lactations at risk for each herd, as per Kelton et al. 1998.

Additionally, the following edits were applied to records of first to fifth parities: first parity (cows calving from 21 to 37 months of age inclusive), second parity (from 34 to 52 months of age), third parity (from 46 to 66 months of age), fourth parity (from 58 to 80 months of age), fifth parity (from 71 to 92 months of age); these edits deleted 4,368 records. These edits left 166,792 records from parity 1 to 5. Then, for each parity-binary trait combination only records from sires with at least 5 daughters in at least 2 herds were kept. In addition, we required herd-year-season of calving (HYS) to have at least 3 cows; two HYS were defined per year, one from September to February, and another from March to August.

Statistical Analyses

A model for each parity-binary trait combination was fitted including the following fixed effects: year of calving and age at calving; herd and sire were included as random effects. Heritability for each trait was estimated as $4*\sigma^2_{sire}/(\sigma^2_{sire} + \sigma^2_{herd} + \sigma^2_{residual})$. Each analysis was performed with the procedure Glimmix of SAS specifying a binomial distribution for the dependant variable, and a logit link function. The procedure Corr of SAS was used to estimate genetic correlations between sires' solutions obtained from each analysis as described above and sires' EBVs obtained from the CDN for several production and conformation traits.

RESULTS AND DISCUSSION

Lactational Incidence Rates

The file with health events retrieved after the merge process consisted of 208,552 lactation records, 757 herds, and 73,532 cows, daughters of 4,451 sires. Tables 1 to 3 show the herd LIR for cows in first, second, and third parity, respectively. The highest LIR was for mastitis (first case) and the lowest for digestive and metabolic problems. Because the database used in this study was obtained from commercial herds it is expected that there may be some bias due to selection, because only cows that exhibit a certain level of production and health status are likely to provide multiple records through the years (Uribe et al. 1995). In addition, some bias may be present due to the different emphasis farmers may apply when recording health events (i.e. some may put more attention to specific groups of diseases). In the case of this study the participation of veterinarians in the recording and validation of health events may help to ameliorate such situation. In our study, digestive problems, displaced abomasum, metabolic problems and second case of mastitis had lower incidences than milk fever,

retained placenta, cystic ovaries, reproductive problems, first case of mastitis, and locomotive problems. In another study (Reinsch et al. 1997), clinical mastitis, retained placenta, metritis and various ovulatory diseases had higher incidence, in contrast, milk fever was found to be one of the diseases with a lower incidence. In our study, mastitis was the disease with higher incidence (9.94 in first parity to 15.29 in fifth parity), which is lower than the incidence of 17.2% reported by McLaren et al (2006) for dairy herds in Ontario. Repeated cases of diseases will increase the relative risk of culling for cows; in this sense, the incidences of cystic ovaries and reproductive problems (10 and 9.8% respectively) are consistent with a trend observed in the province of Québec, reproductive problems and mastitis being the main cause of involuntary culling (Valacta 2006).

For all the diseases under study, incidence increased with parity; which is a trend previously discussed by Reinsch et al (1997). In our study, some diseases showed a larger rate of increase than others. For instance, metabolic problems moderately increased from 1.25% in first parity to 2.44% in fifth parity; mastitis incidence (first case) had an incidence of 9.9% in first parity and increased to 15.3% in fifth parity. Other diseases showed larger increases, for instance, milk fever incidence in first parity was 0.10% and increased to 10.7% in fifth parity. This finding is similar to the observation by Reinsch et al (1997), about milk fever being the disease more affected by age than any other disease; they found an average for at least one occurrence of milk fever (within a complete calving interval) of 0.03% in first lactation and 14.4% in fifth parity and 18.3 in sixth and greater parities. In general, as parity increases cows have higher risk of being affected by diseases. It is possible that as parity increases and selection for higher production is applied, the proportion of high producing cows within the population increases which inherently are more likely to be affected by milk fever. Our finding is consistent with other evidence that indicates higher incidence of milk fever in fourth and higher parities compared to earlier parities (Abdel-Azim et al. 2005).

The joint LIR of first case of mastitis of first-second and third or higher parity cows in this study (25.4) was higher than the 21.8 LIR of clinical mastitis reported by (McLaren et al. 2006); in that study when mastitis persisted in the same quarter for subsequent milkings it was considered the same case. In addition, McLaren et al. (2006), reported LIR for retained placenta, displaced abomasum, and milk fever of 9.1, 4.1, and 4.2%, respectively. These incidences are similar to the LIR estimated in this study.

Heritabilities and Genetic Correlations

Table 4 shows the number of cows, herds, LIR, and h^2 for several diseases under study. Heritabilities ranged from low values (0.05 for locomotive problems and metabolic problems, both in first parity) to moderate values (0.27 for retained placenta in first parity). Although the range of h^2 of diseases in dairy cattle may be from low to relatively high (0 to 0.39), evidence shows that low to moderate values are more frequently reported (Uribe et al. 1995, Van Dorp et al. 1998, Zwald et al. 2004).

In this study, mastitis had an h^2 of 0.09 and 0.19 in first and second parity, respectively. The estimate for first lactation cows is similar to estimates reported in other studies. Heringstad et al (2005) reported h^2 for clinical mastitis of 0.08, 0.07 and 0.07 in first, second and third parities, respectively. Harder et al (2006) reported h^2 for udder diseases within the range of 0.05 to 0.08, and Zwald et al (2004) reported 0.10 in first lactation cows and 0.09 in multiple lactation analysis. In another Canadian study h^2 of clinical mastitis was 0.15 (Uribe et al., 1995), which was similar to the estimate of 0.16 reported by Abdel-Azim et al (2005). All studies referred used threshold models.

Our estimate of h^2 for locomotive problems was 0.05. (Uribe et al., 1995) reported h^2 for culling due to leg problems of 0.15. Another Canadian study (Fatehi et al, 2003) estimated h^2 for several feet and leg traits classified by stall type, flooring type and frequency of trimming, and the ranges were from 0.03 for claw uniformity to 0.29 for bone quality. Other studies (Harder et al., 2006, Huang and Shanks, 1995) estimated h^2 for several foot disorders within a range from 0.02 to 0.13. Another study estimated h^2 for several claw disorders using threshold models, and the range was from 0.07 for digital dermatitis to 0.12 for digital hyperplasia (Koenig et al., 2005)

In our study reproductive diseases showed higher h^2 (0.13 for cystic ovaries in second parity to 0.27 for retained placenta in first parity). (Uribe et al., 1995) reported h^2 of ovarian cysts of 0.13 and 0.08 for first and over all lactations, respectively. (Zwald et al., 2004) reported h^2 of ovarian cysts of 0.08 and 0.05 for first parity and all lactation, respectively, a lower estimate, 0.03 was estimated by (Abdel-Azim et al., 2005). Harder et el (2006) categorized ovarian disorders, endometritis, retained placenta and other fertility disorders in a group called fertility diseases; they found heritabilities between 0.04 and 0.07 for several different threshold models.

In general, our estimates of heritability and others reported in other studies reveal a genetic component for several important diseases in dairy cattle. This suggests that genetic improvement of cattle for several health traits is attainable.

We estimated genetic correlations (data not shown) between the sires' solutions for each health trait and production traits (EBV generated by the Canadian Dairy Network for milk, fat, protein, scs, fertility, conformation traits). Most of the correlations between health traits and production traits were negative. The relationships between disease traits and other productive and conformation traits suggest that genetic selection may have an impact on the incidence of the diseases under study.

CONCLUSIONS

This study showed the feasibility of merging veterinary-recorded health events and lactation records from a milk recording system in Québec. Incidences of diseases were within the ranges reported in the literature. Genetic variation for disease traits is present in the population under study, and several estimates of h^2 were of moderate to low values.

Table 1. Lactational incidence rates (%) per herd after applying validations for records of first parity

Disease	Mean	Median	SD	Minimum	Maximum
Milk fever	0.10	0	0.53	0	7.02
Digestive problems	1.55	0	2.84	0	22.92
Retained placenta	3.49	2.50	5.29	0	100.00
Cystic ovaries	6.55	5.45	6.28	0	50.00
Displaced abomasum	1.63	0.61	2.30	0	15.15
Reproductive problems	8.90	6.52	9.77	0	100.00
Mastitis, first case	9.94	7.69	10.19	0	100.00
Mastitis, second case	1.73	0	3.14	0	24.24
Locomotive problems	4.72	2.13	8.50	0	69.23
Metabolic problems	1.25	0	1.78	0	9.28

Table 2. Lactational incidence rates (%) per herd after applying validations for records of second parity

Disease	Mean	Median	SD	Minimum	Maximum
Milk fever	1.02	0	2.52	0	33.33
Digestive problems	1.84	0	3.58	0	33.33
Retained placenta	5.02	4.04	5.31	0	50.00
Cystic ovaries	9.88	8.33	8.33	0	40.82
Displaced abomasum	1.91	0.43	3.18	0	33.33
Reproductive problems	9.06	6.87	9.98	0	100.00
Mastitis, first case	12.23	10.00	11.40	0	100.00
Mastitis, second case	2.73	0.70	4.55	0	27.91
Locomotive problems	5.31	2.74	8.39	0	72.41
Metabolic problems	1.77	0	6.43	0	100.00

Table 3. Lactational incidence rates (%) per herd after applying validations for records of third parity

Disease	Mean	Median	SD	Minimum	Maximum
Milk fever	2.94	1.59	3.95	0	22.22
Digestive problems	2.13	0	5.02	0	100.00
Retained placenta	5.72	4.61	6.33	0	50.00
Cystic ovaries	11.41	9.52	10.05	0	100.00
Displaced abomasum	3.11	1.85	4.58	0	50.00
Reproductive problems	9.35	6.86	10.92	0	100.00
Mastitis, first case	13.00	11.03	11.25	0	56.52
Mastitis, second case	3.01	0	5.18	0	39.13
Locomotive problems	6.77	3.15	11.57	0	100.00
Metabolic problems	2.24	1.06	4.08	0	50.00

Table 4. Number of cows, herds, lactational incidence rates (%) and heritabilities (SE) of some diseases in Holsteins in Québec

Disease		Cows	Herds	LIR	$h^2 \pm SE$
Digestive problems,	first parity	12,790	136	1.77	0.15
Retained placenta,	first parity	12,790	136	4.03	0.27
Cystic ovaries	first parity	17,181	193	7.76	0.15
	second parity	12,201	149	12.2	0.13
Mastitis	first parity	6,218	80	11.3	0.09
	second parity			14.3	0.19
Locomotive problems	first parity	17,181	193	5.6	0.05
Metabolic problems	first parity	17,181	193	10.27	0.05

SE = standard error

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Milk composition studies in transgenic goats expressing recombinant human butyrylcholinesterase in the mammary gland

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ABSTRACT

The use of the mammary gland of transgenic goats as a bioreactor is a well established platform for the efficient production of recombinant proteins. However, the extraordinary demand placed on the secretory epithelium may result in a compromised mammary physiology. In this study, milk composition was compared between control and transgenic goats expressing high levels (1-5 g L^{-1}) of recombinant human butyrylcholinesterase in the milk. Casein concentration, was significantly reduced in the transgenic compared with the control goats throughout lactation ($P<0.01$). Milk fatty acid composition for transgenic goats, as determined by gas chromatography, was found to have significantly fewer short chain fatty acids ($P<0.01$) and more saturated fatty acids ($P<0.05$) compared to controls, suggesting an overall metabolic stress and/or decreased expression of key enzymes (e.g. fatty acid synthase, acetyl-CoA desaturase). The concentration of Na^+, K^+, and serum albumin, showed substantial changes in transgenic animals as lactation progressed, while in control animals they remained unchanged ($P<0.01$).

Keywords: Cholinesterase, transgenic, tight junction, fatty acid composition, caprine serum albumin, caseins.

INTRODUCTION

The production of high value recombinant proteins in the milk of transgenic farm animals has important applications and has been the subject of numerous reviews in the past few years (Keefer 2004). Briefly, the outstanding protein synthesis capacity of the mammary gland coupled with the ease of collecting milk, and a virtually unlimited ability to scale-up production by simply increasing the herd size, have made the transgenic animal platform a valuable alternative for the production of recombinant proteins of pharmaceutical interest.

Transgene expression, however, may result in new phenotypes with the potential for deviant mammary physiology and milk composition. In a previous study (Baldassarre et al. 2007), we described the lactation parameters of 50 transgenic goats expressing recombinant human butyrylcholinesterase (rBChE). Our findings established that high expression levels of rBChE (range 1-5 g L^{-1}) are produced in these animals at the expense of an impaired lactation performance.

In the current study, our main objectives were to: 1) characterize the fatty acid component of milk triglycerides in transgenic goats, in order to understand whether the disrupted lipid secretion observed in our previous study was associated with changes in lipid composition; 2) determine the levels of total caseins produced by transgenic goats as a potential indicator of competitive restrictions in protein synthesis, that

could result from the expression of recombinant proteins under the regulation of the β-casein promoter; 3) investigate the integrity of epithelial tight junctions (TJ) by measuring the presence of serum albumin and the concentration of Na^+ and K^+ in the milk, as a potential mechanism to explain the shortened lactations and high leukocyte counts previously reported in these transgenic goats expressing rBChE in milk.

MATERIALS AND METHODS

Animals

Five transgenic and 3 nontransgenic (control) goats of the same age and parity provided the 73 milk samples analyzed in the course of this study. They were randomly selected from a herd of 50 transgenic goats produced by artificial insemination with semen from a single transgenic male derived from a selected female founder, as previously described (Baldassarre et al. 2007). Goats were milked twice daily following standard operating procedures.

Casein Concentration

Caseins were analyzed by acid precipitation according to the procedure of the Association of Official Analytical Chemists (AOAC 1999).

Milk fatty acids analysis

Milk fatty acids were determined by gas chromatography. Fat was obtained by mik centrifugation and lipids extracted using the procedure described by Kelly et al. (1998). Methylation of milk fat was conducted by a base-catalyzed procedure. The fatty acid methyl esters (FAME) were quantified using a gas chromatograph and following the procedure described by Zhang et al. (2006).

Determination of Milk Minerals

Mineral content of milk was determined by atomic absorption spectrophotometry following the procedure of the AOAC (1999) with minor modifications.

Determination of Caprine Serum Albumin (cSA)

The concentration of serum albumin was determined by the colorimetric bromcresol green dye (BCG) assay, following the procedure of Guzman et al. (1986). The cSA concentration was determined in comparison with a standard cSA curve prepared using purified lyophilized cSA (Sigma Chemical Co., St. Louis, MO, USA).

Statistical analysis

In all cases, data from transgenic and non-transgenic goats were analyzed by one-way ANOVA using the JMP software (SAS Institute Inc., Cary, NC). Differences were considered to be statistically significant at the 95% confidence level ($P<0.05$).

RESULTS

Casein Concentration in Milk

The milk of transgenic goats contained less casein compared with controls (1.61±0.3 vs. 2.64±0.5 g $100mL^{-1}$, $P<0.01$). The difference was equally significant throughout the entire lactation (Fig. 1).

Fatty Acid Composition

Significant differences were observed in milk fatty acid (FA) composition between transgenic and control goats. As described in Table 1, the milk of transgenic goats contained significantly fewer short - and long - chain FAs and a higher percentage of medium chain FAs than controls. Additionally, the milk of transgenic goats was found to have more saturated FAs and less polyunsaturated FAs than controls.

Concentration of Milk Minerals

Significant differences were observed in both the K^+ and Na^+ concentration in the milk of transgenic and control animals (Fig. 2). K^+ and Na^+ contents were not different between transgenic and control animals at the beginning of lactation ($P>0.05$). However, as lactation progressed, the K^+ concentration in the milk of transgenic goats decreased and that of Na^+ increased ($P<0.01$), resulting in an inverted Na:K ratio compared with the milk of control goats.

No major differences were observed in the average milk concentrations of Ca, Mg, P and S between transgenic and control goats (data not shown).

cSA Content in Milk

The average concentration of cSA in milk increased in transgenic goats as lactation progressed, while it remained relatively stable in control goats (Fig. 3). As measured by the BCG assay, the concentration of cSA was similar for transgenic and control goats in early lactation (0.22±0.04 vs. 0.21±0.04 mg mL^{-1}, $P>0.05$), but became higher in the milk of transgenic goats in the late stage of their lactation (0.50±0.08 vs. 0.17±0.08 mg mL^{-1}, $P<0.05$).

DISCUSSION

The concentration of caseins was found to be significantly lower in transgenic milk for the entire lactation. One plausible explanation for this finding could be a decrease in the transcription of the casein genes, potentially resulting from competition with the transgene, as previously shown in transgenic mice (McClenaghan et al. 1995). Alternatively, a reduced casein concentration could result from a slow-down in the downstream processes (translation and post-translation) of protein synthesis, as a consequence of the extraordinary demand placed by the expression of high levels of rBChE. Indeed, the expression of other recombinant proteins in the mammary gland has been previously reported to have a negative impact on the expression of milk native proteins (McClenaghan et al. 1995; Palmer et al. 2003).

Milk fatty acid composition in the control goats was comparable to that previously reported for goat milk (Bernard et al. 2005). However, significant differences were observed in the milk of transgenic goats. Of particular interest was the finding that the milk of transgenic animals contained lower concentrations of short-chain fatty acids, which are reported to be totally (C4-C10) or mainly (C12) derived from de-novo synthesis in the mammary epithelium, as opposed to medium and long chain FAs that are mainly (C14-C16) or completely (≥C18) derived from the circulation (Gerson et al. 1968). As a consequence of the above-referred excessive protein synthesis demand, the expression/synthesis of other molecules, including the enzymes involved in the synthesis of short chain FAs (e.g. acetyl-CoA carboxylase, fatty acid synthase) could be compromised. Aligned with previously discussed hypothesis, it could be speculated that the increased proportion of saturated FAs observed in transgenic milk might be the result of a depressed activity of the key enzyme stearoyl-CoA desaturase.

Our data shows strong evidence in support of TJ integrity loss in the transgenic goats as the lactation progressed (increased Na^+ and cSA; reduced K^+ concentrations). This is consistent with the observation of shortened lactations and decreased milk production in the transgenic goats compared with the controls, which had a normal lactation during this study. When the integrity of the TJs is lost, the cell loses its polarity and cytoskeleton architecture which is absolutely necessary for the directional process of milk secretion (Nguyen and Neville 1998). Further research is required to establish the possible causes for the loss of TJ integrity observed in the transgenic goats.

CONCLUSION

Differences in milk composition between control and transgenic goats have been identified. Interestingly, some of these differences were constantly present throughout lactation (lower casein content, FA composition), while others develop only after several weeks in milk (increased Na^+ and cSA, decreased K^+ and lactose), coinciding with the collapse in milk production observed in the transgenic goats. The findings reported in this study suggest the development of permeable tight junctions as a potential mechanism to explain the premature cessation of milk secretion observed in these transgenic goats. Further investigation is required to understand the causative mechanisms of these observations, as well as the development of treatments that may help control or overcome this problem.

Table 1: Fatty acid composition of milk triglycerides in transgenic and control goats.

Fatty Acids	Group	Transgenic	Control	P value
Size	Short chain	15.34 ± 4.2^{a}	24.32 ± 3.4^{b}	<0.001
	Medium chain	53.61 ± 9.2^{a}	38.83 ± 6.4^{b}	<0.001
	Long chain	31.01 ± 10.4^{a}	35.53 ± 7.4^{b}	<0.05
Number of double bonds	Saturated	70.76 ± 10^{a}	66.48 ± 7.1^{b}	<0.05
	Monosaturated	24.29 ± 9.4^{a}	25.52 ± 4.7^{a}	0.5026
	Polyunsaturated	4.84 ± 2.3^{a}	7.48 ± 2.7^{b}	<0.001

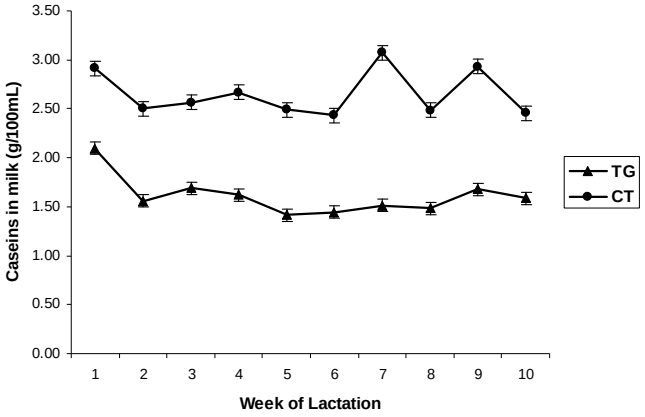
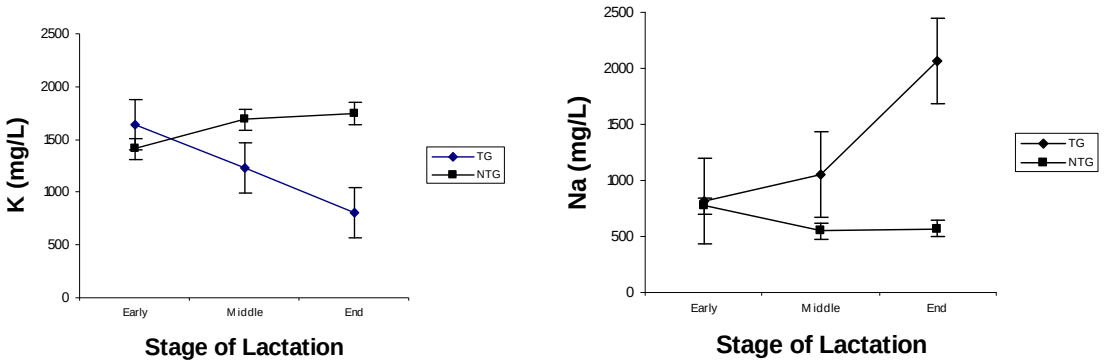


Figure 1. Concentration of caseins in the milk of transgenic (TG) and control (CT) goats.

a **b**



Lactation Stage	TG (mg/L)	CT (mg/L)	P-value
Early	1637±111	1410±128	n.s.
Middle	1227±86	1687±99	p<0.05
End	807±110	1747±127	p<0.01
Overall	1656±321	1244±209	p<0.01

Lactation Stage	TG (mg/L)	CT (mg/L)	P-value
Early	815±53	770±61	n.s.
Middle	1050±44	547±51	p<0.01
End	2067±50	570±221	p<0.01
Overall	1153±453	608±99	p<0.01

Figure 2. Concentration of Na^+ and K^+ in the milk of transgenic (TG) and control (CT) goats.

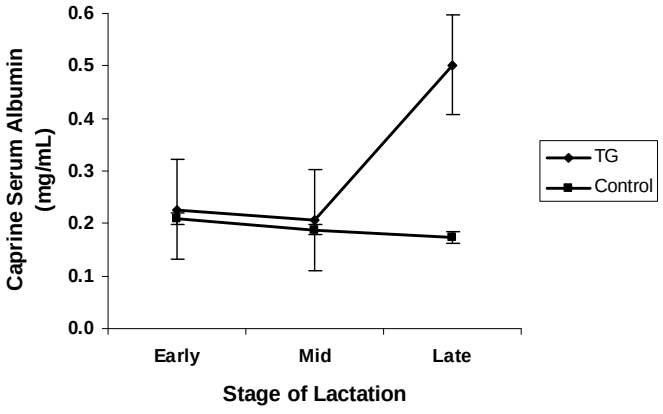


Figure 3. Serum albumin concentration in the milk of transgenic (TG) and control (CT) goats.

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Lactation performance of transgenic goats expressing recombinant human butyryl-cholinesterase in the milk

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ABSTRACT

This study evaluated the lactation performance of a herd of 50 transgenic goats expressing recombinant human butyryl-cholinesterase (rBChE) in the milk. Our findings indicate that high expression levels of rBChE (range 1-5 g L^{-1}) are produced in these animals at the expense of an impaired lactation performance. The key features characterizing these transgenic performances were the decreased milk production, the reduced milk fat content which was associated with an apparent disruption in the lipid secretory mechanism at the mammary epithelium level, and a highly increased presence of leukocytes in milk which is not associated with mammary infection. Despite of having a compromised lactation performance, the amount of rBChE produced per transgenic goat represents several orders of magnitude more than the amount of rBChE present in the blood of hundreds of human donors, the only other available source of rBChE for pharmaceutical and biodefense applications.

Keywords: Cholinesterase, transgenic, goat, lactation, lipid secretion

INTRODUCTION

Transgenic animals, i.e. individuals that have stably incorporated a foreign DNA construct into their genome, have several applications. In addition to the fundamental scientific interest for the study of genes and their regulation (including disease modelling), transgenic animal technology has the potential to accelerate livestock improvement, by means of introducing new genes or modifying the expression of endogenous genes that regulate traits of economic importance (Wheeler 2003) . Moreover, the production of recombinant proteins in the milk of transgenic animals has attracted significant interest in the last decade, due to the outstanding protein synthesis capacity of the mammary gland and the ease to collect milk. As a result, the transgenic animal platform has become a valuable alternative for the production of recombinant proteins that cannot be or are inefficiently produced using conventional systems based on microorganisms or animal cells (Keefer 2004).

Multiple reports of transgenic farm animals expressing recombinant proteins of pharmaceutical and biomedical interest in the milk are available in the literature. Although most of these articles described the production and characterization of founder animals, as well as the chemical characterization of the recombinant protein and its bioactivity, little to no information was reported concerning the lactation performance in these animals, as well as the impact of transgene expression on mammary gland physiology. This paper describes

the natural lactation parameters of 50 transgenic goats expressing recombinant human butyryl-cholinesterase (rBChE), derived from a single transgenic male line. rBChE is considered to be the most suitable bioscavenger for the protection of humans against organophosphate toxicity (Doctor and Saxena 2005). Because the molecule is present in human plasma only in minute quantities (1-2 mg L^{-1}) the transgenic animal platform offers the possibility of large scale production with view to multiple applications including the use as a countermeasure for chemical warfare.

MATERIALS AND METHODS

Animals

Transgenic founder animals were generated by pronuclear micro-injection of in vitro produced zygotes, using a DNA-construct consisting of the human BChE encoding gene, under the regulation of the goat beta-casein promoter, as previously described (Baldassarre et al. 2004). All goats were qualified into production after confirmation of identical transgene integration compared to their transgenic progenitor, which was established to be 10-12 copies of the transgene in a single integration site. Non-transgenic siblings of these goats, served as controls for some of the experiments described hereafter. The lactation performance was evaluated in 50 transgenic goats that completed their first natural lactation and 20 that completed two consecutive lactations.

Goats were milked twice daily following standard operating procedures, including a physical inspection of the mammary gland, visual inspection of first streams of milk, proper hygiene of the teats before starting and sealing of the teats after milking.

Basic Milk Composition Analysis

For determination of percentage of fat, protein and lactose as well as somatic cell count (SCC), 25 mL samples were taken from 20 randomly selected transgenic and non-transgenic goats of the same age and parity, at weekly intervals, and immediately submitted to the processing Laboratory (Valacta, QC).

BChE Assay

The rBChE concentration in milk was determined weekly using the Ellman assay (Ellman et al. 1961).

Milk Somatic Cells Composition

Milk samples (15 mL) were collected weekly from 3 transgenic and 3 non-transgenic goats of the same age, parity and stage of lactation. Sediment samples were produced by centrifugation and subsequently 1) extended on a slide for Giemsa-staining with the objective of establishing cell types, or, 2) fixed with 4% paraformaldehyde in PBS for 20 min, resuspended in PBS and kept at 4 ºC prior to cell sorting. The proportion of inflammatory and non-inflammatory cells in the milk samples were determined by fluorescence-activated cell sorting (FACS) using a leukocyte-specific anti-CD45 antibody raised in mice (VMRD, Pullman, WA).

Mammary Gland Biopsies and Histopathology Studies

Mammary gland tissue samples were collected from 3 transgenic and 2 non-transgenic randomly selected goats of the same age, parity and stage of lactation. Biopsies were collected on day 18, 39 and 52 of lactation by surgery under general anesthesia, and immediately immersed in 10% neutral buffered formalin. Fixed tissues were embedded in paraffin, and 3 µm-sections were cut and stained with hematoxylin, eosin and phloxin.

Statistical Analysis

In all cases, data from transgenic and non-transgenic controls were analyzed by one-way ANOVA using the JMP software (SAS Institute Inc., Cary, NC). Differences were considered to be statistically significant at the 95% confidence level ($P<0.05$).

RESULTS

Lactation Performance and Milk Production

Over the 70-d period of this study, the transgenic animals produced 64 kg $goat^{-1}$ or a daily milk production of 930±258 g per goat. The average milk production at the peak was 1.9±0.5 kg d^{-1}. Non-transgenic goats that were concomitantly milked twice daily over the same period produced an estimated average 132.2 kg per goat, representing an average daily milk production of 1888±430 g per goat. Their average milk production at the peak was 2.3±0.5 kg d^{-1}. Both groups reached their peak milk production around day 10-14 of lactation.

rBChE Expression Levels

The concentration of rBChE in the milk of the transgenic goats ranged between 1 to 5 g L^{-1}. Variability was observed between individuals and stages of lactation,

but not between first and second lactations (data not shown).

Basic Milk Composition

The SCC (Fig. 1D) climbed to an average peak of 19.3±8 x 10^6 cells mL^{-1} compared to an average of 0.99±1 x 10^6 in the non-transgenic controls ($P<0.05$). Physical examination of the udder and milk bacteriology results confirmed that the raise in SCC was not associated with a mammary gland infection (data not shown). The fat content in transgenic milk declined to <2% by the third week of lactation, while it remained at approximately 5% in the control goats (Fig. 1A). The average milk fat was significantly lower ($P<0.01$) in the transgenic (average 2.14±1.1) compared to the control (4.65±0.6%) goats. The total protein content remained relatively stable in the transgenic and control goats (average 3.34±0.9 vs. 3.26±0.3%, $P>0.05$), with a moderate increase in the transgenic goats at the end of their shortened lactations (Fig. 1B). The lactose content of milk (Fig. 1C) remained unchanged in the control goats while it decreased significantly in the transgenic goats concomitantly with the decrease in milk production (average 4.5±0.3 vs. 3.21±1.4%, $P<0.05$).

Somatic Cell Composition in the Milk

Immunostaining with CD45 antibody followed by FACS allowed characterizing the nature of the increased SCC in transgenic milk as a significant increase in white blood cells. Leukocytes (CD45 positive cells) represented >80% of the cells sorted in the milk from transgenic goats after week 3, while the number of leukocytes remained below 25% in the milk from non-transgenic animals (Fig. 1E). In addition, Giemsa-stained slides from milk sediment showed that >95% of the leukocytes were neutrophils and macrophages, in approximately equal quantities (data not shown).

Histopathological Findings

The number of inflammatory cells present increased from the first to the last biopsy, but was not different between transgenic (Fig. 2C, 2D) and control animals (Fig. 2A, 2B). The most striking finding in the mammary gland of the transgenic animals was the presence of large clear intracytoplasmic vacuoles in the epithelial cells of the acini. These vacuoles were present at all time-points and occupied most of the cytoplasm, pushing the nucleus on one side of the cell. Vacuoles were positively stained using Oil Red O, which confirmed their lipid nature (Fig. 2E).

DISCUSSION

This study demonstrates that transgenic goats expressing high levels of rBChE in milk had an impaired lactation performance, which was associated with decreased lipid secretion and increased somatic cells in milk.

The dramatic rise in the somatic cell presence in the milk concomitant with the sharp decrease in production was of particular interest during the course of this study. Histopathology evaluations didn't show a significant presence of phagocytes in the mammary tissues or signs of extensive inflammation that could justify the dramatic increase of leukocytes in milk. Independent of the cause for their presence, leukocytes could be contributing to accelerate the involution process as their increased presence in the milk has been associated with increased activity of milk proteases, typically observed during mammary gland involution (Weng et al. 2006).

The histopathology sections of all transgenic goats sampled at the three points in lactation showed large lipid vacuoles in the majority of the secretory cells. This histopathology finding is consistent with the observation that the fat content of milk decreases dramatically (from >4 to <2%) during the lactation crisis, while it remained relatively unchanged in the non transgenic controls (>3.5%). One possibility to explain this significant drop in milk fat is that rBChE could be somehow interfering with the intracellular lipid secretion pathway.

Despite of having a compromised lactation performance, the amount of rBChE produced per transgenic goat represents several orders of magnitude more than the amount of BChE present in the blood of hundreds of human donors, the only other available source of rBChE for pharmaceutical and biodefense applications.

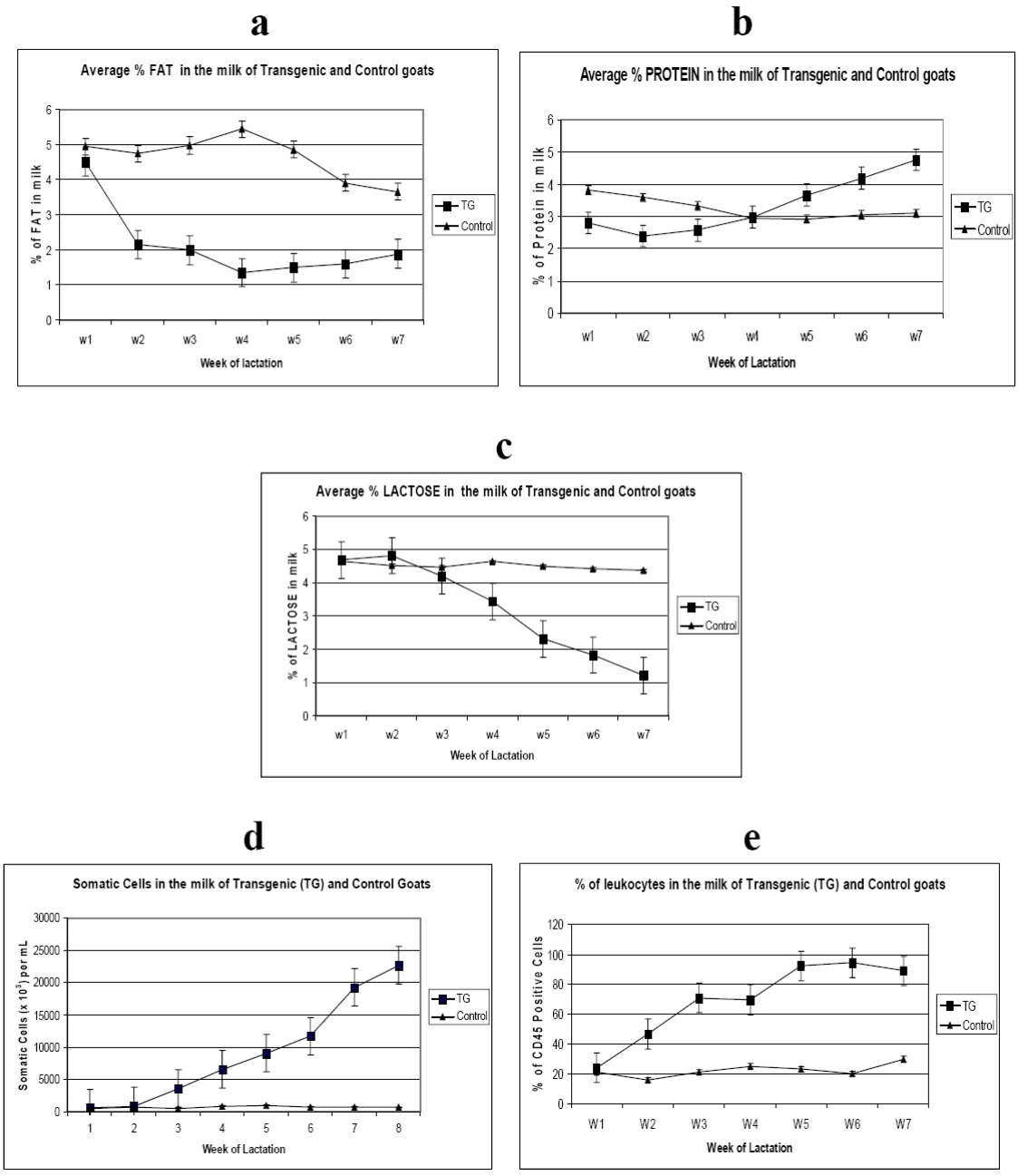

Figure 1: Differences in milk composition between controls and rBChE expressing transgenic goats over a 10-week period.

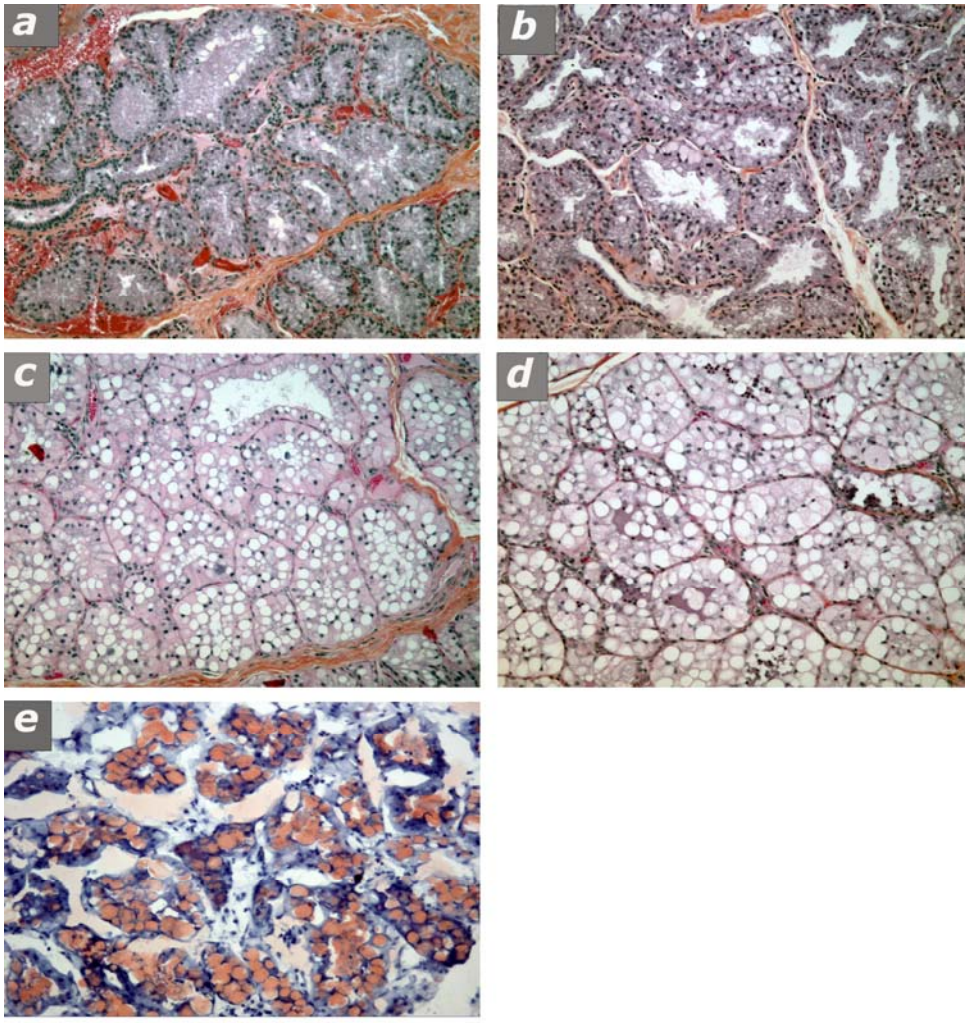

Figure 2: Histological analysis of mammary gland biopsies obtained at two points in the lactation of control **(a-b)** and rBChE-expressing transgenic goats**(c-d)**.

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Epigenetic changes and development of somatic cell nuclear transfer swine embryos after inhibition of histone deacetylases

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ABSTRACT

The present study assessed changes in epigenetic markers and pre- and post- implantation development in somatic cell nuclear transfer (SCNT) porcine embryos after treatment with the inhibitor of histone deacetylases Trichostatin A (TSA). Embryos were generated using either a male foetal fibroblast (FF) cell line or bone marrow cell (BMC) lines derived from adult sows. Reconstructed oocytes were either exposed or not to 10 ng mL^{-1} TSA for 10 h starting 1 h after cell fusion. Reconstructed oocytes were fixed at 18-20 h or 46-48 h after cell fusion and immunocytochemically processed to assess histone H3 acetylation at lysine 14 (H3K14ac), histone H3 dimethylation at lysine 9 (H3K9dime) and global DNA methylation (5-methylcytosine or 5-MeC) patterns using specific primary antibodies. TSA treatment increases both H3K14ac and DNA methylation but did not affect H3K9dime. TSA treatment resulted in higher (45.2% vs. 23.9%; $P<0.05$) development to the blastocyst stage in embryos produced from FF cells but there was no difference (34.9% vs. 34.7%; $P>0.05$) between treated and control groups derived from BMCs. The transfer of D 5-6 blastocysts to synchronized gilts resulted in the birth of piglets from both TSA treated and control embryos. These findings suggest that TSA treatment alters epigenetic reprogramming and in vitro development in SCNT swine embryos but this seems not to improve long term development after embryo transfer to recipient females.

Keywords: SCNT, trichostatin A, nuclear reprogramming, embryo development, swine

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INTRODUCTION

It is thought that developmental success or failure in embryos produced by somatic cell nuclear transfer (SCNT) depends on the proper reprogramming of epigenetic factors in the reconstructed embryo. Epigenetic reprogramming in SCNT embryos refers to the remodelling of the transplanted nucleus, after it is transferred into the cytoplasm of an enucleated oocyte, which involves modifications in chromatin-associated proteins and DNA methylation. These changes in chromatin structure and DNA affect gene expression and are necessary to support normal embryo and fetal development (Riek et al. 2007). Attempts to improve somatic cell reprogramming have shown that treatments with modulators of acetylation and methylation factors might enhance epigenetic reprogramming and improve embryo development in different species including cattle (Enright et al. 2003), mice (Ribouchkin et al. 2006), rabbits (Shi et al. 2008), and pigs (Zang et al. 2007). Among the tested treatments, the exposure of the reconstructed embryos to TSA, an inhibitor of histone deacetylase enzymes, has been receiving special attention because it was shown to increase the pre- and post-implantation development of SCNT embryo in mice (Ribouchkin et al. 2006). Therefore, the main goals of the present study were to assess the

effect of TSA treatment on epigenetic changes using specific markers for histone acetylation and methylation, and DNA methylation, and to determine the effect on the in vitro and in vivo development of SCNT porcine embryos reconstructed with fetal and adult somatic cells.

MATERIALS AND METHODS

Oocyte Preparation and Embryo Production

Oocyte collection, in vitro maturation, nuclear transfer and oocyte activation were performed using standard conditions as previously described (Che et al. 2007). Fetal fibroblasts (FF) or bone marrow cells (BMC) derived from confluent cultures were used for embryo reconstruction. After activation, oocytes were separated into 2 groups and cultured in PZM-3 supplemented or not with 10 nM TSA for a period of 10 h starting after ionomycin treatment. The cleavage and blastocyst rates were determined at 48 h and 6-7 days after fusion. Day 5-6 blastocysts produced from both TSA treated and control embryos derived from FF and BMC were surgically transferred to recipient females.

Immunodetection of H3K14ac, H3K9dime and 5-MeC

Embryos were rinsed in PBS, fixed during 20 min in 4% paraformaldehyde, and then transferred to PBS supplemented with 3% BSA and 0.5% Triton X-100. Samples were exposed overnight at 4 ^{o}C to the primary antibodies (rabbit anti-mouse anti-acetyl histone H3 lysine 14 or anti-dimetyl histone H3 lysine 9) diluted 100 times in blocking solution (3% BSA and 0.2% Tween-20 in PBS). Embryos used to detect DNA methylation (5-MeC) were first placed into a solution of 10 N HCL and 0.1% Triton X-100 for 10 min, then washed in PBS, transferred to 100 mM Tris/HCL buffer (pH 8.5) for 15 min, and finally exposed for 1 h to the primary antibody (mouse anti 5-methylcytidine) in blocking solution. Samples were then washed three times for 20 min each in blocking solution and incubated for 1-2 h at room temperature in the presence of 1:500 diluted Alexa Flour 488 goat anti-rabbit (H3K14ac and H3K9dime) or FITC goat anti-mouse (5-MeC) secondary antibodies. Samples were washed three times (20 min each) in blocking solution and mounted on microscope slides using a drop of Mowiol containing 10 □g mL^{-1} of Hoechst 33342 or propidium iodide for chromatin visualization. Specimens were examined by epifluorescence using a Nikon eclipse 80i microscope (Nikon, Tokyo, Japan).

RESULTS AND DISCUSSION

The present study assessed the effect of Trichostatin A, an inhibitor of histone deacetylases, on the reprogramming of epigenetic markers and pre- and post-implantation development of porcine SCNT embryos derived from fetal fibroblasts and bone marrow cells. We report here that the inhibition of deacetylases affects epigenetic reprogramming in SCNT embryos produced from both cell types. However, pre-implantation development was only improved in embryos derived from FF cells. Viable cloned piglets were produced from both control and TSA treated embryos, which suggests that this treatment has no apparent detrimental effects on post-implantation survival and development of porcine SCNT embryos.

The initial objective in this study was to assess whether the exposure of reconstructed embryos to TSA affects pre-implantation development of SCNT porcine embryos reconstructed from different cell types. Our findings indicate that TSA treatment can improve blastocyst formation, but this effect seems to be dependent of the nuclear donor cell type. When embryos were produced from FF cells, TSA increased the blastocyst formation rate; however, no difference was seen between treated and control embryos derived from BMC (Table 1). Although previous studies have similarly showed a positive effect of TSA treatment on pre-implantation embryo development using cumulus, neural stem cells, skin fibroblasts and spleen cells in mice (Ribouchkin et al. 2006), as well as skin cells in rabbits (Shi et al., 2008) and fetal fibroblast in pigs (Zang et al. 2007), there was no previous information regarding the use of this treatment in embryos produced from BMCs. One possible explanation for the effect of the cell type could be the differentiation state of the cells. Indeed, BMCs are known to be multipotent stem cells that remain capable to differentiate into different cell types (Bosch et al. 2006). It is, therefore, possible that the chromatin of BMCs requires less reprogramming to allow the proper expression of genes necessary to support pre-implantation development. In support of this are previous studies showing that porcine

mesenchymal cells resulted in higher embryo development to the blastocyst stage compared to other cell types (Faast et al. 2006).

Our second goal in this study was to evaluate the reprogramming of epigenetic factors before and after cleavage in embryos that were treated or not with TSA. Although TSA treatment had a cell-dependent effect on pre-implantation development, a similar effect on the presence of epigenetic markers was seen in both cell types. A higher signal for H3K14ac was detected in both pre- and post-cleaved embryos after exposure to TSA (Figure 1). Similarly, other studies have previously reported an increase in the acetylation of lysine 12 in the histone H4 after TSA treatment of mouse SCNT embryos produced from cumulus cell nuclei (Ribouchkin et al. 2006). This confirms the inhibition of histone deacetylases by TSA and supports the idea of the increase in histone acetylation being the potential benefit of this treatment on chromatin reprogramming. For instance, histone acetylation could be important to drive the expression of genes that regulate embryo development. Indeed, a recent study in mice showed that the inhibition of histone dehacetylation by TSA increased the expression of Sox2 and cMyc genes at the blastocyst stage (Li et al. 2008).

Interestingly, TSA treated embryos from both FF and BMC also presented a higher signal for global DNA methylation compared to control embryos. Although no previous studies have evaluated the effect of TSA on global DNA methylation, this surprising observation suggests a detrimental effect of this treatment on 5-MeC reprogramming because a global demethylation is known to occur during early embryo development (Yang et al. 2007). Indeed, higher DNA methylation is correlated with an inactive chromatin and could potentially interfere with normal gene expression required for embryo development (Oda et al. 2008).

The effect of TSA treatment on the post implantation development of SCNT embryos derived from both FF and BMC was evaluated in the last experiment. Day 5-6 blastocysts were surgically transferred to the uterus of 8 estrus synchronized gilts (Table 2). A total of 4 (50%) females became pregnant and naturally delivered 20 cloned piglets after $PGF_{2\alpha}$ injection given at D-115 of pregnancy. Both TSA and control derived embryos resulted in 50% (2/4) pregnancy and farrowing rates. Seventeen male clones were produced from FF cells, 11 derived from the 2 gilts transferred with control embryos and 6 derived from 1 of the 2 gilts transferred with TSA treated embryos. From BMC derived embryos, only one female that was transferred TSA treated embryos became pregnant and delivered 3 cloned females. Despite the limited number of embryo transfers, these results indicate that TSA treatment did not hamper post-implantation development and survival of SCNT piglets. Although not in agreement with previous studies with mice (Ribouchkin et al. 2006), our findings suggest that the exposure of reconstructed embryos to TSA seems not to be an effective approach to improve SCNT efficiency in pigs.

CONCLUSIONS

1. The inhibition of histone deacetylases by TSA treatment increases H3K14 acetylation and 5-MeC at 1 and 2-4 cell stage porcine embryos produced by SCNT.
2. TSA treatment resulted in a cell-type dependent improvement of SCNT embryo development to the blastocyst stage.
3. Inhibition of deacetylases after SCNT allows complete nuclear reprogramming since both male and female cloned piglets were produced from TSA treated embryos.
4. TSA treatment seems not to improve the efficiency of cloning from somatic cells in the pig.

Table 1. Effect of trichostatin A (TSA) treatment and nuclear donor cell type on the *in vitro* development of somatic cell nuclear transfer embryos

Nuclear Donor Cell	TSA treatment	No. of reconstructed oocytes (replicates)	% of embryo development to	
			2-cells	Blastocyst[a]
FF	-	721 (7)	67.1 ± 3.7ab	23.9 ± 4.4a
	+	648 (7)	64.9 ± 3.7b	45.2 ± 4.4b
BMC	-	1226 (22)	77.6 ± 2.1ac	34.9 ± 2.5ab
	+	568 (10)	80.8 ± 3.1c	34.7 ± 3.7ab

[a]% based on the number of cleaved oocytes.
a-c Within columns, means with different superscripts are statistically different ($P \leq 0.05$).

Table 2. Post-implantation development of SCNT embryos produced from foetal fibroblasts (FF) or bone marrow cells (BMCs) after treatment with TSA

Nuclear donor cell type	TSA treatment	No. of embryos transferred to each recipient	Recipient			Cloned piglets	
			n	pregnant	farrow	n	birth weight (kg)
FF	-	81, 50	2	2	2	11	0.874
	+	53, 72	2	1	1	6	1.02
BMC	-	60, 45	2	0	-	-	-
	+	60, 60	2	1	1	3	1.3

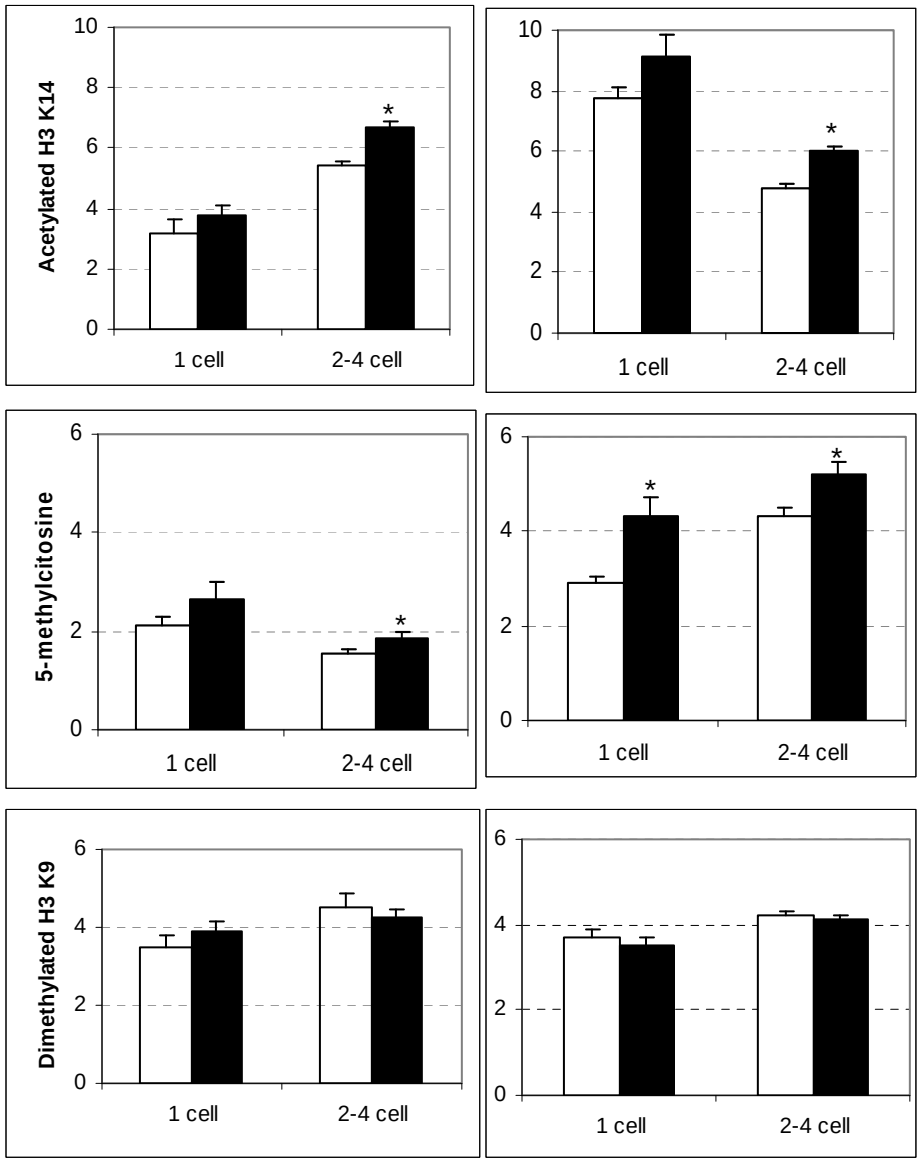

Figure 1. Epigenetic differences in somatic cell nuclear transfer embryos produced from fetal fibroblasts or bone marrow cells after treatment (black) or not (white) with TSA.

* is indicating a significant difference ($P<0.05$) between the TSA-treated and untreated embryos.

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Mastitis-specialized clonal-lineage of *Staphylococcus aureus* revealed by the analysis of repeats in the R-domain of the clumping factor A gene

K. B. Said and X. Zhao

ABSTRACT

The objectives of this study were to determine if the R domain was useful in sub-typing *S. aureus* and whether the repeats were affected by serial passage in bovine milk or nutrient broth (NB), or during invasion of mammary cell line. R-domain is composed of a tandemly repeated 18 bp units that code $[Ser\text{-}Asp]_3$ dipeptides. PCR primers specific to the R domain were designed against the conserved regions flanking the repeats on *S. aureus* subspecies COL sequence. DNA from human and mastitis isolates was amplified. In 55 R domains analyzed, 44 had copy numbers between 44 and 57, and only seven had copy numbers between 63 and 73. Further, while human strains were polymorphic, mastitis isolates were clonal and 16 of the 19 isolates had 52 copies. Phylogenetic trees constructed using the R-domain sequences were congruent; while human strains/isolates formed different clusters, mastitic isolates were assigned to a single cluster indicating a common ancestor. Moreover, repeats were stable during passage in milk, nutrient broth, and invasion of host cells showing suitability for typing. Results showed that R domain is useful for subtyping *S. aureus*. Moreover, existence of highly polymorphic protein motifs in human strains and the dominance of a clonal motif in mastitis strains imply that a selection had occurred in the mammary gland.

Key words: *clfA*-R domain polymorphism, mastitis clone, *S. aureus*

INTRODUCTION

In the dairy industry, bovine mastitis is the most frequent and most costly disease and *S. aureus* is a top causative agent of intramammary infections. *S. aureus* causes contagious bovine mastitis which usually develops into chronic infections. The chronic nature of *S. aureus* intramammary infections and the poor success rates of antibiotic treatment have been attributed to their ability to invade and persist in host cells (Bayles et al. 1998). Genomic analysis and population structure of *S. aureus* indicate that separate host-specific lineages exist (Kapur et al. 1995; van Leeuwen et al. 2005). However, the basis for this specialization is still elusive. Molecular markers capable of differentiating host-specific clones are crucial to trace the sources of strains and to understand the basis for specialization. *spa* gene has been successfully used as a single genetic marker for typing *S. aureus* (Koreen et al. 2004). Nevertheless, the importance of *spa*-repeat variation is unknown, and there is no relationship between *spa*-repeat variants and the host species (Atkins et al. 2008). Therefore, we aimed to explore a coding variable-number tandem-repeat (VNTR)-based typing, using the R-domain of the clumping factor A gene (*clfA*). The R-domain is composed of a hypervariable-variable tandemly-repeated 18 bp units that follow the consensus [GAP (P= pyrimidine), TCN (N=any base), GAP, TCN, GAP, AGP] and code for a [Ser-Asp] dipeptide-motifs. The objectives of this study were to determine if R-domain would be useful in subtyping *S. aureus* from humans and cows and whether the repeat diversity correlates with host species.

MATERIALS AND METHODS

PCR primers were designed against the conserved regions flanking the repeats on the chromosome of *S. aureus* subspecies COL sequence using DNAstar and primer3 programs and were BLAST checked against databases for homologous regions. Conditions for amplification of a single PCR product were established. Genomic DNA from human and mastitis isolates were amplified and resolved in agarose gels and amplicons were tested for polymorphism of R-domain fragment lengths. R-domain repeat-copy-numbers were inferred from PCR product sizes and confirmed by sequencing. All nucleotide sequences, including those of *clfA* from published *S. aureus* strains of human and bovine mastitis were analyzed to determine repeat-copy-numbers, phylogenetic analysis, and alignment patterns of whole R-domain nucleotide sequences using Geneious v.0 3.5.6, DNAstar, Tandem Repeat Finder (Benson 1999) (http://tandem.bu.du/trf/trf.html), and ClustalW program from the European Bioinformatics Institute web site (http://www.ebi.ac.uk/Tools/clustalw/index.html). The stability of the repeats was investigated by passing strains Lowenstein, Wright, and Rosenbach in milk and nutrient broth (NB) incubated at 37ºC with and without shaking for 15 consecutive days, and checking copy-number by PCR every-2-days. Similarly, 19 sporadic bovine mastitis isolates were passed in NB for 15 passages, in a similar manner. DNA was immediately isolated from passages P1, P3, P6, P9, P12, and P15 of milk and NB cultures. The above three strains were also tested for repeat stability after invasion into mammary cells (MAC-T), according to Bayles et al. (1998).

RESULTS AND DISCUSSION

The present study shows that repeats in the *clfA*-R-domain were stable during passage in milk, nutrient broth, and during invasion of mammary epithelial cells, indicating the suitability of the R-domain for typing *S. aureus*. In 55 R-domains analyzed from *S. aureus* isolated from human and bovine mastitis infections, 80% had low-repeat-copies of 44-57 (Tables 1 and 2). This is interesting and correlates well with the invasiveness of these bacteria. These included most of the methicillin-sensitive *S. aureus* (MSSA) isolates of blood and synovial fluids of patients, mastitis isolates, and the majority of the methicillin-resistant *S. aureus* (MRSA) including invasive community-associated strains analyzed. In addition, strains Lowenstein (50 copies) and Wright (57 copies) belonged to this group. Consistent with the finding of Tollersrud et al. (2000) that Lowenstein and Wright dominate human isolates from many sources, the majority of our isolates had copy numbers similar to these two strains. In fact, allele 9 was associated with the highest numbers of isolates (n=5) with 57 copies of repeats identical to that of Wright. Although the overall virulence of the low-copy-range could not be due to the repeat lengths alone, it is reasonable to postulate that it could be a factor. This is further supported by the finding that reducing the number of copies, had resulted in gradual reduction in levels of bacterial cell clumping titers and in binding of anti-ClfA serum and fibrinogen coated plastic surfaces (Hartford et al. 1997). Similarly, the degree of adherence correlated well with the length of the R-domain *in vitro* (Risely et al. 2007). The exact relationship between the copy number and the invasiveness of *S. aureus* is worthy of further investigation.

Furthermore, isolates from human infections were highly polymorphic, while those from mastitis were clonal for the repeat copies. Sixteen of the 19 mastitic isolates had 52 copies (Fig. 1). Two distinct mastitis clones were identified (Tables 1 and 2, Figs. 2 and 3). In addition, phylogenetic trees constructed based on R-domain nucleotide sequences were highly congruent; human and animal isolates were assigned to respective clusters, A, B, C, D, E, F, G1, and G2 (Figs. 2 and 3). In agreement with the earlier findings of comparative genomics (van Leeuwen et al. 2005), population structure studies (Kapur et al. 1995), and genome-based molecular typing, this study has shown that only a limited number of closely-related clones were responsible for bovine mastitis (Fig. 3 F and G1). The clonal natures of mastitis isolates were quite distinctly identified in comparison with the highly polymorphic nature of the human-associated isolates. Interestingly, subclonal populations of mastitis could be successfully further differentiated (Fig. 3, F). This is in agreement with the finding of Smith et al. (2005) that dominant clones belonging to the same global clonal-complex evolved into subclonal populations in different geographical region.

This implies that local environmental conditions of dairy cows select for a specific variants capable of adaptation to that environment. Therefore, the finding of this study could be particularly significant in establishing local strain profiles since strain- or clone-specific factors affect the probability of cure or infection (Barkema et al. 2006) and that these factors vary depending on differences in local selection pressures such as farm or hospital environments. Thus, it is quite reasonable to suggest that in addition to the standardized global protocol for typing, more localized typing methods established for local strain/clone profiling, treatment efficacy testing, and assessment of selection pressures and for routine national or regional interlaboratory use of data on epidemic clones. The study will also support the identification of specific factors associated with host specificity in *S. aureus* (Herron et al. 2002).

CONCLUSION

In this study, application of comparative VNTR analysis, using the hypervariable *clfA*-R-domain, in typing *S. aureus* has provided new perspectives on the genetic relationships and host- and organ specialization of *S. aureus*. This will contribute to a better understanding of *clfA* role in differentiating virulent clones and in identifying basis for selection in the mammary gland. *clfA-R* typing has a potential as a complementary typing method for tracking local and global strain profiles and examining *S. aureus* transmission, ecology, and evolution. This method is simple, sensitive and reproducible. Moreover, the existence of variant repeats in human strains and the dominance of a clonal motif in mastitis isolates imply that selection of a specific R-domain length has occurred in the mammary glands, implying the importance of this domain in the invasiveness and colonization of this pathogen.

Table 1. Sources and addresses of strains and of published *S. aureus* genomes used in the analyses of R domain copy numbers of *clfA*, and their corresponding copy numbers.

Strain	Source	Site	Origin	Copy#
S.aureus subsp. aureus COL (MRSA)	TIGR	http://www.tigr.org	Human	50.5
S.aureus subsp. aureus MRSA252 (HA-MRSA= hospital acquired methicillin resistant *S. aureus*)	Welcome Trust Sanger Institute	http://www.sanger.ac.uk/	Human	66
S.aureus subsp. aureus N315 HA-MRSA	Juntendo Univ.	http://www.staphylo-coccus.org/jp/	Human	59.5
S. aureus subsp. aureus MSSA476 (CA-MSSA= community acquired methicillin sensitive *S. aureus*)	Welcome Trust Sanger Institute	http://www.sanger.ac.uk/	Human	49
S.aureus subsp. aureus MW2 (CA-MRSA)	NITE	http://www.bio.nite.go.jp/	Human	51
S.aureus subsp. aureus Mu50 MRSA-VRSA (MRSA-Vancomycin resistant *S. aureus*)	Juntendo Univ.	http://www.staphylo-coccus.org/jp/	Human	49.5
Staphylococcus aureus subsp. aureus NCTC 8325	University of Oklahoma	http://microgen.ouhsc.edu/s_aureus/s_aureus_home.htm	Human	48
Staphylococcus aureus subsp. *aureus* USA300 invasive-MRSA	University of California, San Francisco	http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=16313	Human	49
Mastitis *Staphylococcus aureus* RF122	Univ. Minnesota	http://pathogenomics.umn.edu/staph_index.htm	Bovine mastitis	46
S. aureus Rosenbach, Noncapsulated Smith Compact (CP)	ATCC	-	-	66
S. aureus Lowenstein	ATCC59521		Human	50
S. aureus Wright	ATCC49525		Human	57

Table 2. Alleles associated with human isolates (H1 to H24) and mastitis isolates *List of 16 mastitis isolates:92M, 4M, 143M, 144M, 145M, 152M, 209M, 358M, 363M, 428M, 429M, 431M, 439M, 758M, 759M, and 764M.

MSSA isolates from human infections		
Allele #	**Copy #**	**Isolates**
1	36	H18
2	44	H5
3	50	H19, H20
4	52	H6, H15, H22
5	52.5	H8
6	53.5	H3, H23
7	54	H4
8	55.5	H12
9	57	H7, H9, H14, H21, H24
10	60	H16
11	61	H17
12	62	H13
13	63	H1
14	64	H2, H11
15	73.5	H10
Bovine Mastitis Isolates		
1	52	(16 mastitis isolates)*
2	50	(Isolates M760, 855M)
3	44	140M

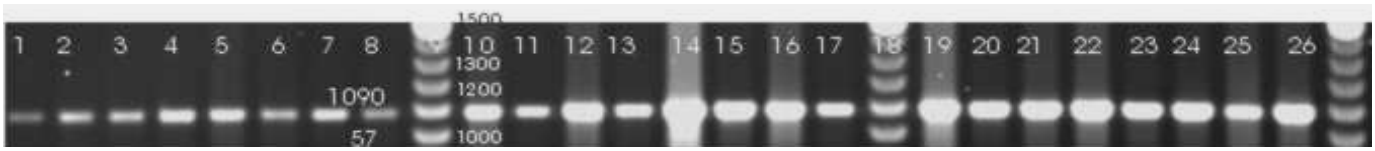

Figure 1. PCR product sizes (above bands) and the corresponding inferred copy numbers (below bands) of strain Wright, after passage in bovine milk and NB for 15 days, incubated at 37ºC with and without shaking. No repeat variations were found during passage/invasion of the three strains used; therefore, only strain Wright results are shown. Wright in NB and milk: P1, lanes1 to 4; P3, 5 to 8; P6, 10 to 13; P9, 14 to 17; P12, 19 to 22; P15, 23 to 26. Lanes 9, 18, and 27 = ladder (100bp Invitrogen Inc Canada).

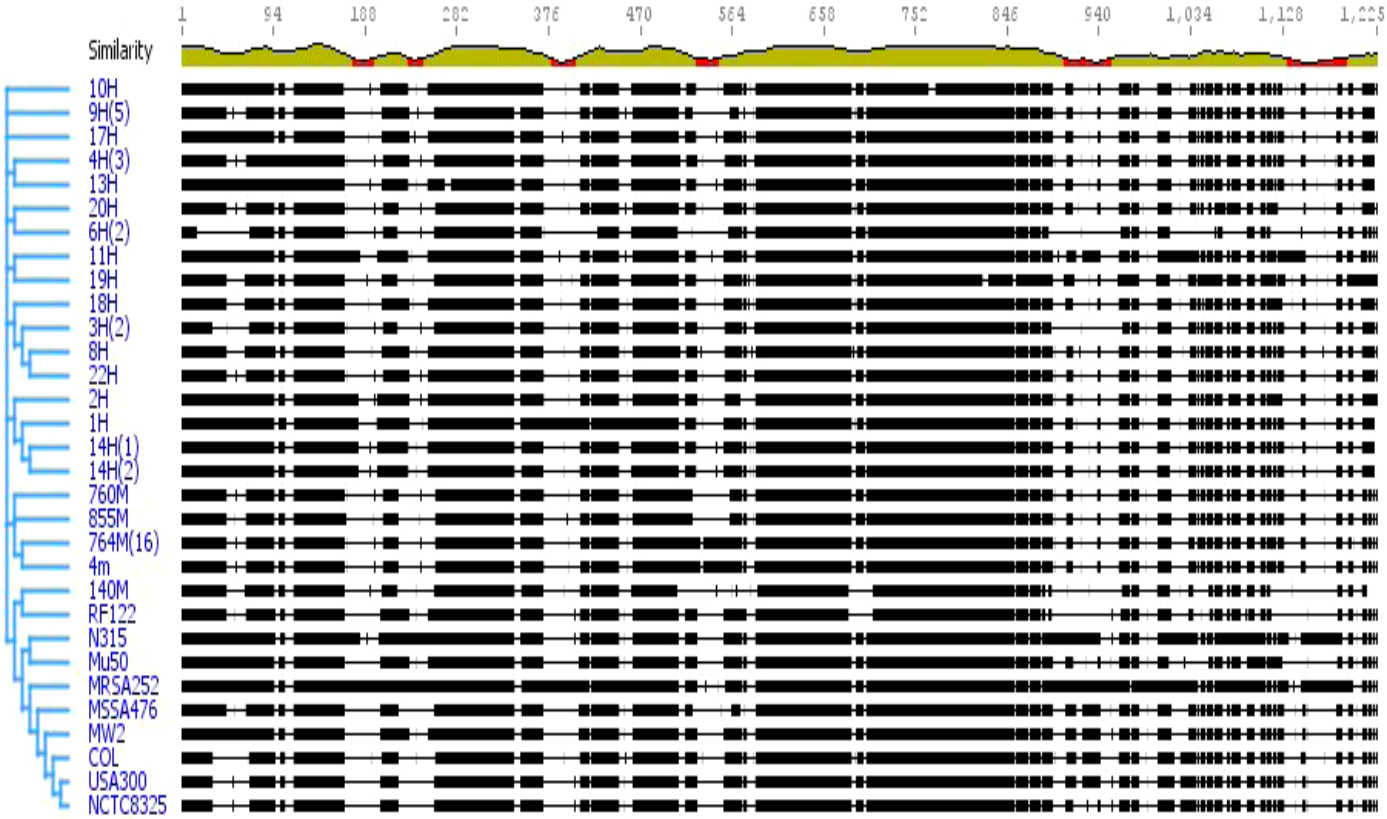

Figure 2. Multiple sequence alignment and Cladogram with a guide-tree using Neighbor-Joining (Distances estimated by the Tamura-Nei) built by Geneious Program (v. 3.5.6) using R-domain nucleotide sequences of representative isolates from human, mastitis, and the published sequences of *S. aureus.*

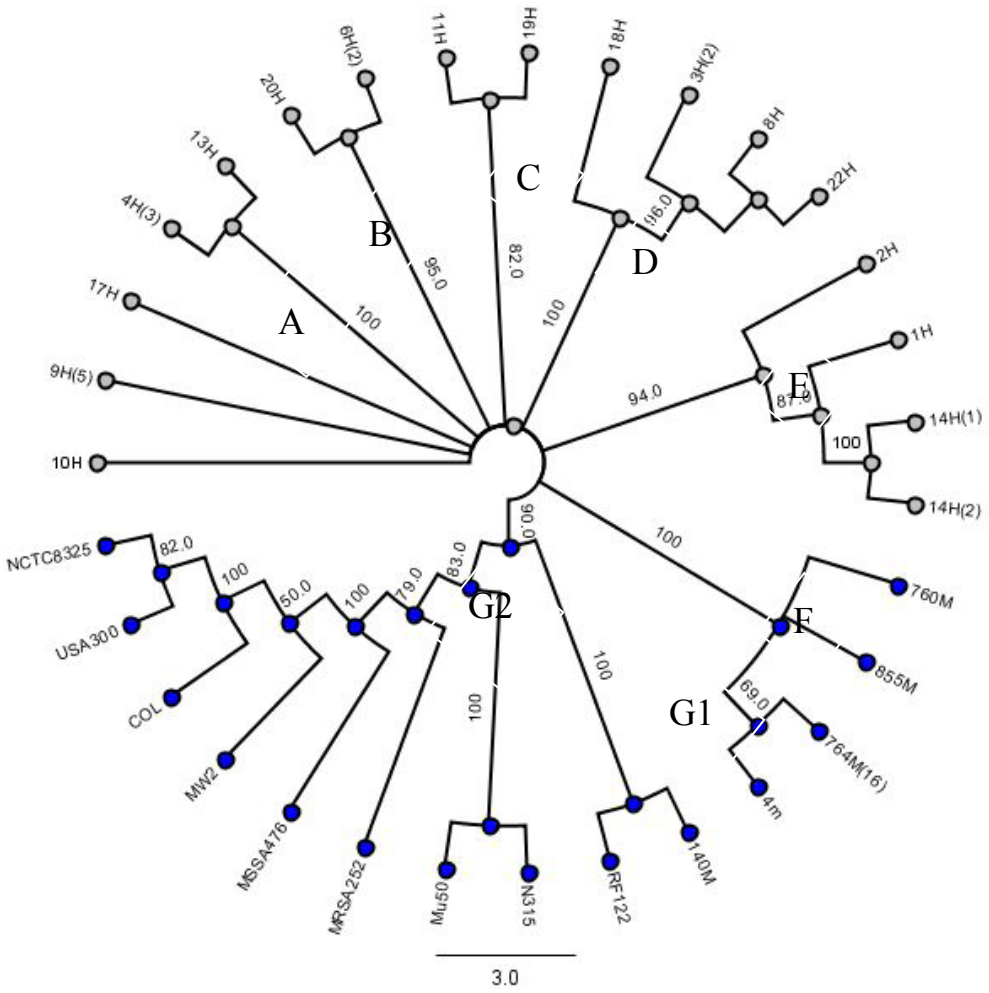

Figure 3. Phylogenetic tree based on Neighbor-Joining (Distances were estimated by the Tamura-Nei, bootstrapping100, threshold of 50%, built by Geneious Program (v. 3.5.6) using R domain nucleotide sequences isolates from human, mastitis, and the published sequences of *S. aureus.*

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Factors altering milk urea nitrogen in dairy cattle

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ABSTRACT

Multiple factors alter concentrations of milk urea nitrogen (MUN) however, little is known about their possible interactions. The objective of this study was to determine multiple factors altering MUN from Quebec dairy cows. Milk test-day and feeding composition records (n = 2,382,043) from Ayrshire, Brown Swiss, Holstein and Jersey cows were analyzed. Descriptive-statistical analysis indicated that MUN concentrations were altered by the effect of stage of lactation, parity, and breed. Decision-trees classifications identified hidden interactions among nutritional and non-nutritional factors influencing specific levels of MUN. Low concentrations of MUN are attained from the group of cows that are producing a low milk fat percentage and fed low levels of dietary crude protein and high non-structural carbohydrates. Contrary, high concentrations of MUN were observed with the association of cows that produced a high milk fat percentage and low somatic cell count, and were fed high levels of dietary crude protein.
Keywords: Dairy cows, machine learning, milk urea nitrogen,

INTRODUCTION

Milk urea nitrogen (MUN) is a non-protein compound directly related with dietary composition. Several reports have evidenced the effect of multiple factors altering MUN however, most studies have determined the effects of specific nutritional and non-nutritional factors independently, and the evaluation of multiple factors influencing MUN in single analysis has modestly been reported.

Dairy Herd Improvement Associations (DHIA) analyze milk and feed samples on a regular basis. Data from DHIA are an invaluable source of information however, limitations of some of the statistical techniques for highly dimensional datasets, represent the largest obstacle for analysis. Machine learning and exploratory data analysis techniques are employed to find hidden interactions among complex datasets (Fayyad et al. 1996). Machine learning techniques enable the user to include multi-dimensional datasets in single analysis, something that classical data analyses often lack. The application of machine-learning techniques can improve the understanding of single and/or multiple factors influencing MUN (Pietersma et al. 2005). The objective of this study was to identify associations among multiple dietary and non-dietary factors altering MUN through the application of machine-learning techniques on multidimensional records.

MATERIALS AND METHODS

Data Description and Pre-Processing

Animal identification, on-farm events, test-day analyses, feed composition analyses, and feeding equipment records of Quebec herds enrolled in Valacta from 2000 to 2005 were collected. Data cleaning was performed using SAS 9.1 (SAS Inst., Inc., Cary, NC). Abnormal tests, redundant attributes, and outliers were removed. The final dataset contained unique test-day and diet composition records (n = 2,382,043) of 404,111 cows belonging to 4,872 herds on a given test-day. Milk composition analysis included milk yield, milk fat and protein percentages,

somatic cells count (SCC), and MUN. Days in milk (DIM) were grouped into (14-d) categories to avoid within cow repeated measurements. Diet composition analysis included dry matter intake (DMI), crude protein (CP), rumen-undegradable protein (RUP), net energy for the lactation (NE_L), acid detergent fiber (ADF), neutral detergent fiber (NDF), fat, nonstructural carbohydrates (NSC), and minerals. Table 1 illustrates milk and diet composition analysis.

Statistical and Decision-Tree Analyses

PROC UNIVARIATE, PROC MEANS, and PROC GPLOT of SAS were used to performed descriptive-statistical analysis of single variables. For the decision tree deployment the target variable (MUN) was classified into three levels (low, medium, and high) using quantile values within breed. InforSense Kensington Discovery Environment 4.0 (InforSense Ltd, London, UK) was implemented for the machine-learning analysis. Decision trees were induced with seventy percent of a random dataset to obtain the training sample; the residual thirty percent of the dataset was used to evaluate the predictive model.

Predictive variables included breed (Ayrshire, Brown Swiss, Holstein, and Jersey), parity, stage of lactation (early, medium-late), milk yield, milk fat percentage, milk protein percentage, SCC, season-year, diet composition, time of milk sampling (morning, afternoon, 24 hour), region, feeding equipment, herd size, and a binary code to determine herds containing multiple or single breeds. After several preliminary decision-tree analyses, a decision-tree was induced from 1,310,124 random records to detect interactions altering low-, medium-, and high-MUN concentrations. The decision-tree performance was evaluated through 10-fold cross-validation techniques and information gain tools of the KDE InforSense Software.

RESULTS AND DISCUSSION

The MUN variations associated with DIM showed that the first months of lactation were characterized by low concentrations of MUN; the lowest point was observed during the 28-42 DIM category (10.37 ± 3.33 mg dL^{-1}) followed by a gradual increase over the rest of the lactation reaching a maximum point towards the end of the lactation period (11.45 ± 3.55 mg dL^{-1}). Other Canadian studies have reported similar results (Miglior et al., 2006b). Early lactation is usually characterized by low concentrations of MUN during the first 45 DIM in most studies. The variations of MUN over the lactation could be related to physiological changes: during pre-partum there is a physiological decrease of DMI that lasts approximately 30-d post-partum; during post-partum there are large differences in milk constituents associated to the colostrums effect, and differences in DMI associated to rumen microbial adaptation and rumen absorptive capacity. During mid-lactation ration nutrient composition and feeding programs stabilized hence milk yield production also stabilizes (de Vries and Veerkamp 2000).

Overall mean MUN concentrations were higher for Jersey than for Brown Swiss, Ayrshire, and Holstein cows (Table 1). Although, the differences between the concentrations of MUN by breed were numerically small, this effect could be related to body weight (BW) differences. Large breeds tended to have lower concentrations of MUN compared to the small breeds.

Table 2 shows mean MUN concentrations by breed and parity. Values showed that mean MUN increased within parity number and the lowest MUN concentrations were found in the first parity for all breeds. However, parity variations among breeds were numerically small. Primiparous cows are likely to have lower milk yield compared to multiparous cows, therefore it is logical to assume that within parity MUN variations could be produced by ration-nutrient formulations commonly based on parity groups and stage of lactation.

Figure 1 shows the final classification tree induced from 1,310,124 completely-randomized records. The target or root of the tree represents the three levels of MUN, branches are arranged in the form such that the first branches (from left to right) correspond to the attributes with the highest predictive value recognized to be associated to MUN levels. The software recognized milk fat percentage as the first variable associated to MUN, and split this continuous variable into two binary tests using cut points as probable thresholds (values less than or greater than the cut point). The decision tree in Figure 1 indicates that the variable with the

second highest predictive percentage recognized to be associated with MUN levels and milk fat percentage was dietary CP. At the third node levels the variables interacting with milk fat and dietary CP were different in all of the interactions. The effect of NSC, SCC and milk protein percentage was observed on specific interactions of the tree.

There were eight different interactions altering specific levels of MUN. Figure 1 shows the predictive percentage values of each interaction on low, medium, and high levels of MUN. The value represents the percentage of data (from the combination of variables or interaction) that was correctly predicted or classified. Comparing the predictive percentages in all interactions, the highest probability percentage at low levels of MUN was obtained from the interaction of the group of cows that produced less than or equal to 3.7% of milk fat, fed less than or equal to 17.5% of CP, and that were fed more than 34.2% of NSC. Medium levels of MUN would be most likely produced from the group of cows that produced less than or equal to 3.7% of milk fat and had more than 693,000 c/ml of SCC, and were fed more than 17.5% of CP. The group of cows that produced more than 3.7% of milk fat and no more than 303,000 c/ml of SCC, and were fed more than 17.0% of CP would most likely produce high levels of MUN and less likely to produce low levels of MUN.

CONCLUSION

We conclude that MUN concentrations are influenced by stage of lactation, parity, and breed. There is a strong interaction between dietary CP and milk fat percentage, altering specific concentrations of MUN: low levels of MUN can be obtained from cows producing low milk fat percentage, which are being fed low dietary CP and high NSC; cows producing high milk fat percentage, low levels of SCC, which are being fed high dietary CP, are most likely to produce high MUN concentrations.

Table 1. Mean and SD of milk and nutrient composition of diets (% of DM) on given test-days in Holstein (n = 2,272,030), Ayrshire (n = 86,729), Brown Swiss (n = 14,253), and Jersey (n = 9,031)

	Ayrshire		Brown Swiss		Holstein		Jersey	
Parameter	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Milk								
Milk (kg)	22.02	7.72	22.48	7.48	27.50	8.89	19.43	6.59
Fat (%)	4.15	0.62	4.29	0.65	3.90	0.70	5.04	0.79
Protein (%)	3.48	0.37	3.62	0.38	3.38	0.37	3.94	0.42
MUN[1]	12.13	3.71	13.52	3.82	11.10	3.43	13.78	3.80
SCC[2]	184.07	280.36	182.19	283.26	194.65	311.94	222.54	307.12
Diet								
DM	32.50	7.49	36.10	10.05	38.50	8.34	30.19	7.59
CP	16.46	1.31	16.13	1.49	16.43	1.29	17.10	1.61
RUP[3]	34.34	3.94	34.03	4.51	34.40	3.60	35.00	3.47
NE_L[4]	1.60	0.14	1.61	0.10	1.63	0.16	1.70	0.25
ADF	23.07	3.41	24.30	3.86	22.09	3.25	21.60	4.36
NDF	38.06	5.41	39.50	6.08	36.20	5.24	35.21	7.12
Fat	3.25	0.45	3.24	0.47	3.40	0.49	4.25	1.57
NSC	34.41	4.98	33.52	5.44	36.10	4.78	35.50	5.64
Ca	0.86	0.14	0.84	0.15	0.84	0.13	0.94	0.17
P	0.45	0.06	0.42	0.06	0.44	0.05	0.47	0.08
Mg	0.28	0.04	0.27	0.05	0.28	0.04	0.30	0.05
K	1.63	0.31	1.72	0.36	1.58	0.29	1.52	0.31
Na	0.24	0.11	0.20	0.11	0.25	0.11	0.33	0.18
Cl	0.60	0.10	0.58	0.12	0.59	0.11	0.64	0.14

[1]mg dL^{-1}
[2]1,000'
[3]% of CP
[4]Mcal kg^{-1}

Table 2. Mean and SD of MUN (mg dL^{-1}) by parities in Ayrshire, Brown Swiss, Holstein and Jersey cows

	1st Parity		2nd Parity		3rd Parity		4th Parity	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Ayrshire	11.82	3.67	12.30	3.70	12.34	3.77	12.39	3.70
Brown Swiss	13.20	3.78	13.70	3.89	13.65	3.74	13.77	3.87
Holstein	10.78	3.39	11.25	3.43	11.29	3.43	11.32	3.43
Jersey	13.51	3.86	13.99	3.78	13.93	3.72	13.80	3.77

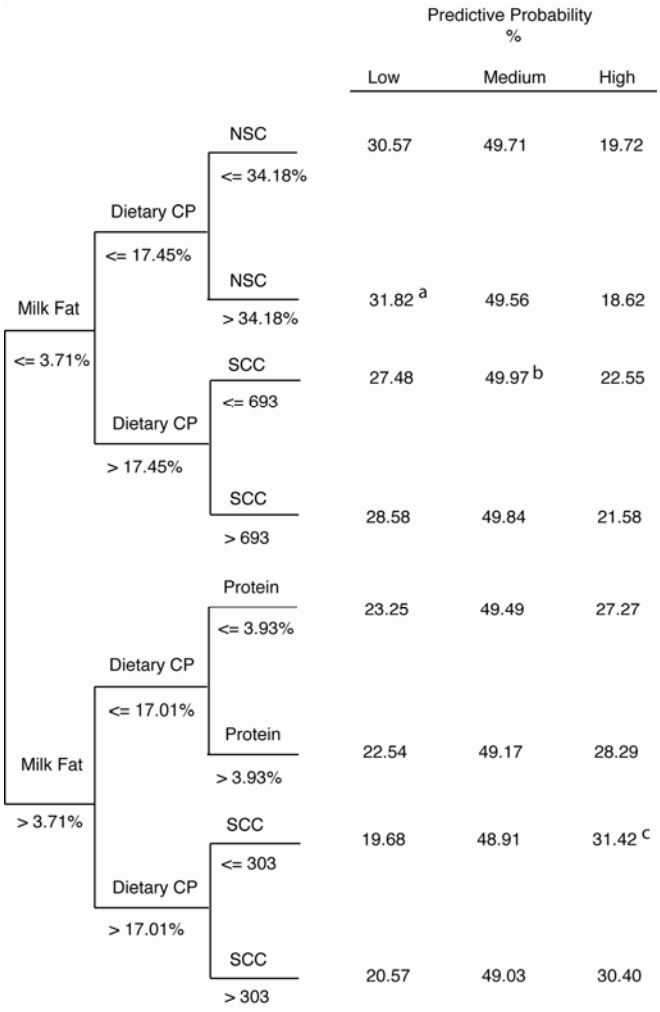

Figure 1. Decision-tree classification of multiple interactions altering low, medium and high concentrations of MUN. The predictive probability values represent the percentage of data correctly classified in low, medium, and high MUN levels at each interaction. The cut points are shown below nodes or variables. The highest predictive probability percentages are denoted at low (a), medium (b), and high (c) levels of MUN.

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Effects of feeding forage soybean silage on milk production, nutrient digestion and ruminal fermentation of lactating dairy cows

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ABSTRACT

The objective of this study was to determine the feeding value of forage soybean silage (SS) for dairy cows relative to a fourth cut alfalfa silage (AS). Two iso-nitrogenous diets were formulated with 48:52 forage:concentrate ratio. Soybean silage and AS comprised 72% of the forage in each diet with corn silage comprising the remaining 28%. Twenty Holsteins cows in early lactation were used in a switchback design. Four cows fitted with ruminal cannulae were used to determine the effects of dietary treatments on ruminal fermentation parameters and total tract nutrient utilization. Dry matter intake (23.5 vs. 25.1 kg/d) and milk yield (35.5 vs. 37.2 kg/d) were lower for cows fed SS than those fed AS. However, energy corrected milk and milk efficiency were similar for both dietary treatments. Milk protein, lactose and total solid concentrations were not influenced by dietary treatments. However, cows fed SS produced milk with higher milk fat and milk urea concentrations compared with cows fed AS. Ruminal pH was lower while ruminal ammonia N concentration was higher in cows fed SS than in cows fed AS. Total tract digestibility of dry matter, crude protein, and neutral detergent fiber were not influenced by silage type. It was concluded that forage SS when compared with AS, had a negative impact on feed intake and milk yield, while energy-corrected milk, milk efficiency and total tract nutrient digestion were similar.

Keywords*:* milk yield and composition, soybean silage, nutrient utilization

INTRODUCTION

Soybean was first introduced and utilized in North America as a forage crop. Until the early 1940's, more than half of the surface area planted to soybeans in the USA were harvested as forage (Hartwig, 1973). In eastern Canada and the northern USA, interest in soybean as a source of forage has increased in recent years, in part due to the recurrence of alfalfa winterkill and the increase in corn acreage, with the resulting preference for shorter crop rotations. Consequently, several new forage type cultivars have been developed specifically for these regions (Sheaffer et al., 2001). Dry matter yields of forage cultivars range between 4.5 and 14 tons/ha in eastern Canada and the northern USA. Forage cultivars are usually taller and later maturing than typical oilseed cultivars (Seiter et al., 2004). To our knowledge no information is available regarding the feeding value of forage soybean silage for dairy cows. Our objectives were to determine effects of feeding forage soybean silage to lactating dairy cows on milk yield and composition, ruminal fermentation and total tract nutrient utilization.

MATERIALS AND METHODS

Silage preparation

Forage soybean (cultivar Kodiac) was seeded in the last week of May 2005 in Ste-Anne-de-Bellevue, QC, (seeding rate of 70 kg/ha, 7,980 seeds/kg, 558,600 seeds/ha). The soybean forage was harvested when lower leaves

started to become yellow. A fourth cut alfalfa forage was harvested at early bloom stage. All forages were wilted to a targeted 30% DM, chopped to a theoretical cut length of 10 mm and then ensiled in tower silos for three months.

Production study

Twenty Holstein cows of mixed parities in early to mid lactation were blocked by parity and divided into two groups of 10 cows each. Two iso-nitrogenous diets were formulated with 48:52 forage:concentrate ratio. The forage part of the diets consisted of 72% soybean silage or 72% alfalfa silage with the remaining 28% consisting of corn silage in both diets (Table 1). Diets were offered *ad-libitum* as TMR twice daily.

Experimental periods (n = 3) consisted of 10 d of diet adaptation and 14 d of data collection in a switchback design. Feed intake and milk yield were measured on d 11 to 24 of each period. Cows were milked twice daily and daily milk samples were pooled by proportion according to milk yield at each milking. Diets were sampled daily during each collection period and composited by period. The composited feed samples were oven-dried at 60 °C for 48 h, ground through a 1-mm screen and stored at room temperature for later analysis.

Ruminal Fermentation Parameters and Total Tract Nutrient Utilization

Four lactating Holstein cows fitted with ruminal cannulae were used in a switchback design with three periods (n = 3) consisting of 14 d of diet adaptation and 7 d of data collection. Dietary treatments were the same as in the production study.

Chromic oxide was used as an inert external marker to determine total fecal output. Grabbed fecal samples were collected 4 times daily during the last 3 d of each period. Feed samples were collected during the fecal collection period and were dried as previously described and pooled by treatment within each period.

Samples of ruminal fluid were collected on d 19 of each prior to morning feeding (0 h) and at 2, 4, 6, 8, 10 and 12 h post-feeding and ruminal pH was determined immediately. Separate samples were collected at the same time for later analysis of volatile fatty acid and NH_3-N.

Chemical Analyses

Ground feed and silage samples were analyzed for DM, ash, and ether extract using standard procedures (AOAC 1990). Neutral detergent fiber and ADF were determined using an Ankom Fiber Analyzer. Acid detergent lignin (ADL) was determined according to AOAC (1990). Crude protein (N x 6.25) was measured using a Leco Nitrogen Analyzer. Soluble protein and NPN were determined according to Licitra et al. (1996) while acid (ADICP) and neutral detergent insoluble protein were determined by analyzing NDF and ADF residues for total N. Gross energy (GE) of feed samples were determined using an oxygen bomb calorimeter Ground fecal samples were analyzed for DM, ash, CP, NDF, and GE as previously described. Chromic oxide in fecal samples was determined according to the procedure of Fenton and Fenton (1979).

Milk samples were analyzed for fat, protein, lactose and MUN using infrared analyzer (Milk-o-Scan, model: Foss 4000, Foss Food Technology, Denmark). Milk total solids were determined according to AOAC (1990).

Ruminal VFA and NH_3-N concentrations were determined as described by Gonthier et al. (2004).

Statistical Analysis

Data of the production study and total tract nutrient utilization were analyzed as Switch-back design using PROC MIXED of SAS (1999). Data of volatile fatty acids, ruminal pH and NH_3-N were analyzed as repeated measurements across time using PROC MIXED of SAS (1999). Data from the *in situ* trial were analyzed as a randomized complete block design using cows as blocks. Significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Chemical Composition and in Situ Ruminal Degradabilities of Soybean Silage

Chemical composition of SS and AS is shown in Table 2. Relative to AS, SS contained 11% more NDF, 40% more ADL and 25% less CP. *In situ* soluble DM and NDF fractions were higher ($P < 0.05$) for AS than SS while *in situ* soluble CP fraction was similar for both silages (Table 3). Slowly degradable DM, CP and NDF fractions were not affected by silage type and averaged 38.6, 30.8, and 44.5%, respectively.

Relative to alfalfa silage, SS had slower ($P < 0.05$) rates of degradation of DM, CP and NDF fractions. Effective ruminal degradability of DM, CP and NDF were all higher ($P < 0.05$) for AS than SS (Table 3).

Differences in ruminal nutrient degradabilities between SS and AS were mainly due to differences in ruminal nutrient degradation rates particularly that of NDF (Table 3). The slower rate of degradation of the potentially degradable NDF fraction of SS relative to AS can be attributed to its higher ADL concentration (Table 2). Lignin has been proposed as a main factor in reducing forage fiber digestibility (Jung et al., 1997). The higher rate of degradation of the slowly degradable CP fraction of AS relative to SS is likely due to its lower ADICP concentration. A strong negative correlation between rate of degradation of slowly degradable CP fraction and concentration of ADICP has been reported for forages (Hoffman et al. 1999).

Intakes and Milk Yield and Composition

Cows fed SS consumed less ($P < 0.05$) DM, CP and NDF than cows fed AS (Table 4). Milk yield and solid corrected milk were higher ($P < 0.05$) for cows fed AS than for those fed SS. However, energy corrected milk, 4% fat-corrected and milk efficiency were similar for both dietary treatments. Diet NDF content and digestibility are major factors affecting DM intake and milk yield of dairy cows particularly in early lactation where DM intake is often limited by rumen fill (Allen 2000). The reduction DM intake and consequently milk yield for cows fed SS diet can be attributed at least in part to the higher (7.6% more) NDF content of SS diet relative to AS diet (Table 1). Neutral detergent fiber has been used as a chemical predictor of DM intake (Waldo 1986).

Neutral detergent fiber of SS was less degradable than that of AS (Table 2) which could also contribute to the reduced DM intake of cows fed SS diet relative to those fed AS. The effect of dietary NDF digestibility on DM intake of dairy cows is well documented and several authors have reported positive relationship between DM intake and NDF digestibility (Oba and Allen, 2000). Despite the negative effect of SS on DM intake and milk yield, milk efficiency was similar for both dietary treatments suggesting efficient utilization of nutrients from SS by dairy cows.

Milk fat and milk urea nitrogen concentrations were higher ($P < 0.05$) in milk of cows fed SS than in milk of cows fed AS (Table 4). However, milk protein and lactose concentrations were unaffected by silage type. Apparent milk fat depression might result from increased milk fluid yield relative to milk fat yield (i.e. dilution effect) which explains the lack of difference in energy-corrected milk between dietary treatments (Table 4).

Ruminal Fermentation and Total Nutrient Digestibilities

Total and molar proportions of volatile fatty acids were not influenced by dietary treatments (Table 5). Ruminal pH and NH_3-N were lower ($P < 0.05$) in cows fed AS than in cows fed SS. However, differences in pH were small and are not expected to be biologically significant. Differences in NH_3-N concentration between dietary treatments are likely due to the addition of urea to the SS diet (Table 1). The higher ruminal NH_3-N concentration for cows fed SS relative to those fed AS help to explain the higher MUN content in their milk.

Total tract digestibility of DM, organic matter, CP, NDF, and GE were similar for the two dietary treatments and averaged 72.1, 72.9, 70.1, 55.7, and 71.0%, respectively (Table 6). The lack of differences in total tact nutrient utilization between SS and AS diets can be attributed, at least in part to differences in DM intake. High DM intake especially for diets containing more digestible fiber is usually associated with decreased total tract nutrient digestibilities due to a reduced ruminal retention time and therefore increased passage rate. It is also possible that cows fed SS diets had higher post-ruminal fermentation than cows fed AS diet to compensate for reduced ruminal fiber degradability.

CONCLUSIONS

Under the conditions of our study, feeding forage soybean silage reduced dry matter intake and milk yield of dairy cows when compared with alfalfa silage likely due to an increased content of neutral detergent fiber and its reduced ruminal degradability. Despite the negative impact on ruminal fiber degradability, forage soybean silage had no effect on energy-corrected milk, milk efficiency and total tract nutrient utilization. More research is needed to determine the optimum stage of development at harvest and the long term effects of feeding forage soybean silage to dairy cows.

Table 1. Ingredients and chemical composition of dietary treatments (DM basis)

	Dietary treatment	
	Soybean silage	Alfalfa silage
Ingredients (%)		
Soybean silage	36.0	0.0
Alfalfa silage	0.0	36.0
Corn silage	12.0	12.0
Crushed corn grain	37.0	37.4
Canola meal	2.2	2.2
Corn gluten meal	1.7	1.7
Corn distiller's grains	4.4	4.4
Protein supplement	4.3	4.4
Sodium bicarbonate	0.8	0.8
Mineral mix	1.1	1.1
Urea	0.5	0.0
Chemical composition		
DM (%)	54.4	56.5
Ash (%)	6.3	6.6
Ether extract (%)	4.6	4.7
NDF (%)	36.7	34.1
ADF (%)	24.3	22.6
Acid detergent lignin (%)	5.4	4.9
CP (%)	18.6	19.0
NE_l (Mcal kg^{-1})	1.59	1.64

Table 2. Chemical composition (mean ± SD) of soybean and alfalfa silages (DM basis).

	Silage	
	Soybean	Alfalfa
NDF (%)	46.9 ± 1.0	42.5 ± 0.4
ADF (%)	37.7 ± 1.4	32.4 ± 0.7
Acid detergent lignin (%)	11.0 ± 0.7	7.6 ± 0.5
CP (%)	18.4 ± 2.6	24.4 ± 0.6
Soluble Protein (% of CP)	58.2 ± 3.6	64.0 ± 2.8
NPN (% of CP)	56.7 ± 2.7	61.2 ± 4.9
Neutral detergent insoluble protein (% of CP)	28.5 ± 2.7	18.6 ± 1.8
Acid detergent insoluble protein (% of CP)	12.3 ± 3.6	8.8 ± 1.6
NE_l (Mcal kg^{-1})	1.20 ± 0.02	1.44 ± 0.06

Table 3. In situ ruminal nutrient degradabilities of soybean silage relative to alfalfa silage

	Silage			
	Soybean	Alfalfa	SEM[1]	P value
DM				
Soluble fraction (%)	33.8	40.0	0.46	< 0.001
Slowly degradable fraction (%)	39.5	37.7	1.14	0.16
Degradation rate (% h^{-1})	6.0	8.0	0.80	< 0.02
Effective degradability (%)	53.3	63.9	0.73	< 0.001
CP				
Soluble fraction (%)	60.1	59.6	2.15	0.18
Slowly degradable fraction (%)	30.4	31.2	2.10	0.73
Degradation rate (% h^{-1})	6.5	9.4	0.83	0.034
Effective degradability (%)	76.9	79.9	0.59	0.004
NDF				
Soluble fraction (%)	10.0	15.3	0.67	< 0.001
Slowly degradable fraction (%)	43.9	45.1	2.27	0.63
Degradation rate (%h^{-1})	4.8	6.1	0.50	0.04
Effective degradability (%)	31.2	40.6	0.75	< 0.001

[1]SEM = Standard error of the mean.

Table 4. Effects of feeding soybean silage on performance of dairy cows

	Dietary treatment			
	Soybean silage	Alfalfa silage	SEM[1]	*P* value
Intake (kg d^{-1})				
DM	23.5	25.1	0.67	0.033
CP	4.0	4.9	0.15	0.001
NDF	7.4	9.3	0.25	< 0.001
Milk composition (%)				
Fat	3.78	3.58	0.051	0.017
Protein	3.17	3.18	0.022	0.76
Lactose	4.69	4.69	0.012	0.89
Total solids	12.65	12.61	0.079	0.73
MUN (mg dL^{-1})	15.67	14.03	0.164	0.017
Yield (kg d^{-1})				
Milk	35.5	37.2	0.47	0.002
Energy-corrected milk	32.1	32.9	0.33	0.33
4% Fat-corrected milk	34.3	34.8	1.14	0.31
Solid-corrected milk	33.5	34.4	1.09	0.035
Fat	1.35	1.33	0.019	0.39
Protein	1.09	1.16	0.006	< 0.001
Lactose	1.67	1.74	0.012	< 0.001
Total solids	4.49	4.71	0.079	0.004
Milk efficiency	1.56	1.52	0.012	0.34

[1]SEM = Standard error of the mean.

Table 5. Effects of feeding soybean silage on ruminal fermentation parameters of ruminally cannulated cows

	Dietary treatment			
	Soybean silage	Alfalfa silage	SEM[1]	*P* value
pH	6.44	6.34	0.03	0.011
NH_3-N (mg dL^{-1})	23.8	18.5	1.36	<0.001
Volatile fatty acids (m*M*)	78.6	81.6	3.08	0.34
Molar proportion				
Acetate	66.2	63.9	2.41	0.34
Propionate	25.2	27.6	1.67	0.18
Butyrate	15.1	16.0	0.78	0.25

[1]SEM = Standard error of the mean.

Table 6. Effects of soybean silage on total tract nutrient utilization by ruminally cannulated cows

	Dietary treatment			
	Soybean silage	Alfalfa silage	SEM[1]	P value
Intake (kg d^{-1})	26.0	27.7	0.19	0.025
Digestibility (%)				
DM	71.1	70.9	8.9	0.88
OM	71.8	71.7	10.3	0.95
CP	70.6	69.5	18.1	0.74
NDF	57.8	53.5	32.2	0.27
Gross energy	71.9	70.0	1.83	0.32
Digestible energy (Mcal kg^{-1})	3.11	3.01	0.03	0.17

[1]SEM = Standard error of the mean.

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Effects of a propionic acid-based additive on short-term ensiling characteristics of corn and on dairy cow performance

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ABSTRACT

Dairy cattle producers occasionally face situations where it is necessary to open silos before the completion of ensiling process due to feed shortage. The objectives of this study were to determine the effects of a propionic acid-based silage additive (i.e., Solution Foin; 70% propionic acid and 30% NH_4OH) on ensiling characteristics, aerobic stability and feeding value of short-term ensiled forage corn. Chopped whole corn was left untreated or treated with Solution Foin. The additive was added prior to ensiling at a rate of 5 L/ton (wet basis). Treated and untreated forages were placed in plastic silo bags, which were opened one day after ensiling and sampled daily for 30 consecutive days. Animal performance were determined using lactating cows fed total mixed ration with the major forage portion consisted of untreated or treated corn. The feeding study started one day post-ensiling. Solution Foin reduced yeast and mold populations between day 5 and 14 post-ensiling. The largest differences were observed on day 10, where yeast and mold populations for untreated ensiled corn were 7.86 and 2.51 log cfu g^{-1}, respectively, the corresponding values for treated corn were 4.35 and 0 log colony forming unit/g, respectively. Solution Foin improved aerobic stability between day 0 (by 159 h) and day 10 (by 33 h) post-ensiling. No differences in pH or concentrations of organic acids were observed between treated and untreated corn. Dry matter intake (average 23 kg^{-1}) and milk yield (average 29 kg^{-1}) were similar for cows fed treated and untreated corn. Solution Foin can be used to improve the aerobic stability of partially ensiled corn, likely by reducing yeast and mold populations.

Keywords: **C**orn silage; Ensiling; Propionic acid; Yeast; Mold; Aerobic stability

INTRODUCTION

Organic acids have been used extensively as forage and grain preservatives. The addition of organic acids can improve silage quality by accelerating the decline in pH during the early stages of the ensiling process and / or by acting as antifungal agents. Lambert and Stratford (1999) showed that inhibition of microbial growth by weak-acids involves rapid diffusion of undissociated molecules through the plasma membrane. The dissociation of these molecules within cells liberates protons, thus acidifying the cytoplasm and preventing fungal growth. Relative to other short chain organic acids, propionic acid have the greatest antifungal activity (Moon 1983). The antifungal property of propionic acid increases as pH declines, which makes it an ideal preservative for cereal forages.

Producers occasionally face situations where it is necessary,

due to feed shortage, to open silos before completion of the ensiling process. Unfermented or partially fermented forages are less stable once exposed to air. Inadequate fermentation frequently accelerates silage deterioration, since aerobic microbes are still active. No studies have been conducted to evaluate the effects of propionic acid-based additives on aerobic stability and feeding value of unfermented or partially fermented silages.

MATERIALS AND METHODS

Forage Preparation

Whole-plant corn was harvested at one-half milkline, rolled and cut to a theoretical length of 0.75 cm with a forage harvester. Chopped forage was left untreated or treated with a silage additive - Solution Foin (Agro-Bio Contrôle inc., Saint-Charles-Sur-Richelieu, QC, Canada) to obtain a final application rate of 5 L/ton of fresh forage weight. The active ingredient in the additive was ammonium propionate (700 g/kg propionic acid and 300 g/kg NH_4OH). Alternate loads of the chopped forage were either treated or left untreated, and then packed into six plastic silo bags (three replicates for each treatment) using a press-bagging machine. The six silo bags were opened one day after ensiling.

Ensiling Parameters

Representative corn samples (3 to 5 kg) were taken daily from each silo bag and kept frozen for later analysis. Temperature was measured daily for 30 days by inserting a glass thermometer 2 and 20 cm inside the ensiled forages at four different locations. Dry matter of sampled corn was determined in a forced-air oven at 60°C for 48 h. Fifty g samples of forage from each silo bag were homogenized for 10 min in 500 ml of distilled water and the pH of the water extract was immediately measured, using a pH meter. Lactic acid and water-soluble carbohydrates (WSC) were determined on the water extract using colorimetric methods. Water extracts were also analyzed for volatile fatty acids by gas chromatography.

Microbial Population

Fifty g of fresh forage samples taken on day 0, 1, 3, 5, 7, 10, 12, 14, 17, 21 and 26 post-ensiling were homogenized in 500 ml of sterile peptone water (0.1%) in a blender for 1 min and used for culturing. Subsequent serial 10-fold dilutions were made, to obtain 30 to 300 colonies per dish. Lactic acid bacteria (LAB) were enumerated in triplicate by pour plating of Rogosa SL agar. Yeasts and molds were enumerated in triplicate pour plates of malt extract agar. Aerobic stability was determined for forage samples taken on day 0, 5, 10, 15 and 20 post-ensiling according to Hassanat et al. (2007)

Dairy Production Study

Two diets with 50:50 forage:concentrate ratio were formulated to meet the nutrient requirements of lactating dairy cows (Table 1). In both diets, treated or untreated ensiled corn was the main source of forage. Equal amounts of ensiled corn were removed daily from each silo bag, one day after ensiling and the silo bags were sealed after. Diets were offered twice daily as total mixed diets.

Thirty lactating Holstein cows were blocked by parity and days in milk, and randomly assigned to one of the dietary treatments (15 cows each) for 30-day period. Animals within each dietary treatment were subdivided into three groups in such a way that every five cows were fed forage from the same silo bag. Cows were milked three times a day and milk yields were recorded at each milking and milk samples were collected once a week from the three milkings, for milk component analysis.

Milk samples were analyzed for fat, protein, lactose and milk urea nitrogen at the Dairy Herd Analysis Service (Valacta, Sainte-Anne-de-Bellevue, QC, Canada) with an infrared system (Milk-O-Scan 4000).

Statistical Analyses

Chemical composition data were analyzed using the GLM procedure of SAS (SAS Institute Inc., 1989) with a randomized complete block model with split-plot restriction and three replications. Main plots were the additive treatments and subplots were the ensiling periods. When interactions were significant, data were also reanalyzed using a randomized complete block model for each ensiling period or treatment. Data from the production study were analyzed using the mixed model procedure (SAS 1989) in completely randomized design with cows as a random effect. When significant effects were detected ($P<0.05$), least significant difference was used to determine significant differences among means.

RESULTS

The pH of treated pre-ensiled corn was lower (P<0.05) than that of untreated one likely due to the addition of propionic acid (Fig. 1). Solution Foin had no effect on WSC concentration during ensiling except at day 7 post-ensiling where residual WSC concentration was higher (P<0.05) for treated than untreated corn (Fig. 2). As expected, propionic corn between day 14 and day 17 post-ensiling with no effect of the additive being observed beyond this point.

Solution Foin improved (P<0.05) aerobic stability of corn removed at day 0, 5 and 10 post-ensiling with the highest effect noted for pre-ensiled forages (i.e. day 0) (Fig. 6). Aerobic stability was also numerically higher (42 vs. 85 h) for treated than untreated corn at day 15 post-ensiling. However, Solution Foin had no effect on aerobic stability at day 20 post-ensiling. Both untreated and treated corn removed at day 20 post-ensiling remained stable for more than a week with no difference between treatments. Solution Foin had no effect on feed intake of dairy cows, which averaged 23.1 kg d^{-1} (Table 2). Milk yield and milk composition were also similar for cows fed treated or untreated corn (Table 2).

DISCUSSION

Overall, Solution Foin had no effect on pH or residual WSC of ensiled corn, which is in agreement with other studies that also reported a lack of effect of propionic acid additives on silage pH (Kleinschmit et al. 2005). Similar to our findings, Mill and Kung (2002) observed a decline in propionic acid concentration from buffered propionic acid additives during ensiling. Factors such as stability can affect the degradation of propionic acid during ensiling and storage (Mill and Kung 2002).

The inhibition of microbial growth by weak acids involves rapid diffusion of undissociated molecules through the plasma membrane (Lambert and Startford 1999). The dissociation of these molecules within cells liberates protons, thus acidifying the cytoplasm and preventing fungal growth. A reasonable explanation for the lack of differences in fungal populations between day 0 to day 5 (Fig. 5) could be attributed to the relatively high pH during that period (Fig. 2) which might have resulted in low concentration of the undissociated molecules. After day 14 of ensiling, the ensiled forages were well fermented, and the lower pH presumably inhibited the fungal growth in the untreated silage. This might explain the lack of difference in yeast and mold populations between d 17 and d 26 post-ensiling.

During the early stages of the ensiling process, fermented forages are aerobically unstable

acid concentration was only detected in treated corn at all ensiling times (Fig. 3). The concentration was highest (P<0.05) at day 0 (i.e. > 0.2 g/kg) and decreased to < 1 g kg^{-1} by day 10 post-ensiling. Concentrations of lactic acid and acetic acid were not influenced by Solution Foin throughout ensiling (Fig. 3). For both treated and untreated ensiled corn, LAB increased (P<0.05) between day 0 and day 12 post-

ensiling then declined rapidly (*P*<0.05) between day 14 and day 21 post-ensiling (Fig. 4). Solution Foin had little impact on the LAB population throughout the ensiling period.

Solution Foin reduced (P<0.05) both yeast and mold populations between day 5 and day 14 post-ensiling (Fig. 5). Yeast and mold populations declined (P<0.05) sharply for untreated and treated due to relatively high yeast and mold population and low lactic acid concentration. It is well documented that yeasts are the main microorganisms responsible for aerobic deterioration (Woolford 1990; Kung et al. 1998). Due to their antimycotic properties, propionic acid-based additives such as Solution Foin can significantly reduce yeast and mold populations and therefore improve aerobic stability of partially fermented forages (Fig. 6). The anaerobic and low pH environment of the stable phase of the ensiling process is hostile to most microorganisms (Pahlow et al. 2003; Kunkle et al. 2006). As a result, yeast and mold populations are expected to decline significantly during this phase, which was evident by the low counts of these microorganisms by day 17 post-ensiling (Fig. 5). This helps to explain the lack of effect of Solution Foin in aerobic stability after day 15 post-ensiling. The effects of propionic acid additives on aerobic stability of fully fermented silages are inconsistent. Aerobic stability was improved in some studies (Kung et al., 1998) but not others (Kung et al. 2000; Mills and Kung 2002; Kung et al. 2004).

Results of the production study are in agreement with other studies, which showed no improvement in DM intake of lactating cows fed silage treated with buffered propionic acid-based additives (Kung et al. 1998). The authors attributed the lack of improvement to the fact that untreated silage was not spoiled to an extent that would have affected feed intake. Furthermore, cows in the present study were not high producers and might have not been sensitive to changes in diets.

CONCLUSIONS

Our results showed that Solution Foin is an effective inhibitor of yeast and mold growth in early stages of the ensiling process and therefore can significantly improve aerobic stability of partially fermented corn. However, in well-fermented corn, Solution Foin had no effect on aerobic stability. Solution Foin also had no impact on the fermentation characteristics of ensiled corn or on lactational performance of dairy cows. Further research is warranted to investigate the effects of Solution Foin on aerobic deterioration of other silages and total mixed diets.

Table 1. Ingredients and chemical composition of diets in the dairy cow trial.

	Ensiled corn	
	Untreated	Treated
Ingredients (g/kg of DM)		
Corn silage	331	331
Alfalfa hay	245	245
Cracked corn	308	308
Commercial protein supplement[a]	61	61
Soy bean meal	39	39
Mineral mix[b]	7	7
Sodium bicarbonate	9	9
Chemical composition (g/kg of DM)		
Dry matter	479	496
Crude protein	166	165
Natural detergent fibre	303	280
Acid detergent fibre	165	149
Acid detergent lignin	22	21

[a]Contained (g/kg) 60 acid detergent fibre, 100 neutral detergent fibre, 40 ether extract, 500 crude protein, 25 Ca, 15 P, 8 Mg, 3 Na, 5 K, 7.5 NaCl, 5 S.
[b]Contained (g/kg) 10 Na, 180 Ca, 90 P, 80 Mg, 12 S; (mg/kg) 144 I, 7,500 Fe, 2,400 Cu, 7,200 Mn, 7,200 Zn, 24 Co, 500 F; (IU/kg) 750 vitamin A, 225 vitamin D, 3 vitamin E.

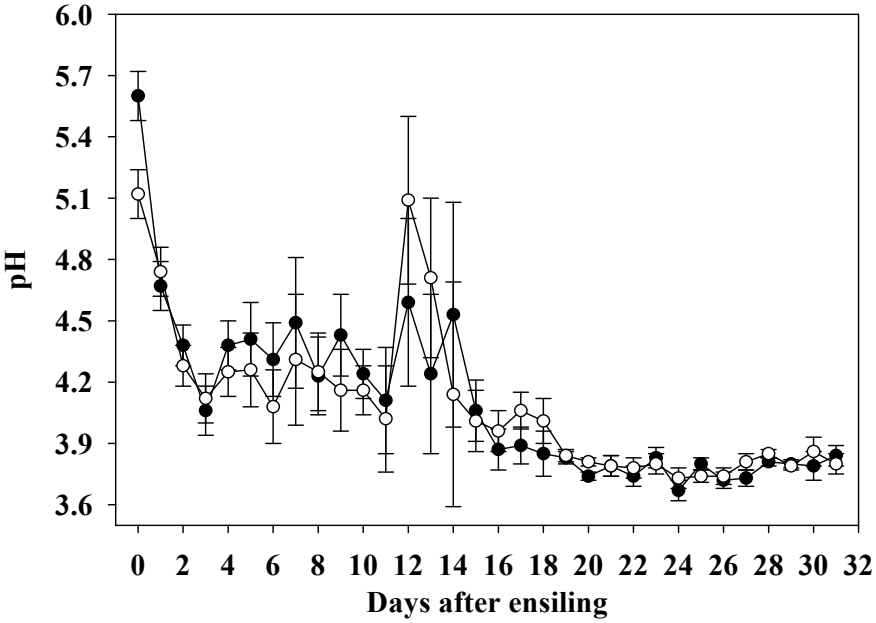

Figure 1. Changes in pH during ensiling of corn treated (○) or not (●) with Solution Foin. Vertical bars represent ± SE.

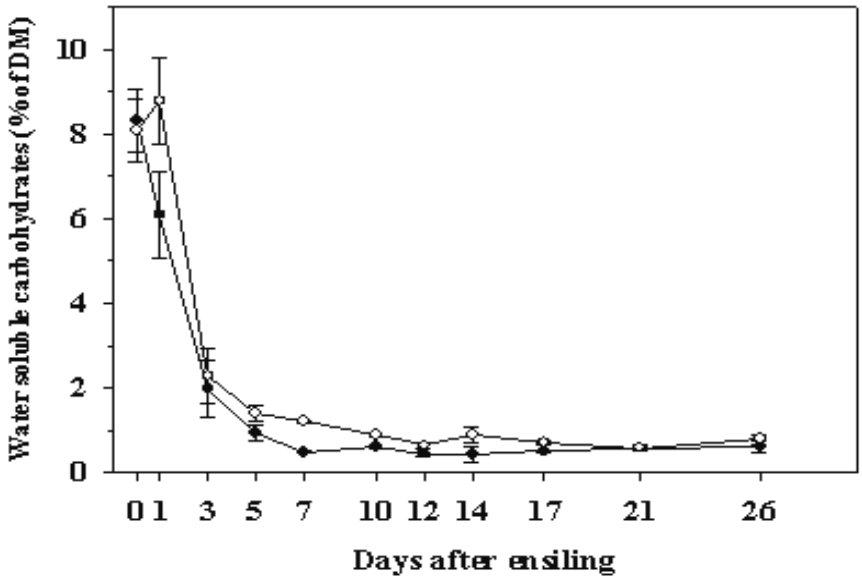

Figure 2. Water-soluble carbohydrates concentration during ensiling of corn treated (○) or not (●) with Solution Foin. Vertical bars represent ± SE.

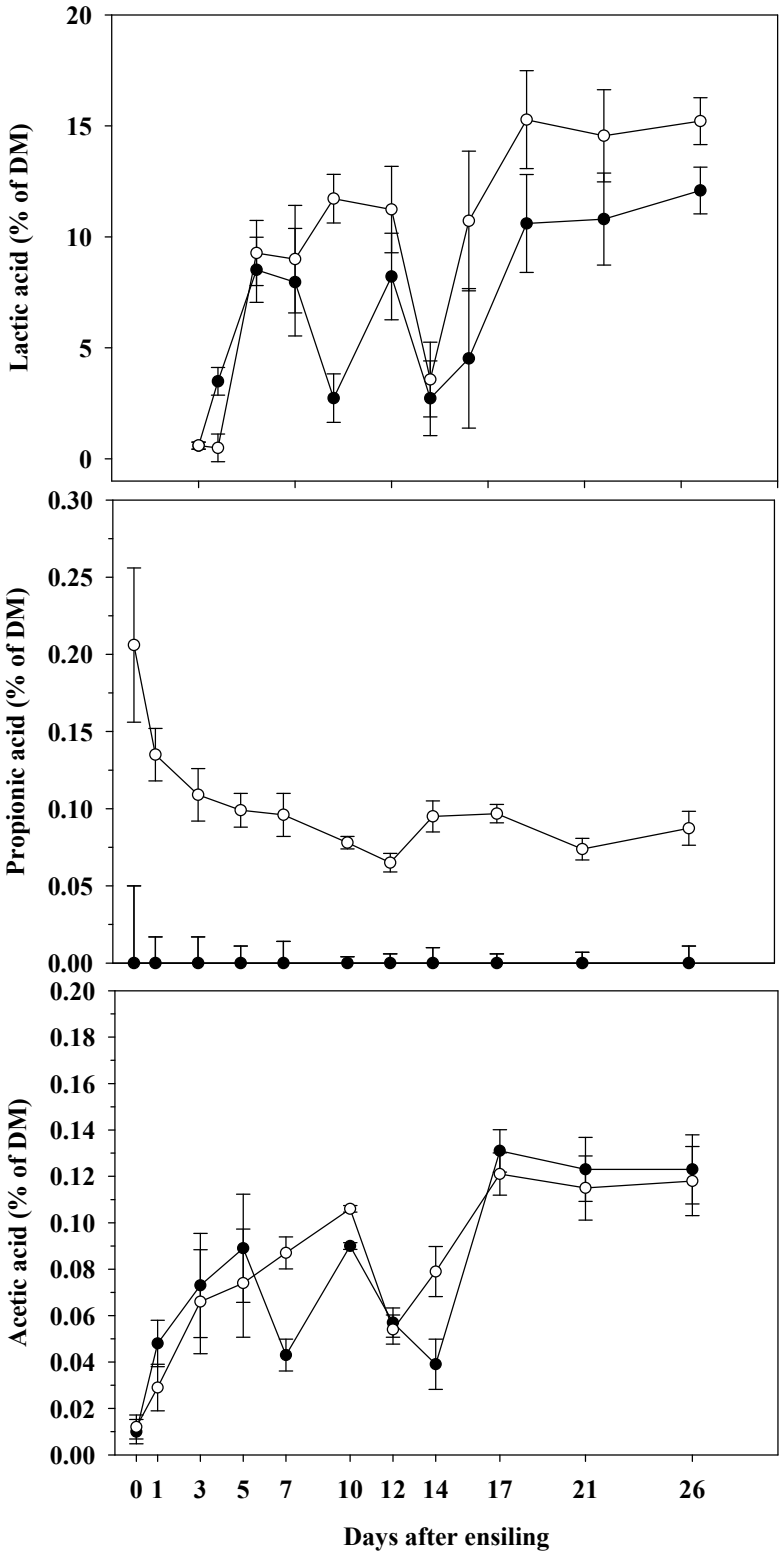

Figure 3. Lactic acid, propionic acid, and acetic acid concentrations during ensiling of corn treated (○) or not (●) with Solution Foin. Vertical bars represent ± SE.

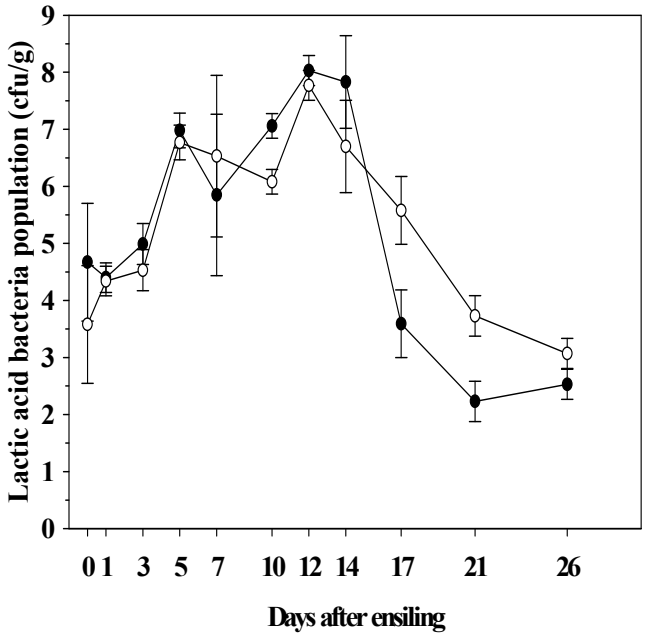

Figure 4. Lactic acid bacteria population during ensiling of corn treated (○) or not (●) with Solution Foin. Vertical bars represent ± SE.

Table 2. Effects of corn treatment with Solution Foin, a propionic acid-based silage additive, on performance of dairy cows.

	Untreated	Treated	SEM[a]
Dry matter intake (kg/d)	22.8	23.4	0.9
Milk yield (kg/d)	28.7	29.3	1.7
Milk fat (%)	3.13	3.39	0.17
Milk protein (%)	3.51	3.41	0.08
Milk lactose (%)	4.56	4.64	0.08
Milk urea nitrogen (mg/dL)	10.3	11.1	0.66

[a]Pooled standard error of the mean.

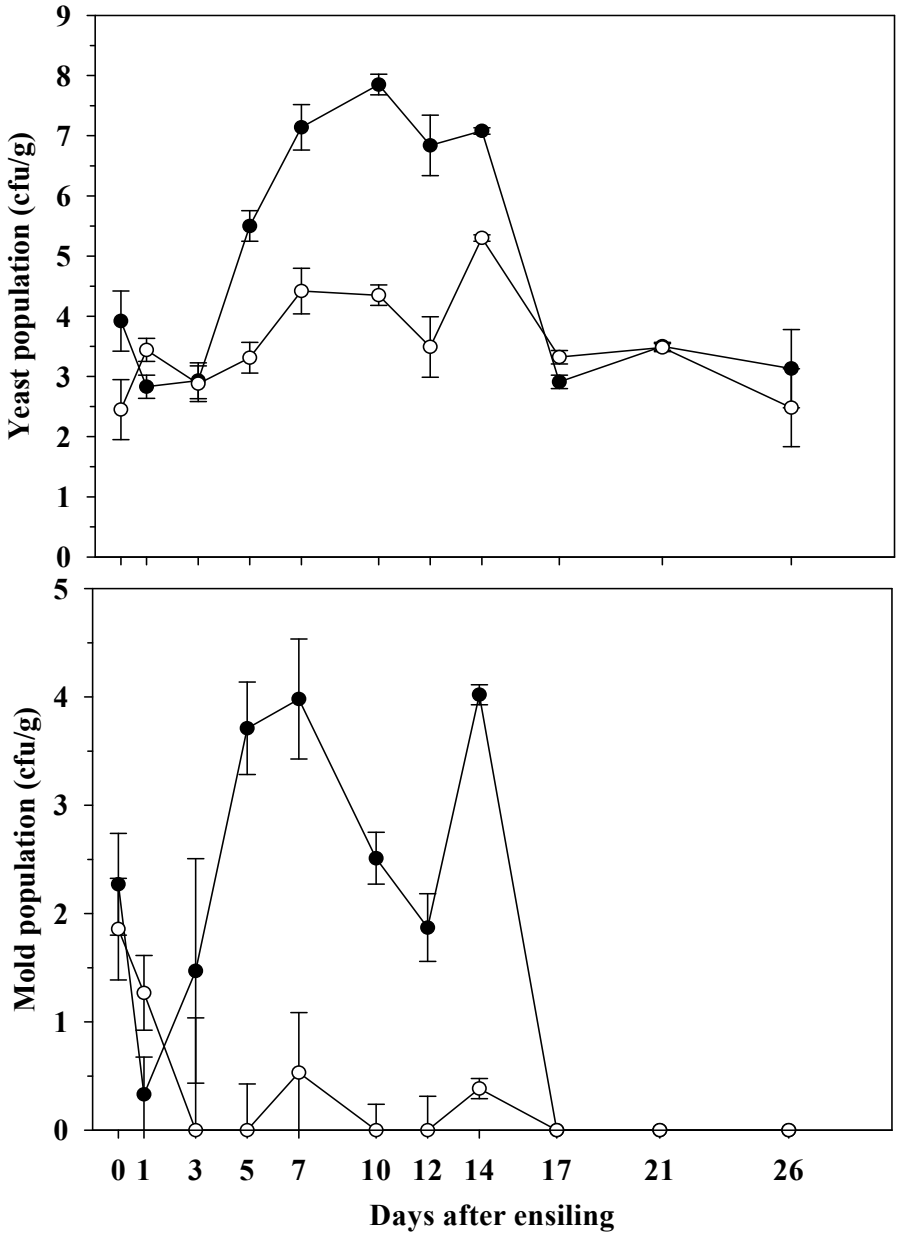

Figure 5. Yeast and mold populations during ensiling of corn treated (○) or not (●) with Solution Foin. Vertical bars represent ± SE.

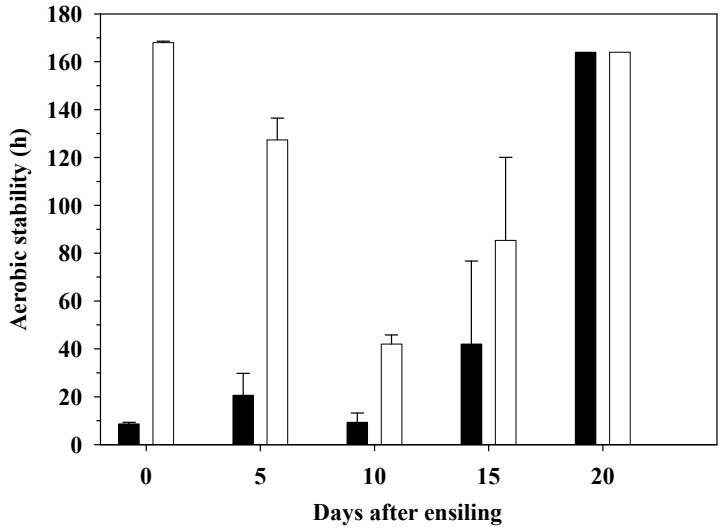

Figure 6. Changes in aerobic stability of corn treated (□) or not (■) with Solution Foin. Vertical bars represent ± SE.

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Effects of feeding high levels of cactus (*Opuntia fícus-indica* Mill) cladodes on urinary output and electrolyte excretion in goats

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ABSTRACT

A study was conducted to determine the effects of feeding spineless cactus cladodes on diuresis and urinary electrolyte excretion in goats. Five bucks were used in a 5 x 5 Latin square experiment with 17-day periods. Experimental diets contained (g kg^{-1} DM basis) 370, 470, 570, 670, and 770 spineless cactus cladodes. Water consumption from feed and urine output increased linearly ($P<0.05$) as the level of cactus cladodes in the diet increased. However, water intake from drinking was low and unaffected by cactus cladode level. Creatinine clearance and urinary Na excretion were similar for all dietary treatments while K excretion decrease linearly ($P<0.05$) as the level of cactus cladodes in the diet increased. Feeding cactus cladodes caused diuresis and reduced urinary K excretion in goats. Possible reasons for these effects include water over-consumption from cactus cladodes and high dietary K intake.

Keywords: Cactus cladodes; Diuresis; Goats; Renal excretion.

INTRODUCTION

Spineless cactus (*Opuntia fícus-indica* Mill and *Nopallea cochenillifera* Salm and Dyck) is an important feed for ruminants in north-east of Brazil especially, during the dry season due to forage shortage. Cactus cultivation in north-east Brazil stretches over 400,000 ha and the most cultivated species are *Opuntia ficus-indica* and *Nopalia cochenillifera*. Cactus cladodes are low in dry matter (DM), P and K and high in Ca, K and ash (Batista et al., 2003). Due to low DM content, high consumption of cactus cladodes may result in water loading and therefore may initiate water diuresis. Studies on sheep showed a negative relationship between cactus cladode intake and consumption of drinking water (Gebremariam et al. 2006). Cactus is also known for its diuretic and relaxant effects on the renal tract. Extracts from different parts of *Opentia spp.* increased urinary output and altered urinary electrolyte excretion in rats (Bwititi et al., 2001a,b; Galati et al., 2002). However, the effects of feeding cactus cladodes on renal function in ruminants have not been investigated. Therefore, the objectives of this study were to provide preliminary information on the effects of feeding high levels of cactus cladodes on urinary output and urinary electrolyte excretion in goats.

MATERIALS AND METHODS

The present study is a part of a larger project with portions of the results reported elsewhere (Vieira et al. 2008). Five adult Alpine bucks fitted with rumen cannulas,

were fed five diets according to a 5 x 5 Latin square design. Each period lasted 17 days with the first 10 days for diet adaptation followed by 7 days for data collection. Bucks were housed in individual pens and had free access to water. Consumption of drinking water was measured daily prior to feeding and corrected for evaporation losses.

All diets contained spineless cactus (*Opuntia fícus-indica* Mill, cultivar Gigante) cladodes, tifton hay (*Cynodon dactylon* Pers.), soybean meal and mineral mix. Ingredients and chemical composition of dietary treatments are reported by Vieira et al. (2008). Two-year-old cactus plants (subterminal cladodes) were harvested weekly from an established cactus plantation. Cladodes were then stored in a shaded area and chopped daily into small pieces using a sharp knife immediately prior to feeding. Chopped (4 mm sieve) tifton hay was the main ingredient in the first diet (contained 370 g/kg cactus of the diet DM) and cactus cladodes were included at 470, 570, 670 and 770 g/kg of the diet DM to replace tifton hay in the other four diets. Diets were offered as total mixed diets *ad libitum* once daily and the amounts fed and refused were recorded daily.

Total urine was collected during the first 24 h of each collection period. Prior to feeding, bucks were fitted with urine collection bags. Bags were attached to the animals with a universal instant adhesive to minimize urine losses. Urine was collected at the end of each urination by cutting one of the lower corners of the bag, which was then sealed with an adhesive tape. Following each collection total volume of urine was recorded, acidified with 100 ml of 1 M sulfuric acid (final pH of the urine < 3) and sub-sampled (0.1 of total volume). Blood samples were collected from the jugular vein using a vacutainer tube 4 h post-feeding.

Urine and blood plasma samples were analyzed for urea, uric acid and creatinine using a Technicon autoanalyzer. Sodium and K were analyzed by a Micronal-B242 flame photometer. Endogenous creatinine clearance (a measure of glomerular filtration rate, GFR) and electrolyte excretion rates were calculated according to Garry et al. (1990).

Data were subjected to analysis of variance for a Latin square design using PROC MIXED of SAS (1989) with the model:

$$Y_{ijk} = \mu + T_i + P_j + C_k + e_{ijk}$$

Where: Y_{ijk} is observation, μ is population mean, T_i is treatment, P_j is period, C_k is random effect of buck, e_{ijk} is residual error. Data were tested for linear and quadratic effect of cactus inclusion. Significant differences were declared if $P<0.05$.

RESULTS

The concentrations of Na and K (g kg^{-1} DM basis) for the 370, 470, 570, 670, and 770 g kg^{-1} cactus cladode diets were 0.9 and 17.5, 0.9 and 18.2, 0.9 and 18.9, 0.9 and 19.6, and 0.8 and 20.4, respectively. Potassium intake increased quadratically ($P<0.05$) while Na intake decreased quadratically ($P<0.05$) as the level of cactus cladodes in the diet increased (Table 1). Water intake from feed and urine output increased linearly ($P<0.05$) as the level of cactus cladodes in the diet increased. Plasma urea concentration decreased linearly ($P<0.05$) as cactus cladode level in the diet increased (Table 1). Urinary concentrations of creatinine, urea and K decreased linearly ($P<0.05$) as the level of cactus cladodes in the diet increased. A tendency for a linear decrease ($P=0.06$) in uric acid concentration was also noted as a result of cactus cladode inclusion.

Daily urinary excretion of urea and K decreased linearly ($P<0.05$) as the level of cactus cladodes in the diet increased (Table 1). However, daily excretion of creatinine, uric acid and Na was not influenced by cactus cladode level. Creatinine clearance and Na excretion rate were similar for all dietary treatments while K excretion rate decreased linearly ($P<0.05$) as the level of cactus cladodes in the diet increased.

DISCUSSION

Differences in Na and K intakes between dietary treatments are likely due to the fact that cactus is high in K and low in Na (Galati et al., 2002). Results for water consumption are consistent with other studies, which reported low water intake from drinking for sheep fed cactus-based diets (Gebremariam et al., 2006). Our results suggest that cactus might have provided more water than required by bucks. That was evident since water intake from feed decreased as the level of hay in the diet increased while water intake from drinking remained the same (Table 1).

Diuresis associated with cactus cladode consumption in the present study can be attributed at least in part to excessive water intake from feed. Bucks in all dietary treatments consumed more than 5 L of water from feed (Table 2). Water overload is usually associated with increased urine output (Fenske 2006). Cacti also possess diuretic properties. Galati et al. (2002) found that infusion of cladode, fruit and flower extract of *O. ficus indica* increased diuresis in rats. Similar effects have also been reported for *O. megacanth* leaves' extract (Bwititi et al. 2001a). Ingestion of *O. megacanth* increased water intake and urine volume (Bwititi et al. 2001b). Diuretic properties of cactus can partly be attributed to the effects its high K concentration exerts on renal epithelia (Galati et al., 2002). Drugs and plants with diuretic activities are characterized by high K:Na ratio (Szentmihalyi et al. 1998). The K:Na ratio for dietary treatments in our study ranged between 19:1 to 26:1 which is within the range reported for diuretic drugs and plants (i.e. 5:1 to 615:1) (Szentmihalyi et al. 1998).

The reduction in plasma and urine urea concentration as the level of cactus cladodes in the diet increased is likely due to the negative effect of cactus cladodes on ruminal ammonia production (Vieira et al. 2008). The lack of significant difference in GFR (measured as creatinine clearance) between dietary treatments suggest that feeding high levels of cactus cladodes to bucks had no adverse effects on renal function. In contrast to our findings, Bwititi et al. (2001a) reported that leaves' extract from *O. megacantha* increased GFR in rats.

In the present study cactus inclusion deceased kaliuresis with no effect on natriuresis. Effects of cacti on urinary electrolyte excretion are inconsistent. Leaves' extract of *O. megacantha* increased natriuresis and reduced kaliuresis in rats while extracts from flowers, fruits and cladodes of *O. ficus indica* had no effect on urinary electrolyte excretion (Galati et al., 2002). Our results suggest that cactus cladodes acted as K-sparing diuretic either by locking Na channels in the luminal membrane of the collecting tube or by antagonizing the action of aldosterone (Puschett, 1981). Potassium-sparing diuretics usually have little or no impact on natriuresis (Puschett, 1981).

CONCLUSIONS

Our results indicated that diuresis associated with feeding high levels of cactus cladodes to goats is mainly due to water over consumption. Cactus cladodes also manipulated renal functions by reducing K excretion. More studies are warranted to elucidate the impact of feeding cactus cladodes on renal functions in ruminants.

Table 1. Ingredient and chemical composition of experimental diets

	Cactus level in the diet (g kg^{-1} of dry matter)				
	370	470	570	670	770
Ingredients (g kg^{-1})					
Spineless cactus	373	473	572	670	765
Tifton hay	450	350	250	150	50
Soybean meal	172	172	173	175	180
Mineral salt[a]	5	5	5	5	5
Chemical composition (g kg^{-1})					
Dry matter	197	159	136	119	106
Ash	88	92	95	98	101
Ether extract	19	19	19	19	19
Crude Protein	132	129	127	125	124
Neutral detergent fiber	493	454	414	374	333
Acid detergent fiber	245	228	210	192	174
Acid detergent lignin	33	33	33	32	32
Non-fiber carbohydrates	267	306	345	384	423
Na	0.9	0.9	0.9	0.9	0.8
K	17.5	18.2	18.9	19.6	20.4

[a]Contained per kg: Ca 130 g, P 75 g, S 14 g, Na 151 g, Cl 245 g, Mg 5 g, Fe 1.500 mg, Co 100 mg, Cu 275 mg, Mn 1000 mg, Zn 2000 mg, I 61 mg, Se 11 mg

Table 2. Effects of cactus level on water intake, blood and urine metabolites and urinary excretions

	Cactus level in the diet (g kg^{-1} of dry matter)						Effect of cactus inclusion[a]	
	370	470	570	670	770	SEM	L	Q
Water intake (g d^{-1})								
From feed	5142	6102	7145	7713	7074	193.7	< 0.01	NS
From drinking	151	142	45	86	69	46.7	NS	NS
Total	5293	7800	7190	7800	7143	209.4	< 0.01	NS
Na intake (g d^{-1})	2.1	2.0	2.0	2.0	1.8	0.05	<0.05	<0.05
K intake (g d^{-1})	18.4	18.3	20.1	20.5	16.5	1.03	NS	<0.05
Urine volume (mL d^{-1})	3097	3011	4397	4706	4658	458.1	<0.01	NS
Plasma metabolites								
Urea (mg dL^{-1})	29.1	23.4	21.4	24.4	15.5	2.73	<0.05	NS
Creatinine (mg dL^{-1})	1.1	1.1	1.1	1.1	1.0	0.04	NS	NS
Uric acid (mg dL^{-1})	0.1	0.1	0.1	0.1	0.1	0.02	NS	NS
Urine metabolites								
Urea (mg dL^{-1})	374.3	409.0	259.0	322.7	162.0	83.54	<0.01	NS
Uric acid (mg dL^{-1})	2.7	3.5	2.2	1.9	1.9	0.39	0.09	NS
Creatinine (mg dL^{-1})	16.2	19.8	13.2	14.8	10.9	1.93	<0.05	NS
Na (mg dL^{-1})	45.2	24.9	24.5	20.7	30.5	8.33	NS	NS
K (mg dL^{-1})	494.9	578.9	394.7	399.8	285.7	61.95	<0.05	NS
Urinary excretion								
Urea (g d^{-1})	11.8	11.2	11.0	11.7	7.2	2.2	< 0.05	NS
Uric acid (mg d^{-1})	78.8	91.9	85.8	77.9	79.3	11.23	NS	NS
Creatinine (mg d^{-1})	474.2	533.1	513.7	480.0	455.5	42.26	NS	NS
Na (g d^{-1})	1.37	1.27	1.13	1.14	1.27	0.330	NS	NS
K (g d^{-1})	14.54	14.71	13.68	12.29	11.51	1.017	<0.05	NS
Urine excretion rate (ml min^{-1})	2.15	2.09	3.05	3.27	3.23	0.32	0.06	NS
Creatinine clearance (ml min^{-1} kg^{-1})	1.9	2.1	2.1	1.9	2.0	0.20	NS	NS
Na excretion rate (mg min^{-1} kg^{-1})	0.02	0.01	0.02	0.02	0.02	0.006	NS	NS
K excretion rate (mg min^{-1} kg^{-1})	0.25	0.25	0.25	0.21	0.21	0.019	< 0.05	NS

[a]L: Linear effect, Q: Quadratic effect
[b]NS: Not significant (P>0.05)

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Chemical composition and ruminal degradability of spineless cactus grown in northeast Brazil

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ABSTRACT

A study was conducted to determine chemical composition and ruminal nutrient degradability of eight spineless cactus cultivars grown in northeast Brazil. Results showed that neutral detergent fiber was similar for all cultivars and averaged 249 ± 7.3 g kg^{-1}. Acid detergent fiber ranged between 148 and 207 g kg^{-1} with some significant differences between cultivars. Starch and water soluble carbohydrates were similar for all cultivars and averaged 198 ± 6.3 and 155 ± 9.0 g kg^{-1}, respectively. Protein content was less than 50 g kg^{-1} with some significant differences between cultivars. Calcium was the mineral with the highest concentration followed by K and Mg with no differences between cultivars. Effective ruminal degradability of dry matter and neutral detergent fiber were unaffected by cultivar and averaged 701 ± 8.4 and 503 ± 5.8 g kg^{-1}, respectively. It was concluded that cultivars had little impact on chemical composition and ruminal degradability of spineless cactus. Based on chemical composition and in situ ruminal degradability, spineless cactus can be considered an excellent source of fermentable carbohydrates for grazing and non grazing ruminants. Because of its high carbohydrate quality, spineless cactus can be used as an emergency feed or as part of a complete diet providing that the diet contains adequate amount of degradable protein.

Keywords: Spineless cactus, cultivars, chemical composition, ruminal degradability

INTRODUCTION

Forage cacti consist of several species of the genera *Opuntia* and *Nopalea* which belong to the Cactaceae family. It is an important forage in several arid and semi arid regions in the world due to its high productivity under harsh environments. Benefits of cactus include high biomass yield, drought resistance, salinity tolerance and soil adaptability. There are more than 500,000 ha under cactus cultivation in the northeast region of Brazil where large quantities are fed during the dry season to ruminants due to the lack of other forages. Several factors can affect chemical composition and therefore nutritive value of cactus. These include type of cultivar and species, stage of maturity, soil moisture and nutrient composition, fertilization, and agronomical practices such as population density (Dubeux et al. 2006). Criteria for breeding programs of forage cactus include high yield, insect tolerance and adaptability to local conditions. New high yielding and insect (*Dactylopius coccus*) resistant cultivars have recently been propagated by Empresa Pernambucana de Pesquisa Agropecuária (IPA) in Pernambuco, Brazil. The objective of this study was to determine effects of cultivar on chemical composition and ruminal degradability of spineless cactus cladodes grown in the northeast region of Brazil.

MATERIALS AND METHODS

Eight cactus cultivars were grown in Arcoverde in the semi-arid region of Pernambuco state, northeast Brazil. Each cultivar was planted in three plots (1.0 x 0.5 m each) as a randomized complete block design (i.e. eight cultivars within each block, n = 24). Cultivars included the two most commonly grown cultivars in northeast of Brazil (i.e. Gigante and Miúda), two insect (*Dactylopius coccus*) resistant cultivars (i.e. 1317 Chile and 1267 Algeria), and four high producing cultivars (i.e. IPA-20, IPA-90-92, IPA-90-155, and additional 1258). All cultivars belonged to the genus *Opuntia* except for Miúda, which belonged to the genus *Nopalea*. Plants were manually harvested when they were two years old (early flowering stage) by cutting cladodes at the base using a sharp knife. Three plants per plot were harvested and composited as one replicate.

Following harvest, cladodes were cut horizontally into two halves which were further cut into small pieces and dried in a forced-air oven. Dried samples were ground through a 2-mm screen and subsamples were reground through a 1-mm screen. Ground samples were then analyzed for dry matter (DM), ash, ether extract, crude protein (CP, Kjeldahl N x 6.25) and acid detergent lignin according to the procedures of AOAC (1990). Neutral and acid detergent fiber were determined using an Ankom Fiber Analyzer. Starch was determined according to the procedure of McCleary et al. (1997) and water-soluble carbohydrates (WSC) according to Dubois et al. (1956).

Mineral concentrations were determined following digestion with a perchloric–nitric acid mixture (AOAC 1999) using a Perkin–Elmer Model 500 atomic absorption spectrophotometer. Oxalate concentration was determined using an enzymatic kit (Trinity Biotech, Newark, NJ).

Three mature bucks fitted with ruminal cannulae and fed chopped tifton hay *ad libitum* were used for in situ ruminal incubations. Equal portions (200 g) from the three replicates of each cultivar were composited to obtain a single sample for each cultivar. Duplicate samples weighing approximately 4 g (air basis) were placed into nylon bags and incubated in the rumens of the three bucks for 2, 4, 8, 12, 24, 48, and 72 h.

Residues from the nylon bags at each incubation time were analyzed for DM and NDF as described previously. The disappearance at each incubation time was calculated from the concentrations of DM and NDF in the original samples and the residues were used to estimate DM, CP and NDF degradation parameters using the equation of Dhanoa (1988).

All data were analyzed using PROC GLM of SAS (SAS Institute, 1989). Data of chemical composition were analyzed as a randomized complete block design (eight treatments and three blocks) while data of the in situ study were analyzed as a randomized complete block design using animals as blocks. Treatment differences were declared significant when $P < 0.05$.

RESULTS

Chemical composition of the cactus cultivars is shown in Table 1. Dry matter content was higher ($P < 0.05$) for Miúda than Gigante, APA-20, Additional 1258, Algeria and Chile cultivars. Ash content ranged between 68 and 94 g kg^{-1} with the largest differences observed between Algeria and IPA-90-155 cultivars. Ether extract was low and similar for all cultivars. Neutral detergent fiber was not influenced by cultivar and averaged 249 g kg^{-1}. Acid detergent fiber was higher ($P < 0.05$) for IPA-90-155 than Additional 1258, Chile and Miúda and was higher ($P < 0.05$) for Algeria, IPA-90-92, IPA-20, and Gigante than Miúda. Acid detergent lignin was low for all cultivars and was higher ($P < 0.05$) for IPA-90-155 than Additional 1258, Algeria and Chile cultivars. Starch and WSC concentrations were not influenced by cultivar and averaged 198 and 155 g kg^{-1}, respectively. Total carbohydrates constituted more than 60% of the DM and were higher ($P < 0.05$) for the IPA cultivars than Gigante and Algeria cultivars. Non-structural carbohydrates made up more than 50% of the total carbohydrates and was higher ($P < 0.05$) for IPA-90-155 than Algeria and Gigante cultivars.

Crude protein content of all cultivars was less than 50 g kg^{-1} and was higher ($P < 0.05$) for Gigante and Algeria than the other cultivars (Table 1). Neutral and acid detergent insoluble protein concentrations were similar for all cultivars and averaged 223 and 87 g kg^{-1} of CP, respectively. Cultivar had no effect on mineral composition of cactus (Table 1). Calcium

was the mineral in the highest concentration in all cultivars followed by Mg and K. Oxalate concentration was also not influenced by cultivar and averaged 1.69 g kg^{-1}.

In situ soluble DM fraction ranged between 269 and 331 g kg^{-1} of DM and was higher ($P < 0.05$) for Gigante than IPA-20, IPA-90-92 and Chile cultivars. Rate of degradation of the slowly degradable DM fraction was higher ($P < 0.05$) for Chile than the other cultivars (Table 2). However, effective ruminal degradability of DM was similar for all cultivars and averaged 701 g kg^{-1}.

Differences in NDF kinetic parameters between cactus cultivars were small (Table 2). In situ soluble NDF fraction was smaller ($P < 0.05$) for Algeria than Gigante cultivar while rate of degradation of slowly degradable NDF fraction was greater ($P < 0.05$) for Miúda than the other cultivars. However, slowly degradable NDF fraction and ruminal effective degradability of NDF were not influenced by cultivar and averaged 697 and 503 g kg^{-1}, respectively.

DISCUSSION

Values of ash and mineral concentrations in the present study were lower than those reported for spineless cactus cultivars from other locations in northeast Brazil (Batista et al. 2003a,b) and Tunisia (Ben Salem et al. 1996). Ben Thlija (1987) indicated that ash content of spineless cactus could be as high as 300 g·kg^{-1} DM. Factors such as soil mineral and moisture contents can significantly affect ash and mineral concentrations of spineless cactus. Water deficiency and high levels of Ca compounds in the soil tend to push cacti to accumulate Ca in their cladodes (Nobel 2002). The low Ca concentrations for the cactus cultivars in our study were reflected in the oxalate content, which was lower than the value reported by Ben Salem et al. (2002b). Oxalate synthesis is considered means of reducing the negative impact of excessive Ca uptake by cactus (Libert and Franceschi 1987).

Our NDF, ADF and lignin values were in good agreement with those reported by Batista et al. (2003b). Neutral detergent fiber values for spineless cactus ranged between 186 and 392 g kg^{-1} while ADF concentration ranged between 113 to 248 g kg^{-1} (Ben Thlija 1987; Ben Salem et al. 1996; Batista et al. 2003a,b). Variations in fiber values between studies can be due to differences in cactus species and cultivar and stage of maturity.

All cactus cultivars contained moderate levels of starch, which were higher than the values reported by Batista et al. (2003b) but within the range reported by Retamal et al. (1987). Retamal et al. (1987) found that starch content of cactus varies according to stage of maturity with young cladodes containing more starch than mature ones. Cactus can be considered unique forage because of its moderate starch and WSC concentrations. Batista et al. (2003b) reported that rapidly (i.e. sugars and organic acids) and intermediately (i.e. starch) degradable carbohydrate fractions constituted 42 and 19%, respectively of the total carbohydrates in cactus.

Cactus is characterized by a low CP concentration. Average values of 38 and 58 g kg^{-1} have been reported by Ben Salem et al. (1994) and Ben Salem et al. (1996), respectively. However, Batista et al. (2003a,b) reported higher CP values (60 to 77 g kg^{-1}) for several cactus cultivars from northeast of Brazil. Crude protein content of cactus is affected by factors such as soil moisture and N contents and N fertilization (Dubeux et al. 2006).

All cactus cultivars had high ruminal DM degradability with little differences between cultivars. (Table 2). In agreement with our results, Ben Salem et al. (2002) and Batista et al. (2003a) reported in situ DM disappearance of 760 to 800 g kg^{-1} for spineless cactus following 48 h of ruminal incubation. High ruminal DM degradability of cacti is likely due to their high non-structural carbohydrate and low lignin contents, which may explain the high degradation rate of the slowly degradable DM fraction. High degradation rates (7.0 to 10.0 % h^{-1}) of potentially degradable DM for different cactus cultivars have also been reported by Batista et al. (2003a). Values of in situ soluble DM fraction in our study were higher while values of potentially degradable DM fraction were lower than those reported for 10 cultivars of spineless cacti (Batista et al. 2003a). Data of ruminal degradability of cactus fiber are limited. Batista et al. (2003b) reported that 63% of NDF of three cactus cultivars disappeared following 48 h of in situ ruminal incubation. High rate of degrada-

tion on NDF can be attributed to low lignin concentration.

CONCLUSION

Our results showed that cultivar had minimal effects on chemical composition and ruminal degradability of cactus. Because of its low fiber and moderate starch and water-soluble carbohydrate concentrations, cactus can be considered a good source of readily fermentable non-fiber carbohydrates. The cell wall fraction is also expected to be highly degradable in the rumen because of its low lignin content. Feeding programs for cactus should focus on adequate supplementation with protein and effective fiber sources.

Table 1. Effect of cultivar on chemical composition of spineless cactus cladodes.

	Cactus cultivar								
	Gigante	Miúda	IPA-20	IPA-90-92	IPA-90-155	Additional1258	Algeria	Chile	SEM
DM ($g\,kg^{-1}$)	153bc	187a	156bc	165ab	170ab	148bc	126c	138bc	7.1
Ash ($g\,kg^{-1}$)	81b	81b	72bc	78bc	68c	76bc	94a	79bc	2.6
Ether extract ($g\,kg^{-1}$)	24	20	20	23	23	19	17	23	1.9
NDF ($g\,kg^{-1}$)	248	241	247	255	263	241	246	249	6.3
ADF($g\,kg^{-1}$)	179ab	148c	185ab	178ab	207a	162bc	183ab	158bc	7.1
Lignin ($g\cdot kg^{-1}$)	13ab	13ab	12ab	13ab	17a	7b	10b	8b	1.4
Starch ($g\,kg^{-1}$)	207	205	198	194	204	190	193	195	14.2
Water soluble carbohydrates ($g\,kg^{-1}$)	154	146	166	150	153	161	141	165	12.2
Total carbohydrates ($g\,kg^{-1}$)	625b	660ab	690a	690a	711a	666ab	617b	633b	14.0
Non-structural carbohydrates ($g\,kg^{-1}$)	398bc	439abc	464ab	462ab	468a	445abc	381c	404abc	15.0
Crude protein (CP, $g\,kg^{-1}$)	44a	33b	37b	34b	37b	36b	44a	39b	1.3
NDICP[1] ($g\cdot kg^{-1}$ of CP)	195	208	218	234	241	248	213	228	10.4
ADICP[2] ($g\cdot kg^{-1}$ of CP)	82	81	95	100	93	80	89	75	7.4
Ca ($mg\,kg^{-1}$)	18.73	21.41	16.76	17.26	17.57	21.31	23.11	20.67	1.36
P ($mg\,kg^{-1}$)	1.25	1.02	1.14	1.44	1.02	1.24	1.18	1.05	0.124
Mg ($mg\,kg^{-1}$)	8.88	10.13	9.21	7.91	9.01	9.40	9.90	10.72	0.740
K ($mg\,kg^{-1}$)	8.52	8.64	8.00	8.10	7.56	10.33	8.49	7.82	1.20
Na ($mg\,kg^{-1}$)	0.05	0.06	0.03	0.05	0.06	0.01	0.01	0.01	0.021
Zn ($mg\,kg^{-1}$)	0.04	0.02	0.04	0.03	0.08	0.03	0.05	0.08	0.016
Oxalate ($mg\,kg^{-1}$)	1.23	1.95	2.10	1.30	1.60	1.90	1.50	1.90	0.298

[1]Neutral detergent insoluble protein
[2]Acid detergent insoluble protein.
Means in the same row with different letters are different ($P < 0.05$)

Table 2. Effects of cultivar on ruminal dry matter and neutral detergent fiber degradability of spineless cactus cladodes.

	Cactus cultivar								
	Gigante	Miúda	IPA-20	IPA-90-92	IPA-90-155	Additional 1258	Algeria	Chile	SEM
Dry matter (DM)									
Soluble fraction (g kg^{-1} of DM)	331a	318ab	300b	306b	315b	308b	310b	269c	4.6
Slowly degradable fraction (g kg^{-1} of DM)	560b	573b	582b	575b	563b	578b	568b	627a	5.1
Degradation rate (%h^{-1})	10.7b	10.9b	11.1b	10.5b	10.8b	10.1b	10.0 b	12.3a	0.25
Lag time (h)	0.2	0.2	0.1	0.1	0.1	0.2	0.1	0.1	0.04
Effective degradability (g kg^{-1} of DM)	712	711	700	696	701	693	689	707	5.7
Neutral detergent fiber (NDF)									
Soluble fraction (g kg^{-1} of NDF)	109a	99ab	101ab	105ab	106ab	99ab	98b	107ab	2.5
Slowly degradable fraction (g kg^{-1} of NDF)	704	690	693	705	701	692	700	694	4.9
Degradation rate (%h^{-1})	6.5b	7.6a	7.0b	6.7b	6.5b	6.7b	6.7b	6.4b	0.18
Lag time (h)	0.1	0.3	0.2	0.2	0.2	0.2	0.1	0.1	0.08
Effective degradability (g kg^{-1} of NDF)	506	514	504	507	502	496	499	498	5.0

Means in the same row with different letters are different ($P < 0.05$)

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Effects of mannanoligosaccharides at two different concentrations on intestinal morphology, cecal and litter microbial populations, and carcass parameters of broiler chickens

B. Baurhoo and X. Zhao

ABSTRACT

This study was conducted to compare the effects of a mannanoligosaccharide (MOS) prebiotic with commonly used antibiotic growth promoters (AGP) on growth performance, microbiology and morphology of the intestines, and carcass parameters in broiler chickens. Dietary treatments included: 1) AGP-free diet (CTL); 2) VIRG (virginiamycin); 3) BACT (bacitracin); 4) LMOS (0.2% MOS); 5) HMOS (0.5% MOS). BW, feed conversion, and carcass yields (breast, thigh, drumstick and wing) did not differ among dietary treatments at weekly intervals. In contrast to birds fed the VIRG and BACT diets, LMOS and HMOS increased villi height and goblet cell number per villus at d 24 and 34. At d 14, 24 and 34, *Bifidobacteria* concentrations were higher in LMOS and HMOS fed birds. None of the dietary additives altered *E. coli* and *Campylobacter* loads in the ceca at d 14 and 24. In comparison to birds fed CTL, at d 34, *E. coli* load was reduced in BACT, LMOS and HMOS fed birds and *Campylobacter* load was reduced in VIRG, BACT and LMOS fed birds. There was no benefit in using HMOS than LMOS in any of the parameters assessed. These results indicate that the recommended MOS level (0.2%) can enhance development of morphological structures and preferentially stimulate growth of beneficial bacteria in the broiler intestines.

Keywords: Antibiotic, mannanoligosaccharides, intestinal microflora, goblet cells, broiler

INTRODUCTION

The gastrointestinal tract of chickens is inhabited by a flora of pathogenic bacteria that results in productivity losses and contamination of poultry products. For decades, sub-therapeutic antibiotics have been used for the control of these pathogens. However, the link between antibiotic-resistance in human and animal bacteria have led to scientific reconsideration of usage of such antibiotics in animal agriculture. Nowadays, there is an urge for natural alternatives that can sustain farm performance and safety of poultry products in the absence of antibiotics.

Mannan-oligosaccharide (MOS) is a prebiotic that competitively binds to gram-negative pathogens, such as *Salmonella* and *E. coli*, possessing Type 1 fimbriae and reduces their colonization of the intestine (Newman 1994). MOS has also been shown to alter the morphology of chicken intestines by increasing goblet cell number of the villi membrane (Baurhoo et al. 2007). These cells secrete mucins, glycoprotein compounds, which bind pathogenic microorganisms and reduce their adherence to the intestinal mucosa (Bloomberg et al. 1993). But, so far, MOS has only been studied at 0.1 or 0.2 % of the diet in broiler chickens. In turkeys, however, dietary inclusion of higher MOS levels resulted in greater BW responses (Zdunczyk et al. 2005) and goblet cell number (Solis de los Santos et al. 2007). We, therefore, hypothesized that a higher level of MOS could further improve the microbial ecology and morphology of broiler intestines thereby providing greater protection against enteric pathogens.

The objectives of this study were to evaluate the effects of a recommended (0.2 %) and a higher (0.5 %) dietary level of MOS on growth performance, microbial populations in the ceca, intestinal morphology, and carcass yield parameters. These effects of MOS were then compared to those of an antibiotic-free diet and one containing commonly used antibiotics, namely virginiamycin and bacitracin.

MATERIALS AND METHODS

Eight hundred and twenty five one-day-old male broilers (3 pen replicates; 55 birds per pen) were randomly assigned to 5 dietary treatments: 1) control diet (**CTL**, AGP-free); 2) **VIRG** (diet 1 + 16.5 mg kg^{-1} virginiamycin); 3) **BACT** (diet 1 + 55 mg / kg^{-1} bacitracin); 4) **LMOS** (diet 1 + 0.2% MOS); 5) **HMOS** (diet 1 + 0.5% MOS). Feed offered included a starter diet from 1 to 21 d and a grower diet from 22 to 38 d. Birds were group weighed by pen and feed consumption was determined at weekly intervals.

At d 14, 24 and 34, three birds per pen (n = 9 / treatment) were euthanized and 1cm segment of the jejunum was dissected, fixed in 10% buffered formalin and used for measurement of villus height and goblet cell number into villi membrane as previously described (Baurhoo et al. 2007). Ten measurements were taken for each parameter per bird and the average of these values was used in statistical analysis.

At d 14, 24 and 34, 1 g of fresh cecal contents was serially diluted in 0.85 % sterile saline solution and used for enumeration of *Lactobacilli* (Lactobacilli MRS Agar), *Bifidobacteria* (Wilkins-Chalgren agar modified with glacial acetic acid (1 mL L^{-1}) and mupirocin (100 mg L^{-1}), *Salmonella* (Brilliant Green Agar), Campylobacter (Campylobacter Agar Base with Lysed Horse Blood, Preston Campylobacter Selective Supplement and Campylobacter Growth Supplement), and *E. coli* (Rapid *E. coli* 2 Agar).

On d 38, 3 birds per pen (n = 9 / treatment) were euthanized and yields of eviscerated carcass, whole breast, breast fillet, breast tenders, thigh, drumstick and wing were determined.

Data were analyzed as a one-way ANOVA using the MIXED procedure of SAS. A completely randomized design was used for performance parameters with pen as experimental unit. For microbiology, histology and carcass parameters, a Nested Model Design was used with birds nested within treatments; birds served as the experimental unit. Treatment means were separated using the least square means option of SAS. Differences between treatment means were tested using Scheffe's Multiple Comparison test and statistical significance was declared at $P<0.05$. All microbiological concentrations were subjected to $\log_{10}$ transformation prior to statistical analysis.

RESULTS AND DISCUSSION

There was no significant effect of virginiamycin, bacitracin, LMOS and HMOS in comparison to the CTL diet on BW, feed intake or feed conversion ratio throughout the study (data not shown). Similar results were obtained when broilers were fed MOS (0.1 or 0.2%) or antibiotics containing diets (Hooge 2004). But, this is the first report indicating no production benefits due to usage of higher dietary MOS (0.5%) than the recommended MOS (0.2%) level in broilers and would hence preclude the use of higher dietary MOS levels in broiler production. In addition, at d 38, none of the dietary additives influenced relative carcass weight and carcass yields when compared to broilers fed the CTL diet (Tables 1 and 2); this may be the consequence of similarity in BW responses among dietary treatments.

Goblet cell number was consistently increased in LMOS and HMOS fed birds than birds fed the other dietary treatments at d 24 and 34 (Table 3). At d 24, LMOS and to a lesser extent HMOS increased villi height when compared to CTL or BACT fed birds. Villi tended to be longer in LMOS and HMOS fed birds than those fed the CTL, VIRG or BACT diet at d 34. Despite slight variations in treatment effects, LMOS and HMOS diets generally tended to enhance villi and goblet cell development when compared to the CTL, VIRG and BACT diets. Similar findings were observed when MOS (0.2%) was added to broiler and turkey diets (Baurhoo et al. 2007; Solis de los Santos et al. 2007). Our results demonstrate no further benefit of using MOS at higher dietary level (0.5%) than the recommended level (0.2 %) on the measured histological parameters.

There is evidence that increases in the intestinal populations of

Lactobacilli and *Bifidobacteria* due to MOS usage resulted in longer villi and increased goblet cell number in broilers (Baurhoo et al. 2007). In the present study, the inability of HMOS to more profoundly increase *Lactobacilli* and *Bifidobacteria* concentrations than the LMOS diet was accompanied by lack of greater responses in morphological development of the intestines. MOS only modestly increased villi length at later stages in this study and may not be sufficient to induce improvement in BW. Unfortunately, in comparison with LMOS, feeding broilers the HMOS diet was neither accompanied by greater goblet cell number nor higher concentrations of these bacteria.

There was no treatment effect, at d 14, on cecal *Lactobacilli* concentration (Fig. 1). In comparison to birds fed the CTL or BACT diet, *Lactobacilli* concentration was increased in LMOS fed birds at d 24 and HMOS fed birds at d 34. But, LMOS and HMOS tended to increase *Lactobacilli* load when compared to birds fed the other dietary treatments at both d 24 and 34. *Lactobacilli* concentrations did not differ between birds fed LMOS and HMOS nor between those fed the two antibiotics at all times.

At d 14, 24 and 34, LMOS and HMOS significantly increased *Bifidobacteria* concentrations when compared to birds fed the VIRG or BACT diet (Fig. 2). Moreover, HMOS fed birds harbored higher *Bifidobacteria* loads than those fed the CTL diet at all times. In comparison to CTL fed birds, LMOS significantly increased *Bifidobacteria* load at d 34 only. At d 24, *Bifidobacteria* concentration was greater in birds fed HMOS than those fed LMOS; similar trend was observed at d 34.

The successful establishment of an intestinal population of *Lactobacilli* and *Bifidobacteria* is reported to improve health parameters including intestinal maturation (Solis de los Santos et al. 2007), competitive exclusion of pathogens (van der Wielen et al. 2002), secrete antimicrobial substances against pathogens (Jin et al. 1996), and immune modulation (Lan et al. 2005). MOS, therefore, represents a dietary strategy that could favour intestinal colonization by beneficial bacteria thereby conferring health benefits to chickens.

Birds were free from *Salmonella* similar to our previous trials. Results of this study indicate similarity between VIRG, BACT, LMOS and HMOS in the control of intestinal *E. coli* and *Campylobacter* of broiler chickens (Fig. 3 and 4). Neither of these dietary additives significantly altered the cecal concentrations of *E. coli* and *Campylobacter* when compared to the CTL diet at d 14 and 24.

However, at d 34, the BACT, LMOS and HMOS diets significantly reduced *E. coli* concentrations when compared to CTL fed birds. Birds fed the VIRG diet also tended to contain lower *E. coli* concentration than those fed CTL. When similar comparisons were made, the VIRG, BACT and LMOS diets significantly reduced *Campylobacter* concentrations at d 34. At same bird age, the HMOS diet also reduced *Campylobacter* load than CTL fed birds, but the effect was not statistically significant. This study shows that there was no difference between LMOS and HMOS or between VIRG and BACT in the control of intestinal *E. coli* and *Campylobacter*. These pathogenic bacteria are known to cause detrimental effects to the host such as loss of appetite, competition for vital nutrients and reducing nutrient absorption, and also compromise safety of food products for human consumption. Hence, MOS as well as the antibiotics represent dietary strategies that can be used to control intestinal *E. coli* and *Campylobacter*.

It was expected that improving the microflora (increased beneficial bacteria and reduced pathogens loads) and morphological parameters (longer villi and greater goblet cell number) in the chicken intestines due to MOS usage, would lead to improvement in nutrient absorption and partitioning thereby improving BW and lean muscle weights. However, such hypothesis was not evidenced in this trial.

CONCLUSIONS

Results of the current study indicate that MOS may significantly enhance development of morphological structures, favor growth of beneficial bacteria and limit growth of gram-negative pathogens in the intestines, thereby imparting important health benefits in broiler chickens. There is, however, no additional benefit of using MOS at higher doses (0.5%) than the recommended dose (0.2%).

Table 1. Effects of antibiotics and MOS on weights and yields of broiler carcasses and parts at 38 d of age[1]

		Eviscerated carcass		Thigh		Drumstick		Wing	
Treatments[2]	Live weight (g)	Weight (g)	Yield[3] (%)	Weight (g)	Yield[4] (%)	Weight (g)	Yield[4] (%)	Weight (g)	Yield[4] (%)
CTL	2101.78	1391.19	66.14	288.68	20.79	228.64	16.46	129.91	9.36
VIRG	2037.56	1366.69	67.04	312.40	22.83	224.02	16.36	128.22	9.39
BACT	2033.56	1379.12	67.73	303.92	21.94	228.66	16.64	127.56	9.27
LMOS	2027.67	1347.77	66.49	288.77	21.41	218.04	16.17	129.72	9.62
HMOS	1957.78	1289.43	65.88	275.13	21.39	224.84	17.51	123.12	9.61
SEM	59.11	44.34	0.72	11.95	0.44	8.20	0.44	3.86	0.22

[1]Mean ± SE of 9 replicates.
[2]CTL: antibiotic free diet; VIRG: virginiamycin; BACT: bacitracin; LMOS: 0.2% MOS; HMOS: 0.5% MOS.
[3]Carcass yields are expressed as a percentage of live weight.
[4]Thigh, drumstick and wing yields are expressed as a percentage of eviscerated carcass weight.

Table 2. Effects of antibiotics and MOS on breast weights and yields at 38 d of age[1]

	Whole breast		Fillet		Tenders	
Treatments[2]	Weight (g)	Yield[3] (%)	Weight (g)	Yield[3] (%)	Weight (g)	Yield[3] (%)
CTL	670.98	48.46	315.90	22.65	67.10	4.83
VIRG	614.37	44.99	292.24	21.39	65.41	4.79
BACT	641.73	46.51	302.74	21.85	68.38	4.97
LMOS	636.80	47.26	291.32	21.66	62.64	4.65
HMOS	609.41	47.47	283.26	22.04	67.12	5.23
SEM	21.36	0.69	13.67	0.64	2.50	0.16

[1]Mean ± SE of 9 replicates.
[2]CTL: antibiotic free diet; VIRG: virginiamycin; BACT: bacitracin; LMOS: 0.2 % MOS; HMOS: 0.5 % MOS.
[3]Whole breast, fillet and tender yields are expressed as a percentage of eviscerated carcass weight.

Table 3. Effects of antibiotics and MOS on villi height and number of goblet cells per villus in the jejunum of broiler chickens[1]

	Treatments[2]					
Age	CTL	VIRG	BACT	LMOS	HMOS	SEM
			Villi height (μm)			
Day 14	1069.74	1120.65	1107.91	1155.19	1128.74	37.84
Day 24	1099.34[b]	1201.2[ab]	1115.49[b]	1371.85[a]	1222.82[ab]	44.83
Day 34	1187.57	1166.86	1199.41	1393.19	1375.09	44.93
			Number of goblet cells / villus			
Day 14	131.57	133.57	157.91	159.91	177.67	10.62
Day 24	135.59[b]	127.54[b]	144.56[b]	240.11[a]	217.42[a]	14.84
Day 34	171.26[b]	140.1[b]	156.02[b]	236.64[a]	228.00[a]	10.58

[a,b] Means with different superscript within the same row differ (Scheffe t-test, $P<0.05$).
[1]Mean ± SE of 9 replicates.
[2] CTL: antibiotic free diet; VIRG: virginiamycin; BACT: bacitracin; LMOS: 0.2% MOS; HMOS: 0.5% MOS.

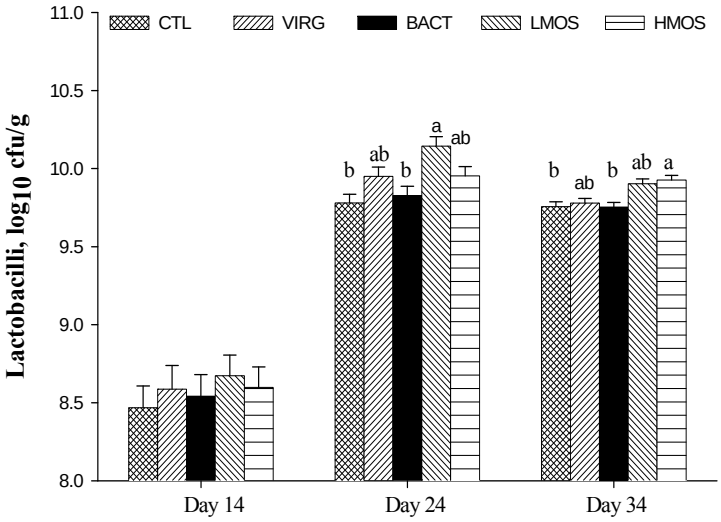

Figure 4. Cecal concentrations (log_{10} CFU g^{-1}) of *Lactobacilli*[1]

[a,b] Means with different superscript within a group differ (Scheffe t-test, P<0.05).
[1]Treatments: CTL: antibiotic free diet; VIRG: virginiamycin; BACT: bacitracin; LMOS: 0.2% MOS; HMOS: 0.5% MOS.

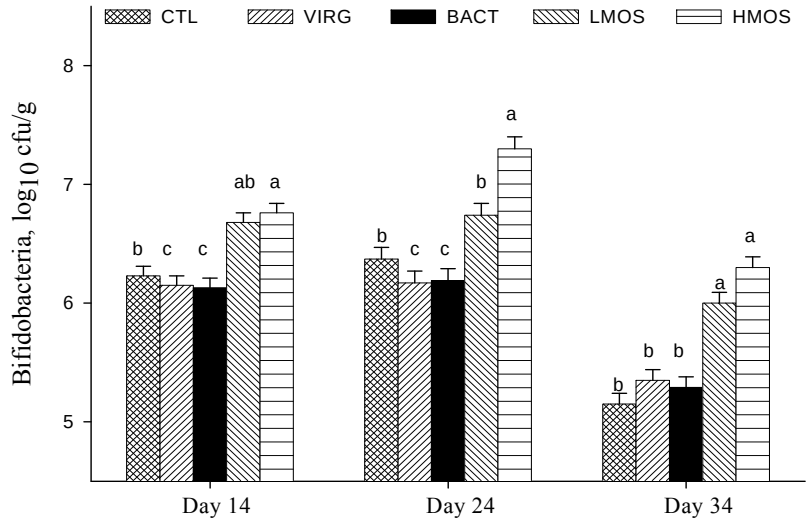

Figure 2. Cecal concentrations (log_{10} CFU g^{-1}) of *Bifidobacteria*[1]

[a,b,c] Means with different superscript within a group differ (Scheffe t-test, P<0.05).
[1]Treatments: CTL: antibiotic free diet; VIRG: virginiamycin; BACT: bacitracin; LMOS: 0.2% MOS; HMOS: 0.5% MOS.

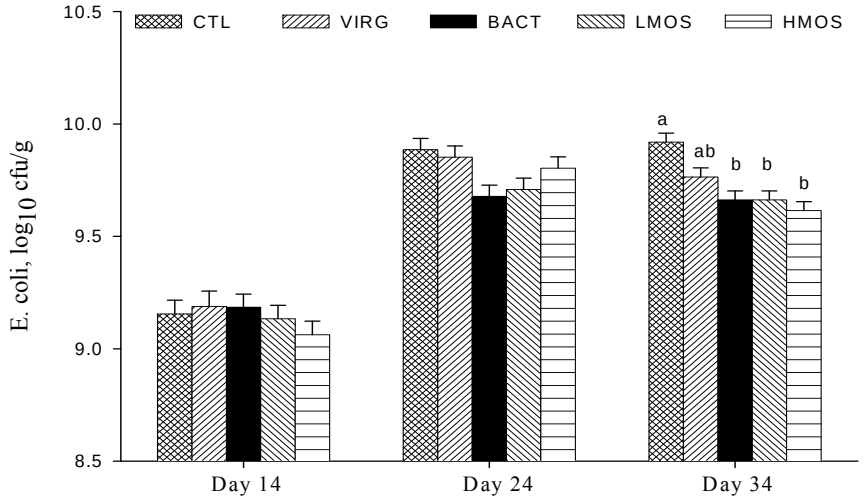

Figure 3. Cecal concentrations (log_{10} CFU g^{-1}) of *E. coli* [1]

[a,b] Means with different superscript within a group differ (Scheffe t-test, P<0.05).
[1]Treatments: CTL: antibiotic free diet; VIRG: virginiamycin; BACT: bacitracin; LMOS: 0.2 % MOS; HMOS: 0.5 % MOS.

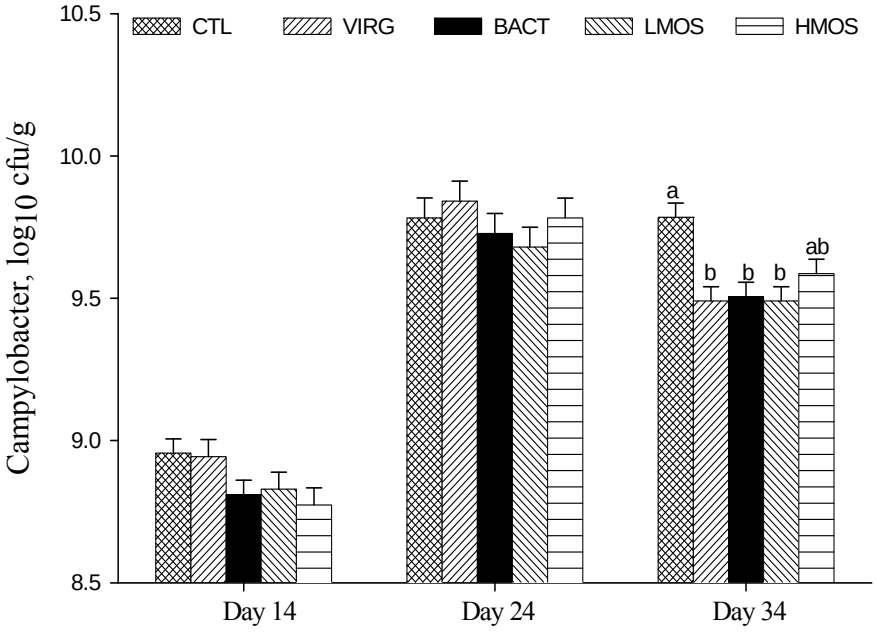

Figure 4. Cecal concentrations (log_{10} CFU g^{-1}) of *Campylobacter* [1]

[a,b] Means with different superscript within a group differ (Scheffe t-test, P < 0.05).
[1]Treatments: CTL: antibiotic free diet; VIRG: virginiamycin; BACT: bacitracin; LMOS: 0.2 % MOS; HMOS: 0.5 % MOS.

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Fate of chlortetracycline and tylosine resistant bacteria in an aerobic thermophilic sequencing batch reactor treating swine waste

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ABSTRACT

Food animals and their wastes constitute a reservoir of antibiotic resistant bacteria. The objective of this work was to characterize the impact of an aerobic thermophilic biotreatment on antibiotic-resistant bacteria in swine waste. The proportion of tylosine- and chlortetracycline-resistant bacteria decreased after treatment but they were still abundant in the treated swine waste. In 22 cases, genes conferring resistance to tylosine and chlortetracycline were detected by PCR before but not after treatment. The overall gene diversity was wider before [*tet*(ABLMOSY), *erm*(AB)] than after [*tet*(LMOS), *erm*(B)] treatment. Analysis by DGGE of amplified 16S rDNA fragments also showed a reduction of the bacterial diversity exept for Tyl^R populations grown at 37°C. The latter were further investigated by cloning and sequencing its 16S rDNA. Phylotypes found before treatment were all closely associated to *Enterococcus hirae*, whereas there was a little more diversity after treatment. This work demonstrated that the aerobic thermophilic biotreatment cannot be considered as a means for preventing the dissemination of aerobic antibiotic resistant bacteria and their resistance genes to the environment.

Keywords: Antibiotic resistance, swine waste, biological treatment, aerobic bacteria.

INTRODUCTION

Field application of wastes from antibiotic-treated swines is a means of introduction of resistant bacteria into agricultural ecosystems, with the consequence that resistance determinants are found in soils, on crops, and in surface waters and groundwaters. Antibiotic resistance genes and resistant bacteria may then get in contact with workers and consumers throughout the food production system, thus contributing to the dissemination of antibiotic resistance in humans. This increases the risks of infectious diseases caused by antibiotic-resistant pathogenic bacteria. For example, one study showed that clinical strains of *Salmonella enterica* resistant to at least one antibiotic originated from a swine herd (Molback et al. 1999).

This context shows the importance of treating swine wastes prior to their application onto agricultural fields. However, available technologies are scarce, costly and incomplete, and generally do not allow for the elimination of antibiotic-resistant bacteria. In our laboratory, an aerobic thermophilic sequencing batch reactor (AT-SBR) was developed for the biological treatment of swine wastes. The AT-SBR biotreatment eliminates odors and pathogenic microorganisms as well as reduces the N and P content in the treated material (Juteau et al. 2004). However, the fate of antibiotic resistance in this AT-SBR biotreatment remains to be determined.

We hypothesize that, among the bioprocesses available for treating swine waste, those that are thermophilic should be more efficient to reduce antibiotic-resistant bacteria since the enteric mesophilic microflora should not survive. Also, horizontal transfert of genetic material during the

process should be limited due to the phylogenetic distance between aerobic thermophiles that are promoted in the bioreactor and the anaerobic mesophiles that dominate the enteric microflora. Consequently, the goal of this work was to study the impact of the AT-SBR swine waste biotreatment on the chlortetracycline- and tylosine-resistant aerobic bacterial populations and on the occurrence of antibiotic resistance genes.

MATERIALS AND METHODS

Swine Waste Biotreatment

Swine waste was sampled from the collecting pit of a growing-finishing farm of about 600 swines using chlortetracycline and tylosine as growth promoters. The AT-SBR used to treat swine waste had the same constituent elements than those described previously (Juteau et al. 2004).

Microbial Enumerations

Untreated swine waste was sampled from a drum kept at 6°C and used to feed the AT-SBR, whereas treated swine waste was sampled at the outlet of the AT-SBR after the 3-day biotreatment. The aerobic microbial populations were determined using an adapted Most Probable Number procedure (MPN) (APHA 1998) with either 20 mg L^{-1} chlortetracycline HCl (Tet^R) or 10 mg L^{-1} tylosine tartrate (Tyl^R), or without antibiotic (Tot). All vials were incubated in the dark at 200 rpm and incubated at 25°C, 37°C and 60°C.

DNA Analyses

DNA was extracted from MPN vials using a physical-chemical procedure (Juteau et al. 2004). PCR amplifications were carried out in a GeneAmp PCR System 2700 (version 2.06, Applied Biosystems, Foster City, CA) (Table 1**).** DGGE on amplified 16S rDNA was used to analyze the biodiversity of bacterial populations (Juteau et al. 2004). Comparisons of the bacterial community composition were performed by cluster analysis of the DGGE banding patterns using Dendron software (version 2.4; Solltech Inc., Oakdale, IA).

Clone libraries of 16S rDNA

Samples taken from MPN vials incubated at 37°C with 10 mg L^{-1} tylosine and inoculated with untreated or treated swine waste were used to construct clone libraries of 16S rDNA (Juteau et al. 2004). Clones migrating at the same position on DGGE gels were grouped and their 16S rDNA was sequenced. After excluding chimeric clones using ClustalW (Huber et al. 2004), the 16S rRNA gene sequences were compared to those in the GenBank using BLASTn (Altschul et al. 1990). Sequences sharing ≥ 98% identity were assigned to the same phylotype. Rarefaction analysis was performed and diversity indices were calculated to characterize both libraries (Good 1953; Krebs 1989; Perrault et al. 2007). A phylogenetic tree was constructed by the Phylip 3.66 package programs.

RESULTS

Despite the reductions observed in the mesophilic populations, the treated swine waste contained high concentrations of antibiotic resistant bacteria (Fig. 1). Overall, the proportion of antibiotic-resistant bacteria decreased with increasing temperature (Table 2). The biotreatment dramatically stimulated the growth of the thermophilic populations and decreased the proportion of the thermophilic Tyl^R population.

Five to 6 different genes conferring resistance to tetracycline or tylosine were detected at each incubation temperature, and the occurrence of resistance genes increased with increasing temperature (Table 3). The occurrence of each detected resistance gene was reduced by 50% or more by the AT-SBR biotreatment, and the diversity of resistance genes was wider before [*tet*(ABLMOSY) *erm*(AB): 9 genes] than after [*tet*(LMOS) *erm*(B): 5 genes] the biotreatment (Fig. 2). Overall, 35 detection events were recorded before the AT-SBR biotreatment, whereas 13 detection events were recorded after the biotreatment (63% reduction).

Before the AT-SBR biotreatment, the thermophilic Tet^R and Tyl^R populations showed almost identical DGGE banding patterns (Fig. 3). DGGE banding patterns divided in two clusters based on the source of the samples, i.e. before or after the AT-SBR biotreatment (Fig. 4). More diversity was found in the sample after than before the biotreatment for the clone two libraries constructed from Tyl^R populations incubated at 37°C (Fig. 5). In agreement with the DGGE observations, calculated indices showed that the clone library was more diverse after than before the AT-SBR biotreatment (Table 5).

DISCUSSION

Our starting hypothesis was that an aerobic thermophilic process

should reduce significantly the antibioresistance. This is partially supported by the negative values of the competitive index, which mean that there was a reduction in the proportion of tetracycline- and tylosine-resistant populations. However, in terms of absolute numbers, antibiotic resistant bacterial populations were still high in the treated swine waste. Those that are thermophilic were even more abundant after than before the AT-SBR biotreatment. A likely possibility is that residual antibiotics were absent or unsignificant in the swine waste, but that resistance genes were already present in the bioreactor bacteria and were stable enough in their genomes to follow the total population trend, which is an increase in the case of thermophilic microflora.

Thermophilic bioprocesses still have advantages over other treatment options concerning antibiotic resistance issues. First, thermophilic bioprocesses will better prevent the dissemination of antibioresistant pathogens since they kill more efficiently pathogenic microorganisms in comparison to bioprocesses operated at a lower temperature. Thus, the resistant bacteria that survive the biotreatment do not represent a direct animal or human health risk. Second, an important proportion of the bacteria leaving the bioreactor are thermophilic. These are less likely to be active in the environment than mesophilic bacteria, so that the former may not play a major role in the horizontal transfer of resistance genes once spread onto agricultural fields.

Our thermophilic treatment was efficient in eliminating antibiotic resistance genes present in the incoming waste, but the resistance determinants to tetracycline and tylosine are so diversified and ubiquitous in the environment that the outcoming population is still highly resistant. Consequently, it is possible that the process would be more efficient to fight against antibioresistance dissemination in the case of newer antiobiotics for which resistance genes are more limited in terms of diversity and distribution.

CONCLUSIONS

This work demonstrated that the AT-SBR biotreatment under study cannot be considered as a means for preventing the dissemination of aerobic antibiotic resistant bacteria and their resistance genes from swine wastes to the environment. This may probably apply to biotreatments in general, especially those operated at lower temperatures. This implies that the disposal of biologically treated swine wastes onto agricultural fields can contaminate crops, soils and water resources with antibiotic resistance determinants.

Table 1. Oligonucleotide primers, annealing temperatures and positive control strains used for PCR amplification of bacterial genes.

Target gene	Primers	Sequence (5'→3')	Amplicon size (bp)	Reference	Annealing temp. (°C)	Positive control
16S rDNA	341-F[a]	CCT ACG GGA GGC AGC AG	~ 233	(32)	55	*Pseudomonas aeruginosa* ATCC 27853
	518-R	ATT ACC GCG GCT GCT GG				
16S rDNA	pA	AGA GTT TGA TCC TGG CTC AG	~ 1533	(15)	55	*Pseudomonas aeruginosa* ATCC 27853
	pH	AAG GAG GTG ATC CAG CCG CA				
tet(A)	tetB-F	GCG CGA TCT GGT TCA CTC G	~ 164	(3)	61	
	tetB-R	AGT CGA CAG YRG CGC CGG C				strain SAS1393
tet(B)	tetB-F	TAC GTG AAT TTA TTG CTT CGG	~ 206	(3)	59	
	tetB-R	ATA CAG CAT CCA AAG CGC AC				strain Ct4afooB (Tn10)
tet(C)	tetC-F	GCG GGA TAT CGT CCA TTC CG	~ 207	(3)	63	
	tetC-R	GCG TAG AGG ATC CAC AGG ACG				pBR322
tet(D)	tetD-F	GGA ATA TCT CCC GGA AGC GG	~ 187	(3)	58	
	tetD-R	CAC ATT GGA CAG TGC CAG CAG				Strain D7-5
tet(E)	tetE-F	GTT ATT ACG GGA GTT TGT TGG	~ 199	(3)	61	
	tetE-R	AAT ACA ACA CCC ACA CTA CGC				pSL1504
tet(K)	tetK-F	TTA TGG TGG TTG TAG CTA GAA A	~ 348	(20)	55	
	tetK-R	AAA GGG TTA GAA ACT CTT GAA A				pAT102
tet(L)	tetL-F	GTM GTT GCG CGC TAT ATT CC	~ 696	(20)	57	
	tetL-R	GTG AAM GRW AGC CCA CCT AA				pVB.A15
tet(M)	tetM-F	ACA GAA AGC TTA TTA TAT AAC	~ 171	(4)	53	
	tetM-R	TGG CGT GTC TAT GAT GTT CAC				pJ13

Target gene	Primers	Sequence (5'→3')	Amplicon size (bp)	Reference	Annealing temp. (°C)	Positive control
tet(O)	tetO-F	ACG GAR AGT TTA TTG TAT ACC	~ 171	(4)	57	
	tetO-R	TGG CGT ATC TAT AAT GTT GAC				pU0A1
tet(S)	tetS-F	GAA AGC TTA CTA TAC AGT AGC	~ 169	(4)	50	
	tetS-R	AGG AGT ATC TAC AAT ATT TAC				pAT451
tet(Y)	tetY-F	ATT TGT ACC GGC AGA GCA AAC	~ 181	(3)	56	
	tetY-R	GGC GCT GCC GCC ATT ATG C				AF070999
erm(A)	ermA-F	TCT AAA AAG CAT GTA AAA GAA	~ 645	(42), (43)	54	
	ermA-R	CTT CGA TAG TTT ATT AAT ATT AGT				CCRI-9930
erm(B)	ermB-F	GAA AAG GTA CTC AAC CAA ATA	~ 639	(42), (43)	52	
	ermB-R	AGT AAC GGT ACT TAA ATT GTT TAC				CCRI-1317
erm(C)	ermC-F	TCA AAA CAT AAT ATA GAT AAA	~ 642	(42), (43)	47	
	ermC-R	GCT AAT ATT GTT TAA ATC GTC AAT				CCRI-1317

[a] : Preceeded by a GC clamp of 40 nucleotides for DGGE: CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G.

Table 2. Impact of the AT-SBR biotreatment on the Tot, Tet^R and Tyl^R aerobic microbial populations at 25°C, 37 °C and 60 °C in the swine waste.

Temp. (°C)	Population	$\Delta \log_{10}$	Competitive Index
25	Tot	- 0.36	-
	Tet^R	- 3.00	- 2.64
	Tyl^R	- 0.67	- 0.32
37	Tot	0.43	-
	Tet^R	- 0.75	- 1.18
	Tyl^R	- 0.21	- 0.64
60	Tot	>2.46	-
	Tet^R	>3.32	N.C.[a]
	Tyl^R	1.26	< - 1.20

[a] : Not calculated.

Table 3. PCR detection of selected chlortetracycline and tylosine resistance genes in the Tot, Tet^R and Tyl^R aerobic bacterial populations at 25°C, 37 °C and 60 °C from the swine waste before and after the AT-SBR biotreatment.

			Chlortetracycline resistance genes		Tylosine resistance genes
Temp. (°C)	Population	Treatment	Efflux genes *tet*(ABCDEKLY)	RM [a] genes *tet*(MOS)	RM[a] genes *erm*(ABC)
25	Tot	Before	*tet*(– – – – – – – –) [b]	*tet*(M – –)	*erm*(– – –)
		After	*tet*(– – – – – – – –)	*tet*(– – –)	*erm*(– – –)
	Tet^R	Before	*tet*(– – – – – – – –)	*tet*(M – –)	*erm*(– B –)
		After	*tet*(– – – – – – – –)	*tet*(– – –)	*erm*(– – –)
	Tyl^R	Before	*tet*(– B – D – – –)	*tet*(M – S)	*erm*(– B –)
		After	*tet*(– – – – – – – –)	*tet*(– – –)	*erm*(– – –)
37	Tot	Before	*tet*(– – – – – – LY)	*tet*(MOS)	*erm*(– B –)
		After	*tet*(– – – – – – – –)	*tet*(– – –)	*erm*(– – –)
	Tet^R	Before	*tet*(– – – – – – L –)	*tet*(MOS)	*erm*(– B –)
		After	*tet*(– – – – – – – –)	*tet*(– – –)	*erm*(– – –)
	Tyl^R	Before	*tet*(– – – – – – L –)	*tet*(MO –)	*erm*(– B –)
		After	*tet*(– – – – – – – –)	*tet*(M – –)	*erm*(– – –)
60	Tot	Before	*tet*(– – – – – – – –)	*tet*(– O –)	*erm*(– – –)
		After	*tet*(– – – – – – – –)	*tet*(– O –)	*erm*(– B –)
	Tet^R	Before	*tet*(– – – – – – L –)	*tet*(MOS)	*erm*(– B –)
		After	*tet*(– – – – – – L –)	*tet*(MOS)	*erm*(– B –)
	Tyl^R	Before	*tet*(– – – – – – L –)	*tet*(MOS)	*erm*(AB –)
		After	*tet*(– – – – – – L –)	*tet*(MOS)	*erm*(– B –)

[a] : RM = ribosomal methylation.
[b] : A dash (–) indicates that no PCR product was detected.

Table 4. Number of phylotypes (dominant / total) detected on a DGGE gel of PCR-amplified Bacteria 16S rDNA fragments from the Tot, Tet^R and Tyl^R aerobic bacterial populations at 25°C, 37 °C and 60 °C in the swine waste before and after the AT-SBR biotreatment.

Temp. (°C)	Population	Number of phylotypes	
		Before	After
25	Tot	2 / 9	2 / 2
	Tet^R	2 / 14	2 / 4
	Tyl^R	1 / 11	2 / 6
37	Tot	2 / 10	2 / 7
	Tet^R	2 / 6	1 / 4
	Tyl^R	1 / 1	2 / 7
60	Tot	1 / 4	1 / 14
	Tet^R	4 / 24	1 / 12
	Tyl^R	4 / 24	2 / 11

Table 5. Numbers of clones and phylotypes analyzed for the two Bacteria 16S rDNA gene clone libraries and their diversity indices[a].

Clone library	No. of clones	No. of phylotypes	Coverage (%)	Shannon index (*H'*)	Simpson's index (1/*D*)	Evenness (*E*)	Chao1	Sorensen index (*S*)
Before	22	1	100	0.51	1.51	1.67	4.50	0.00
After	48	6	96	1.32	3.05	0.62	9.00	

[a] : Libraries were constructed for samples taken from aerobic MPN vials incubated at 37°C with 10 mg tylosine l^{-1} and inoculated with untreated (before AT-SBR biotreatment) or treated (after AT-SBR biotreatment) swine waste.

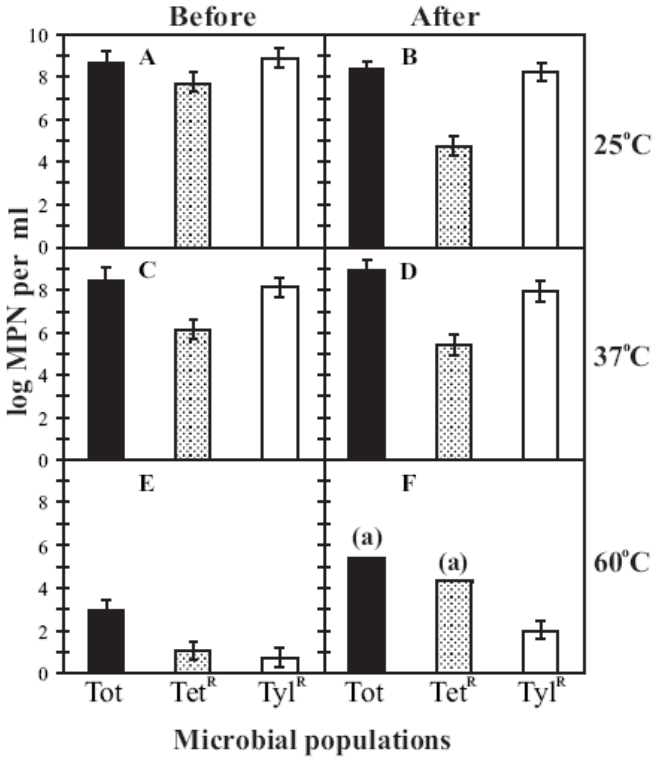

Figure 1. Enumeration of the Tot, Tet^R and Tyl^R aerobic microbial populations at 25°C (A and B), 37 °C (C and D) and 60 °C (E and F) in the swine waste before (A, C and E) and after (B, D and F) the AT-SBR biotreatment. (a) : in these series, growth was observed in all dilutions; consequently, reported MPN values represent a minimum.

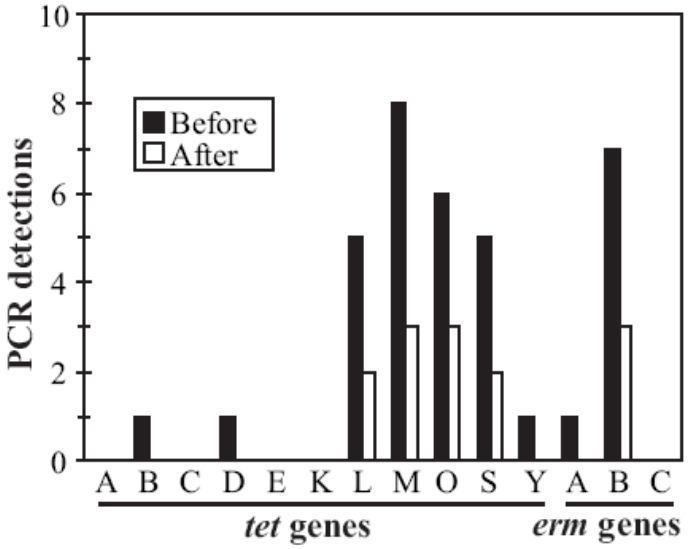

Figure 2. Impact of the AT-SBR biotreatment on the occurrence of selected chlortetracycline and tylosine resistance genes in the aerobic microbial populations. Results are the sum of PCR detections in the Tot, Tet^R and Tyl^R populations at 25, 37 and 60°C (maximum 9 detection events for each resistance gene).

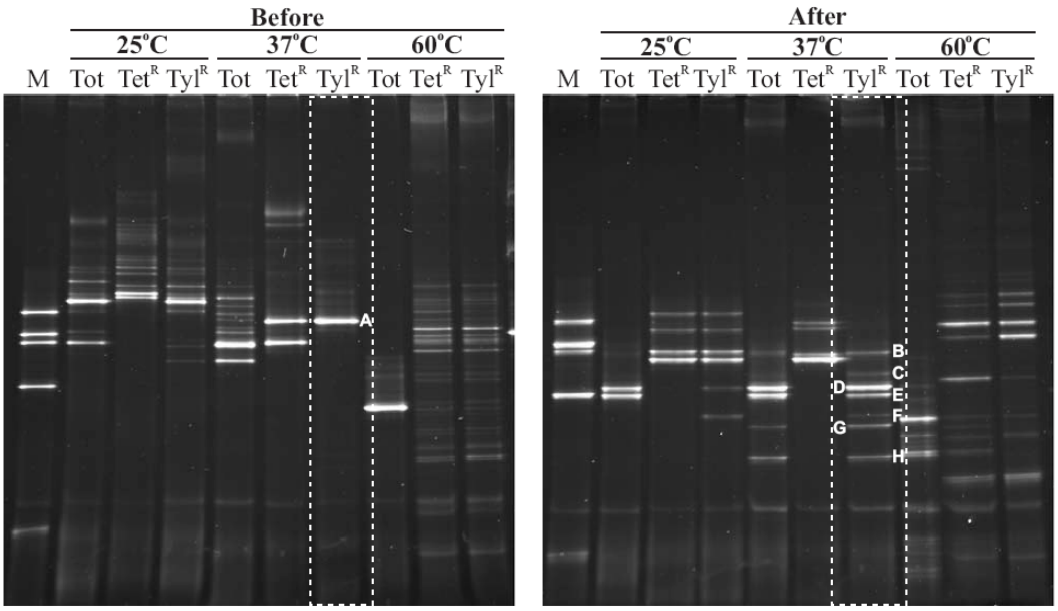

Figure 3. DGGE (20-70% denaturant) of PCR-amplified Bacteria 16S rDNA fragments (V3 region) from the Tot, Tet^R and Tyl^R aerobic bacterial populations at 25°C, 37 °C and 60 °C in the swine waste before and after the AT-SBR biotreatment. M = marker. Dotted boxes indicate the samples that were used for a futher phylogenetic analysis. Letters near bands are used to identify phylotypes (see Fig. 5).

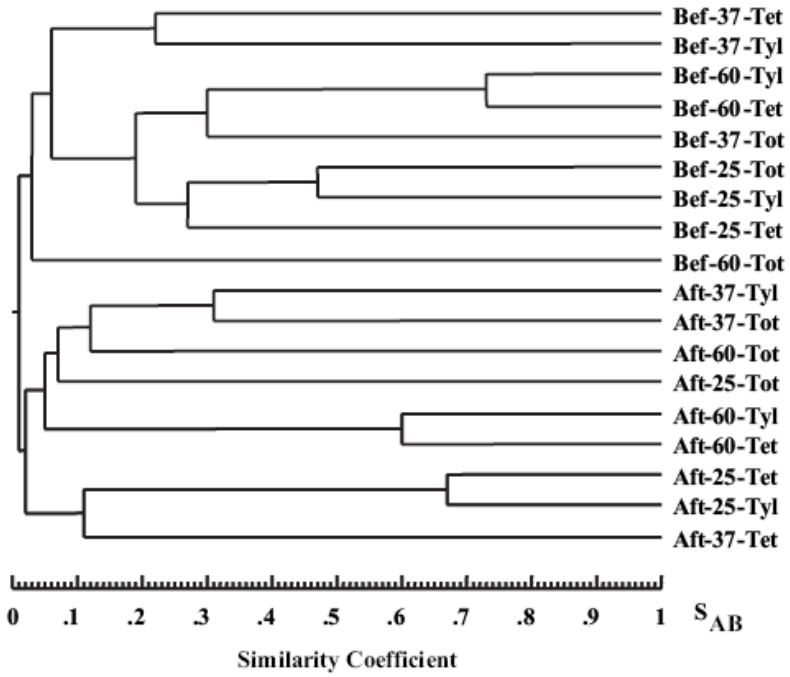

Figure 4. Cluster analysis of DGGE banding patterns from the Tot, Tet^R and Tyl^R aerobic bacterial populations at 25°C, 37 °C and 60 °C in the swine waste before and after the AT-SBR biotreatment. Dendrograms were constructed and similarity coefficients (S_{AB}) were calculated by the Absolute Difference/Maximum Area method.

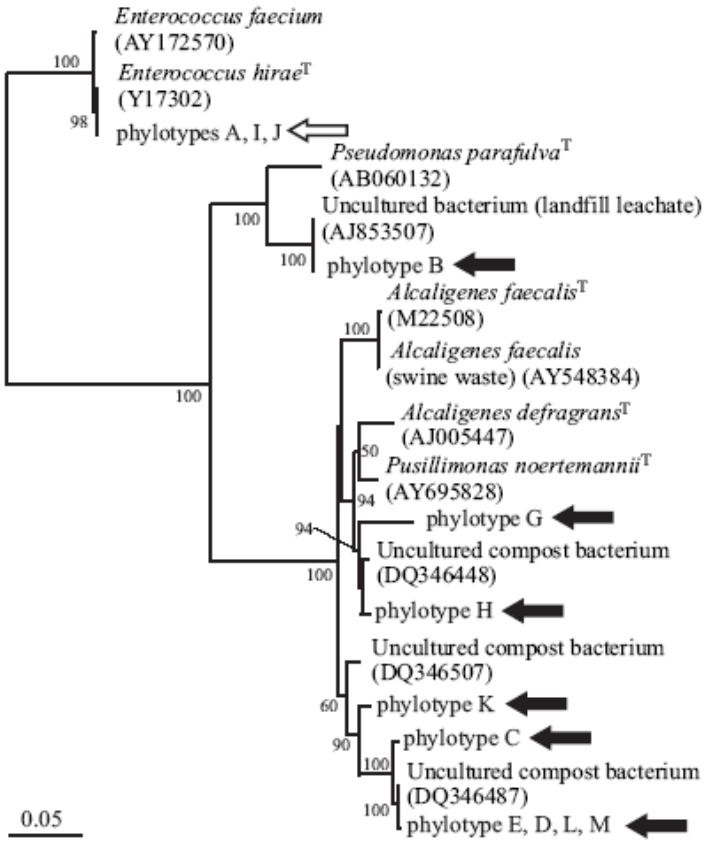

Figure 5. Phylogenetic position of cloned 16S rDNA sequences derived from Tyl^R aerobic bacterial populations at 37 ^oC. White and black arrows indicate phylotypes obtained in the populations before and after treatment, respectively. Bootstrap values (expressed as percentage of 1000 replications) greater than 50% are shown at branch points. Bar = 0.05 nucleotide substitution per position.

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Students who Graduated
2007-2008

Name	Supervisor	Thesis Title
	M.Sc. Degree	
Mohammed Al Abri	R. Cue	Genetic variability of health disorders in Ontario Holstein cows
Marie-Claude Ferland	K.M. Wade	Effects of different feeding systems and sources of grain on lactation characteristics and milk components in dairy cattle
Sunil Kawthekar	X. Zhao	Effects of Arginine, Vitamin E and Vitamin C oncardiopulmonary function and ascites parameters in broilers exposed to cold temperature
Benjamen Olaniyan Benjamen Olaniyan	A. Mustafa	Determination of caprine serum albumin in milk using bromocresol green dye
Jessica Ramirez	K.M. Wade	Use of machine-learning and visualization techniques in the evaluation of factors affecting milk urea nitrogen
Sarah Squires	H. Monardes	M.Sc. Applied
Einar Vargas	A. Mustafa	Performance of dairy cows fed soybean silage
	Ph.D. Degree	
Fadi Hassanat	A. Mustafa P. Seguin	Evaluation of pearl millet forage
Dana Praslickova	U. Kuhnlein X. Zhao	Association of markers in the vitamin D receptor with MHC Class II expression and Marek's Disease resistance

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Publications

Publications in Refereed Journals

Bordignon, V.

Che, L., A. Lalonde and **V. Bordignon**. 2007. Chemical activation of parthenogenetic and nuclear transfer porcine oocytes using ionomycin and strontium chloride. Theriogenology 67: 1297-1304.

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