Effects of dietary inclusion of clinoptilolite in colostrum and milk of dairy calves on absorption of antibodies against *Escherichia coli* and the incidence of diarrhea

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A B S T R A C T

This study investigated effects of administration of two levels of clinoptilolite via colostrum and milk to dairy calves on blood serum antibody levels against *Escherichia coli*, and on the incidence of diarrhea. Eighty-four clinically healthy Holstein calves were divided into three groups according to body weight (BW), sex and the parity of their dams. Group Z1 ($n = 28$) was fed clinoptilolite at 1 g/kg BW/d via colostrum initially, and milk afterwards. Group Z2 ($n = 28$) was fed clinoptilolite at 2 g/kg BW/d via colostrum and milk and Group C ($n = 28$) was fed colostrum and milk without clinoptilolite supplementation. The experiment started at the day of parturition and lasted for 10 d. All calves were fed with the same mixture of frozen colostrum for the first 36 h after calving and thereafter with bulk tank milk twice a day. Specific antibody levels against *E. coli* were determined in blood serum samples of calves at birth, 12, 24 and 48 h after calving. All calves were monitored daily for incidence of diarrhea throughout the experiment. Blood serum antibody levels were higher (P<0.05) in calves that were fed clinoptilolite compared to controls, and those of Z2 were higher (P<0.05) than Z1 throughout the experiment. Administration of clinoptilolite reduced (P<0.05) the incidence of diarrhea. Supplementation of clinoptilolite at 1 g/kg or 2 g/kg BW/d in the colostrum initially, and milk afterwards, during the first 10 d after calving can be effectively used to enhance intestinal absorption of antibodies against enterotoxigenic strains of *E. coli*, and to reduce the incidence and duration of diarrhea in calves.

1. Introduction

Neonatal diarrhea in calves is a syndrome which frequently occurs worldwide and is considered as an important cause of financial losses, especially in winter (Barragry, 1997). Enterotoxigenic strains of *Escherichia coli* (ETEC) is one of the main causative agents of diarrhea in calves during their first days of life, as they adhere to the small intestinal microvilli without inducing morphological lesions and produce enterotoxins which act locally on the enterocytes resulting in hypersecretion and reduced absorption (Nagy and Fekete, 1999). The most common adhesins of ETEC for newborn calves are the fimbriae K-99 and F-41 (Nagy and Fekete, 1999). Prevention of the disease on commercial dairy farms relies on immunization of dairy cows during late pregnancy with vaccines containing K-99 and F-41 antigens (Barragry, 1997; Nagy and Fekete, 1999).

Newborn calves are protected against environmental pathogens, such as ETEC, through passive immunity acquired by absorbing immunoglobulins from the colostrum (Korhonen et al., 2000; Weaver et al., 2000). Absorption of colostal immunoglobulins depends on factors such as the time that colostrum is first ingested, the quantity of colostrum fed, its content of specific immunoglobulins and the health status of the calf (Arthington et al., 2000; Korhonen et al., 2000; Rauprich et al., 2000; Franklin et al., 2003). Due to the importance of colostral immunoglobulins to the humoral immunity in newborn calves during the first few weeks of life, research has focused on finding methods to increase absorption of colostral immunoglobulins.

Clinoptilolite is a natural clay mineral that belongs to the zeolite group. Zeolites are crystalline, hydrated aluminosilicates of alkali and alkaline earth cations that have infinite three-dimensional structures. These materials have unique properties and are characterized by the ability to lose and gain water reversibly, to absorb molecules of appropriate diameter (*i.e.*, adsorption property or acting as molecule sieves), and to exchange their constituent cations without major change of their structure (*i.e.*, ion-exchange property). Due to their physical and chemical properties zeolites, especially clinoptilolite, are used in animal nutrition mainly to improve performance and health status (Mumpton, 1999; Papaioannou et al., 2005). It has been shown in calves that addition of clinoptilolite in the colostrum increases blood serum total immunoglobulins concentration (Stojic et al., 1995; Fratric et al., 2005, 2007; Gvozdic et al., 2008, 2010) and reduces the incidence of diarrhea (Stojic et al., 1995; Nik-Khah and Sadeghi, 2002; Sadeghi and Shawrag, 2008). However the duration of administration, and the quantity of clinoptilolite added, differed among studies, and effects of clinoptilolite administration on absorption of specific immunoglobulins against some infectious diseases was not evaluated.

The objective was to evaluate effects of administration of two levels of clinoptilolite via colostrum and milk in dairy calves on blood serum levels of antibodies against *E. coli*, and on the incidence of diarrhea.

2. Materials and methods

2.1. Animals and experimental design

The study was conducted on a commercial dairy farm in Northern Greece with 84 Holstein calves judged to be healthy before the onset of the experiment. Calves were assigned in three groups according to body weight (BW), sex and the parity of their dams. The first group (Group Z1; $n = 28$) consisted of calves fed clinoptilolite at 1 g/kg BW/d via colostrum initially, and milk afterwards. Calves of the second group (Group Z2; $n = 28$) were fed clinoptilolite at 2 g/kg BW/d via colostrum and milk, and those of the third group (Group C; $n = 28$) were fed colostrum and milk without clinoptilolite supplementation and served as controls.

The experiment for each calf started at the day of calving and lasted for 10 d. Each calf was separated from its dam immediately after calving, weighed and housed in an individual box with straw bedding. Within the first hour of birth, each was offered 2 kg of colostrum via bottle with or without clinoptilolite supplementation according to their experimental group. Colostrum was also fed at 12, 24 and 36 h after calving at the same quantity via plastic buckets that were unique by calf. Afterwards, and until the end of the experiment, calves were fed bulk tank milk twice a day (0.10 of BW). Blood was collected at birth, 12, 24 and 48 h after calving, before each feeding, via jugular vein-puncture (21 – gauge needle) into evacuated glass tubes for determination of antibody levels against *E. coli*. All calves were monitored twice daily by the same animal technician who was blind to treatment for the incidence of liquid feces throughout the experiment. In any case of diarrhea, a fecal sample was taken for determination of the causative agent (ETEC, Rotavirus, Cryptosporidium and Salmonella). Affected animals received appropriate treatment with oral fluid, electrolytes (Diaproof®) and antibacterials (Baytril®) parenterically according to results of the bacterial cultures. Duration of diarrhea (days until regained normal consistency) was recorded.

2.2. Colostrum mixture

Before the experiment, 15 cows of the farm at 4th, 5th and 6th parity that were vaccinated during pregnancy against K-99 and F-41 antigens of *E. coli* were selected to provide the colostrum mixtures that the calves of the experiment were fed. In particular, each cow was milked immediately after calving (1st milking) and colostrum obtained was separated into aliquots of 150 ml. Each aliquot was transferred into plastic vials of 200 ml and kept frozen on the farm. The same procedure was followed for the colostrum obtained 12 (2nd milking), 24 (3rd milking) and 36 h (4th milking) after calving. Within the first hour after birth, each calf was offered a mixture of 15 aliquots (1 from each cow) of the 1st milking. Similarly, at the age of 12, 24 and 36 h, they were offered the mixture of 15 aliquots from the 2nd, 3rd and 4th milkings, respectively.

2.3. Clinoptilolitic material

The zeolitic material used in the experiment had particle size <0.80 mm and contained 920 g/kg clinoptilolite and the mixture was 8 g/kg opal ($SiO_2 \cdot nH_2O$), as determined by X-ray powder diffraction. The material's cation exchange capacity was 220 meq/100 g and its chemical composition is in Table 1.

Table 1
Chemical composition (g/kg) of the clinoptilolitic material used as the supplement.

Zeolite composition	Amount (g/kg)
SiO_2	689.7
Al_2O_3	112.7
CaO	30.2
MgO	6.0
Na_2O	7.5
K_2O	22.3
Fe_2O_3	1.1
H_2O	130.5

2.4. Antibody determination

Blood samples were allowed to clot and serum was separated by low speed centrifugation ($1600 \times g$ for 15 min at $4\,^{\circ}C$), transferred into plastic vials and stored at $2–4\,^{\circ}C$ until further analysis. Antibody levels were determined by ELISA as described previously (Panousis et al., 2001). In brief, ELISA microtitre plates were coated with 50 μl of *E. coli* K-99 pili antigen suspension, provided by the Bacteriology Laboratory of Veterinary School of Bern (Switzerland) and incubated for 12 h at room temperature ($20–22\,^{\circ}C$). After washing the plates with phosphate buffer saline (PBS) containing 0.05 mg/dl Tween-20, 100 μl of serum (1/10 dilution in PBS and Tween-20) was added and incubated for 1 h at $37\,^{\circ}C$. After a wash with PBS and Tween-20, 100 μl of anti-bovine antibody developed in rabbit and marked with horseradish peroxidase (use dilution = 1:8000) was added. The plate was incubated for 30 min at $20–22\,^{\circ}C$ and washed as described previously. 100 μl of substrate TMB-SIGMA was then added and the plate was incubated for 10 min in a dark room. The reaction ended when 50 μl of sulfuric acid 2 M was added. Bovine foetus serum was used as the control. The absorbance value was measured on a spectrophotometer at 450 nm wavelength. The antibody levels at each sample are expressed as the absorbance value obtained at this wavelength.

2.5. Statistical analysis

Data were analyzed with SPSS® 15 (2006). Before analysis, they were tested for normality with Kolmogorov–Smirnov test and the homogeneity of variances was tested with Leven's test. Effects of group, time and their interaction on the blood serum levels of antibodies were evaluated by means of repeated measures analysis with group used as the between subject factor. For each sampling day, data were additionally analyzed, with group used as a fixed factor. In both models, the 0 h antibody levels immediately after calving were used as covariates. Significance of the differences among groups was assessed with Bonferroni test and the associated data are summarized as marginal mean ± SE. Chi-square test was used to determine whether the incidence of diarrhea was significant among groups. In all cases, a significance level of $P \leq 0.05$ was used.

3. Results

3.1. E. coli antibody levels

Blood serum antibody levels against *E. coli* of the three groups of calves were affected by group ($P<0.05$) and there was an interaction group × time ($P<0.05$). Antibody levels differed ($P<0.05$) among experimental groups (2.251, 2.408 and 1.787, for groups Z1, Z2 and C, respectively) as well as age ($P<0.05$), as shown in Table 2. Antibody levels were not significantly affected by age within any group.

Table 2
Blood serum levels of specific antibodies against *E. coli* expressed as the absorbance value obtained by ELISA at the 405 nm of the three groups of calves that were determined at the age of 12, 24 and 48 h. Calves were offered colostrum supplemented with either 1 g/kg BW (Group Z1) or 2 g/kg BW (Group Z2) clinoptilolite/d, whereas those in Group C were offered the unsupplemented colostrum and served as controls.

Age of calves	Group[a]			SEM
	Z1	Z2	Controls	
12 h	2.283a	2.409b	1.810c	0.0187
24 h	2.230a	2.321b	1.748c	0.0214
48 h	2.240a	2.493b	1.804c	0.0469

Different letters (a,b,c) at the same row denote significant difference at $P<0.05$.
Treatment × time: $P<0.05$.

[a] $n = 28$ per treatment.

Table 3
Clinical incidence of diarrhea (number of cases and cases/100 calves) throughout the experiment in the three groups of calves and average duration of the disease in days. Calves were offered colostrum supplemented with either 1 g/kg BW (Group Z1) or 2 g/kg BW (Group Z2) clinoptilolite/d, whereas those in Group C were offered the unsupplemented colostrum and served as controls.

Group[a]	Incidence of Diarrhea Number of cases (cases/100 calves)	Duration (Days ± SD)
Z1	2 (7.0)a	3 ± 1
Z2	1 (3.5)a	2 ± 0
Controls	7 (25.0)b	7 ± 1

Different letters (a,b) at the same column denote significant difference at P<0.05.

[a] n = 28 per treatment.

3.2. Incidence of diarrhea

Diarrhea was observed in 10 calves during the first week of their life (Table 3). ETEC strains were identified at the causative agents for diarrhea in all cases. The incidence of diarrhea was higher in the control group compared to groups Z1 and Z2 (P<0.05). All calves with diarrhea were successfully treated and no death occured. The duration of treatment was shorter in groups Z1 and Z2 *versus* Group C.

4. Discussion

The objective was to determine whether the dietary supplementation of clinoptilolite in colostrum and milk has any effect on the intestinal absorption of antibodies against *E. coli*. Results indicate that the dietary administration of clinoptilolite was associated with higher antibody levels against *E. coli* in blood serum of calves compared to the controls, and that administration rate of 2 g/kg BW was more effective in increasing the antibody levels than 1 g/kg BW. Although effects of clinoptilolite on absorption of specific antibodies against infectious diseases have not been tested in calves to date, it is well documented that administration of clinoptilolite via colostrum in newborn calves increases blood serum concentrations of immunoglobulins (Stojic et al., 1995; Fratric et al., 2005, 2007; Gvozdic et al., 2008, 2010). The mechanisms by which clinoptilolite may enhance intestinal antibody absorption by dairy calves is not clearly understood. However, there are hypotheses. The first hypothesis is that clinoptilolite binds to degradation products of colostral proteins in the intestine, such as ammonia and, by preventing their negative effects on intestinal epithelial cells, increases intestinal absorption efficiency of immunoglobulins (Gvozdic et al., 2010). Clinoptilolite may also enhance antibody absorption due to its retarding effect on the intestinal passage rate (Mumpton and Fishman, 1977) thereby increasing the time that immunoglobulins are available to specific receptors of epithelial cells. As most macromolecules such as immunoglobulins, are pinocytosed by intestinal epithelial cells, another explanation is that clinoptilolite increases the pinocytotic activity of intestinal epithelial cells. Although there is no such evidence in calves, increased pinocytosis of the intestinal epithelial cells has been proven in pigs fed clinoptilolite (Nestorov, 1984).

Blood serum antibody levels remained practically stable after the age of 12 h in all groups of calves. However, other researchers suggest that antibodies peak at about 24 h with two colostrum feedings with constant immunoglobulins content (Morin et al., 1997). This is probably due to the fact that calves at the present study were offered colostrum mixtures with gradually reduced immunoglobulin content representing the natural changes of the mammary secretion during the first 36 h after calving.

Our results confirm previous reports that clinoptilolite supplementation in colostrum and milk reduces the incidence of diarrhea syndrome in calves (Stojic et al., 1995; Nik-Khah and Sadeghi, 2002; Sadeghi and Shawrag, 2008). Calves that received clinoptilolite had lower incidence of diarrhea and a shorter course of the disease compared to controls. This suggests that these calves, due to their higher blood serum antibody titers against ETEC, could respond faster and more efficiently to the ETEC infection. The shorter duration of the illness in these calves may also be due to the following factors: alteration of metabolic acidosis caused by clinoptilolite, through its effects on osmotic pressure in the intestinal lumen (Vrzgula et al., 1988) and/or to adsorption by clinoptilolite of bile acids, one of the endogenic causes of diarrhea, and of glucose whose high content in intestinal fluid acts as an irritant and whose transport through the intestinal cells is reversed during diarrhea (Rodríguez-Fuentes et al., 1997).

5. Conclusions

Results provide the first evidence that administration of 1 g/kg BW, and especially of 2 g/kg BW/d of the natural zeolite clinoptilolite via colostrum and milk during the first 10 after calving increases intestinal absorption of antibodies against ETEC and reduces the incidence and duration of diarrhea in calves. Further research is necessary to determine the exact mechanisms of the antibody absorption enhancement.

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