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Treatment of induced enterotoxigenic colibacillosis (scours) in calves by the lactoperoxidase system and lactoferrin

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Summary — The clinical efficacy of a preparation based on the lactoperoxidase system (LP-s) and lactoferrin (LF) was tested in calves experimentally infected with *E coli* K99⁺, Ent⁺. Mortality, occurrence and duration of diarrhoea were significantly lower ($P < 0.05$) and general clinical status significantly better ($P < 0.05$) in infected calves treated with LP-s and LF preparation than in infected but non-treated calves. Results suggest that LP-s and lactoferrin are effective in the treatment of enteric colibacillosis in calves.

calf / diarrhoea / *Escherichia coli* / lactoperoxidase / lactoferrin

Résumé — **Traitement de la diarrhée colibacillaire entérottoxique chez le veau par le système lactoperoxydase et la lactoferrine.** L'efficacité clinique d'une préparation à base du système lactoperoxydase (LP-s) et de la lactoferrine (LF) a été évaluée chez des veaux inoculés expérimentalement avec *E coli* K99⁺, Ent⁺ et atteints de diarrhées. Il s'est avéré que la mortalité, l'occurrence et la durée des diarrhées étaient significativement plus faibles ($P < 0,05$) et l'état clinique général significativement meilleur ($P < 0,05$) chez les veaux infectés et traités avec le système lactoperoxydase et la lactoferrine que chez les veaux infectés non traités. Les résultats obtenus permettent d'affirmer que le système lactoperoxydase et la lactoferrine sont efficaces dans le traitement de la diarrhée colibacillaire entérottoxique chez le veau.

veau / diarrhée / *Escherichia coli* / lactoperoxydase / lactoferrine

* Correspondence and reprints.

INTRODUCTION

Diarrhoea remains one of the most important causes of mortality and economic loss in calf rearing, but it is difficult to treat effectively because of its complex aetiology (Roy, 1981; Tzipori, 1981). Morbidity and mortality due to calf diarrhoea have been estimated as high as 8–75% and 3–25% respectively in various countries of Europe and North America (Radostits *et al*, 1975; Mornet and Espinasse, 1977; Reynolds *et al*, 1981).

Various microbial pathogens and protozoal agents are involved in neonatal calf diarrhoea. Enterotoxigenic *Escherichia coli* (ETEC), rotavirus, coronavirus and *Cryptosporidium* are the most important pathogens (Roy, 1980; Tzipori, 1981; Fedida *et al*, 1983). These and other microorganisms may provoke acute neonatal calf diarrhoea, either alone or in various combinations (Tzipori, 1981). ETEC is considered as the principal pathogen of early neonatal diarrhoea during the first 4–5 days of life, although it may contribute to diarrhoea up to the age of 3 weeks (Roy, 1980, 1981). Rehydration with electrolyte solutions is generally considered as an important therapeutic measure in this clinical condition. Although antibiotics are also frequently administered their value is more than doubtful due to the emergence of multiple antibiotic-resistant bacterial strains, including *E coli* (Guillot *et al*, 1983; Hinton, 1986) and the general lack of aetiological diagnosis (Radostits *et al*, 1975). Research and clinical application of new aetiological therapies for ETEC diarrhoea is of primary importance under these circumstances (Ramisse *et al*, 1979).

An entirely new therapeutic approach to neonatal bacterial diarrhoea (scouring) has now been developed; it is based on

the antibacterial activity of lactoferrin (LF) and the lactoperoxidase system (LP-s), first reported in 1963 and 1966 respectively (for recent reviews, see Pruitt and Tenovuo, 1985; Reiter, 1985). The aforementioned occur naturally in bovine milk and constitute some of the non-immunoglobulin antibacterial proteins in milk. *In vitro* the LP-s, consisting of lactoperoxidase (LP), thiocyanate ion and hydrogen peroxide only temporarily inhibits some lactic acid bacteria but kills Gram-negative organisms such as *E coli* and eventually lyses them. In both instances the metabolic and synthesising activity is inhibited within minutes of exposure to the LP-s. *In vivo* using cannulated calves, *E coli* is killed in the abomasum and upper duodenum (Reiter *et al*, 1981).

The antibacterial activity of LF was confirmed by Bullen *et al* (1972) who demonstrated that *E coli* was temporarily inhibited in human milk containing a naturally significant quantity of LF. A temporary suppression of the growth of *E coli* by LF *in vitro* was also confirmed (Law and Reiter, 1977).

The aim of this study was to test the clinical effectiveness of Safilac[®], which contains both LF and LP-s, (produced by Synfina-Oleofina SA, Brussels, Belgium) in therapy for acute neonatal calf diarrhoea provoked by experimental infection of ETEC.

MATERIALS AND METHODS

Experimental animals

Forty-eight dairy calves were purchased from different farms in Belgium. The calves were given colostrum on the farms and transported rapidly to the experimental centre (Centre of Rural Economy, Marloie, Belgium).

Housing facilities

Calves were placed in individual pens with straw bedding. Environmental parameters including a stable microclimate were as recommended by Roy (1980). Strict hygienic measures were applied and ETEC infected and non-infected calves were housed separately.

Nutrition

Commercial milk-replacer (Crema Elevage, produced by Sanders Co) was used in the recommended dilution and quantity. Calves were fed twice a day from a nipple bottle and attention was paid not to overfeed them and therefore not to precipitate an alimentary diarrhoea (Roy, 1980).

Treatment of calves with LP-s and LF

Calves were treated after appearance of diarrhoea with 5 or 10 g of a preparation (Safilac®, Synfina-Oleofina SA, Brussels, Belgium) containing 35 mg lactoferrin, 19.3 mg lactoperoxidase, 19.3 mg thiocyanate, 1.55 mg glucose oxidase, 1 g glucose and 9.9 g lactoserum) per litre of milk. The treatment was continued until the end of the experiment at day 6. The product was also preventively administered to non-infected calves without any evidence of diarrhoea at 10 g/l during the entire experimental period as prophylaxis and to establish the preparation's lack of toxicity.

Experimental groups

Calves were distributed at random into 5 experimental groups immediately after their transport to the experimental centre. Eleven calves were infected with *E coli* 510, 12 calves were infected and treated with the preparation of LP-s and LF at 10 g/l, 11 calves were infected and treated with the preparation of LP-s and LF at 5 g/l, 7

calves were not infected or treated, 7 calves were not infected but fed with the preparation of LP-s and LF at 10 g/l.

Bacteria

The *Escherichia coli* strain 510 (0101: K30, K99, F5, F41, Nm*) was obtained from the Department of Bacteriology, Veterinary Faculty, University of Liège, Belgium. The strain produces the heat-stable enterotoxin ST_aP (Mainil and Kaeckenbeeck, 1986) and has been shown to provoke severe diarrhoea in calves under experimental conditions (Schoenaers and Kaeckenbeeck, 1975). The strain was cultured on brain heart infusion agar (Difco Co, USA) for 18 h at 37 °C. The organisms were washed off with 0.9% physiological saline solution (pH 6.8) and suspended to contain 15 x 10⁹ colony forming units/milliliter (CFU/ml). This strain has been shown to be sensitive to LP-s and LF *in vitro*.

Infection of calves

At the age of 8–38 h, 34 calves were given *per os* 10 ml of a suspension containing 15 x 10¹⁰ CFU.

Clinical evaluation

All calves were observed daily over a 6-day period. Simple blinding was applied as an objective method for clinical evaluation of calves. Rectal temperature was measured once a day (morning): values of over 39 °C were considered as indicative of hyperthermia. Rapidity of disappearance of skin fold in the neck region was used as a criterion to evaluate degree of dehydration. Degree of clinical depression was determined by observation of animals (Sherman *et al*, 1983). Depression was considered as the loss of normal alert behaviour, *ie* the animal was predominantly recumbent, with no apparent reaction upon arrival of the clinician, the facial expression was sleepy, there was evidence of

* Non-mobile.

anorexia and reduced individual hygiene (ruffled and dirty coat). Gastrointestinal disorders were rated according to consistency of faeces and manner of excretion: normal (no apparent abnormalities), mild diarrhoea (more frequent emissions of bulky and apparently softer faeces), severe watery diarrhoea (frequent and voluminous emissions of malodorous watery faeces). The calves were weighed before the beginning of the experiment, and on the third and sixth day.

Identification of infectious agents

E Coli 510

E coli 510 was identified in the faeces using 3 different methods for each sample: ELISA technique kits produced by Center of Rural Economy, Marloie, Belgium, using specific K99 antibody; direct immunofluorescence (Kawamura, 1977) using a hyperimmune rabbit anti-serum against the reference strain *E coli* K30; agglutination of *E coli* isolated from the faeces and grown on Gassner medium (Merck Co) for 24 h

at 37 °C, using the rabbit anti-serum against the reference strain K30. The test was performed with 50 lactose positive colonies from each sample of faeces.

Rotavirus, coronavirus and *Cryptosporidia*

They were diagnosed by the ELISA diagnostic method produced by Centre of Rural Economy, Marloie, Belgium.

All the quoted suspected pathogens (Tzipori, 1981; Tzipori *et al*, 1983)

They were recorded in calves upon arrival and then daily during the experimental period.

Evaluation of immune status of calves

The immune status of the calves was estimated by the level of serum immunoglobulins (Ig) using laser immunonephelometry. Values below 8 mg Ig per ml of serum were considered as insuffi-

Table I. Prevalence and duration of diarrhoea ^a, mortality rate and occurrence of severe pathologies ^b in calves. In this study, "Diarrhoea" indicated diarrhoea for less than 1 day, "Severe diarrhoea" indicated watery diarrhoea for more than 2 days and "Severe pathologies" indicated severe watery diarrhoea, mild to severe dehydration, depression and mortal cases.

	Dose of LP-s and LF administered (g/l)				
	Infected calves			Non-infected calves	
	0	10	5	0	10
No (%) of calves with:					
Diarrhoea	10 (91)	12 (100)	11 (100)	3 (43)	3 (43)
Severe diarrhoea	5 (45)	1 (8)	3 (27)	0 (0)	0 (0)
No (m ± SD) of days with:					
Diarrhoea	2,6 ± 1,7	1,8 ± 1,3	2,8 ± 1,3	0,4 ± 0,5	0,7 ± 1,1
Severe diarrhoea	1,6 ± 1,3	0,4 ± 1,3	1,2 ± 1,1	0,1 ± 0,4	0,1 ± 0,4
Calf mortality (No)	4 (36)	0 (0)	3 (27)	0 (0)	0 (0)
No (%) of calves with severe pathologies	8 (73)	1 (8)	4 (36)	0 (0)	0 (0)

Table II. Occurrence of hypogammaglobulinaemia and its relationship to mortality in calves. ^a Calves were given *per os* 15×10^{10} CFU of *E coli* 510.

<i>Dose of LP-s and LF administered (g/l)</i>	<i>Hypogammaglobulinemia No of calves (%)</i>	<i>Mortality rate in hypogammaglobulinemic calves (No of calves) (% of total mortality)</i>
<i>Infected calves</i> ^a		
0	5 (45)	3 (75)
10	5 (42)	0 (0)
5	4 (36)	2 (67)
<i>Non-infected calves</i>		
0	0 (0)	0 (0)
10	2 (29)	0 (0)
Total	16 (33)	5 (71)

cient and recorded as indicative of hypogammaglobulinemia (Pellerin, 1982).

Statistical analysis of results

The Chi-square test and one-way analysis of variance were utilised (Dagnelie, 1983), for the statistical analysis of results.

RESULTS

Diarrhoea occurred significantly ($P < 0.01$) more frequently in infected than in non-infected animals. Diarrhoea was induced in 10/11 (91%) non-treated animals and in all treated animals within 1–2 days post-infection. Occurrence of severe watery diarrhoea lasting 2 or more days was signifi-

Table III. Clinical evaluation of calves during the experiment. ^a Rectal temperature higher than 39.3 °C. ^b Calves were given *per os* 15×10^{10} CFU of *E coli* 510.

<i>Dose of LP-s and LF administered (g/l)</i>	<i>Hyperthermia</i> ^a	<i>Dehydration and clinical depression</i>
	No of calves (%)	
<i>Infected calves</i> ^b		
0	2 (18)	4 (36)
10	0 (0)	0 (0)
5	0 (0)	3 (27)
<i>Non-infected calves</i>		
0	0 (0)	0 (0)
10	2 (29)	0 (0)

Table IV. Live weight changes in calves during the first 3 and 6 days of the experiment. ^a Calves were given *per os* 15×10^{10} CFU of *E coli* 510.

Dose of LP-s and LF administered (g/l)	1st 3 days	6 days
Variation in live body weight (kg), mean \pm SD		
<i>Infected calves</i>		
0	-1.42 \pm 1.82	-1.14 \pm 2.63
10	-0.375 \pm 1.26	-0.7 \pm 1.6
5	-1.39 \pm 1.63	-0.56 \pm 1.53
<i>Non-infected calves</i>		
0	-0.36 \pm 0.58	+0.5 \pm 0.96
10	\pm 0.36 \pm 1.22	\pm 0.79 \pm 1.16

Table V. Elimination of several infectious gastrointestinal pathogens in calf faeces. Each animal was considered to shed a pathogen, if this was confirmed by at least 2 positive samples (*E coli* 510) or by one positive sample (rotavirus, coronavirus and cryptosporidium) during the experimental period. ^a Calves were given *per os* 15×10^{10} CFU *E coli* 510.

Dose of LP-s and LF administered (g/l)	Infectious agents				
	<i>E coli</i> 510	<i>Rotavirus</i>	<i>Coronavirus</i>	Cryptosporidium	<i>Virus species and/or Cryptosporidium</i>
Number of calves (%)					
<i>Infected calves</i>					
0	11 (100)	2 (18)	1 (9)	2 (18)	2 (18)
10	12 (100)	6 (50)	3 (25)	3 (25)	4 (33)
5	11 (100)	8 (73)	2 (18)	6 (55)	5 (55)
<i>Non-infected calves</i>					
0	2 (29)	3 (43)	2 (29)	3 (43)	1 (14)
10	2 (29)	4 (57)	1 (14)	2 (29)	1 (14)

cantly ($P < 0.05$) reduced in the scouring subjects treated with 10 g of preparation per l of milk, but not in the animals treated with 5 g/l as compared with the non-treated diarrhoeic calves. No severe diarrhoea (> 2 days) was observed in non-infected calves (table I).

Duration of diarrhoea was significantly ($P < 0.05$) reduced in infected and treated calves (10 g/l), as compared to infected non-treated animals. The results suggest a positive effect of 10 g/l but not 5 g/l of the product in the treatment of diarrhoea in calves.

Although the distribution of immunodeficient animals was comparable ($P < 0.05$) in all 3 groups of infected calves, mortality was significantly lower ($P < 0.05$) in calves treated with 10 g/l of LP-s and LF than in non-treated diarrhoeic animals (table II).

Clinical data are summarized in table III. Transient periods of hyperthermia (under two days) were occasionally detected in 2 infected and non-infected animals. Mild to severe dehydration associated with clinical depression were observed in 7 calves shortly before their death. In the infected animals, clinical status was significantly ($P < 0.05$) better in treated calves (10 g/l) than in non-treated animals.

Variations in live body weight are presented in table IV. On average, infected calves showed higher weight loss than non-infected animals. However, statistical analysis did not confirm any significant difference ($P > 0.05$) between the groups. Initial live weight of animals before the experiment was comparable among the groups ($P > 0.05$).

Table V summarises data on 4 major gastrointestinal pathogens in calf faeces. *E coli* 510 was confirmed in all infected animals from the second to the fourth experimental day, but was only occasionally found in non-infected animals ($P < 0.01$) although they did not scour. Rotavirus, coronavirus and *Cryptosporidium* were detected in some animals of each group (9–73%), predominantly during the fifth and sixth day of the experiment.

Statistical treatment did not confirm ($P > 0.05$) any significant difference between the groups as far as prevalence of rotavirus, coronavirus or *Cryptosporidia* was concerned.

DISCUSSION AND CONCLUSION

The experiments were designed to study the therapeutic effects of LP-s and LF under controlled clinical conditions resembling modern calf-rearing units (high standard of zoohygiene, nutrition and handling of calves). Colostrum was administered before the experiment but probably not in sufficient quantities in all calves as indicated by the relatively high percentage of hypogammaglobulinaemic animals. The calves were purchased from farms where no diarrhoea associated with ETEC had been detected previously and vaccination against pathogens was not practiced. These two factors may have increased the resistance of calves to the infection (Kaeckenbeek, 1980; Sherman *et al.*, 1983).

ETEC alone produces a profuse diarrhoea only in very young calves and its clinical importance depends on the interaction between the number of organisms (ETEC), age of host and level of immunity (Tzipori, 1981). In the case of calves, a high dose of a pathogen strain of ETEC (Schoenaers and Kaeckenbeek, 1975) before calves are 2 days old is required as they become resistant to experimentally induced intestinal colonisation by ETEC after that age (Smith and Halls, 1967).

In similar experimental studies on ETEC diarrhoea in calves better (Espinasse *et al.*, 1983; Sherman *et al.*, 1983) and worse (Bywater, 1977) results (morbidity and mortality) have been obtained, which were probably due to different ETEC strains and doses, as well as to varying effects of age and immune status (colostrum-fed or deprived calves) of the animals used in the studies.

Monoclonal antibodies to ETEC K99 antigen (Sherman *et al*, 1983), a glucose-glycine electrolyte solution alone or in combination with amoxicillin (Bywater, 1977), amoxicillin (Bywater, 1977; Palmer *et al*, 1977) cephamycin C (Jacks, 1980) or parenteral administration of flumequine (Espinasse, 1983) were found to be therapeutically effective ($P < 0.05$ or less) in colostrum-fed or colostrum-deprived calves infected by ETEC in similar studies. However, the real values of these recommended therapies under normal clinical conditions have been questioned by several authors. For instance, Radostits *et al* (1975) did not confirm a significantly superior therapeutic effect of peroral administration of antimicrobial drugs alone or in combination with parenterally administered antibiotics and/or peroral rehydration solutions, as compared to the non-treated milk-fed animals. Rollin *et al* (1986) experimentally confirmed that several currently used antibiotics (ampicillin, neomycin, chloramphenicol, tetracycline) actually provoked diarrhoea in a majority of normal colostrum-fed calves when they were administered orally for 3 to 5 days in maximally recommended therapeutic doses. Iatrogenic diarrhoea was associated with significantly ($P < 0.05$) decreased and delayed glucose absorption during oral glucose tolerance tests.

LP and LF are antibacterial non-immunoglobulin proteins, which occur naturally in milk of all mammalian species, but in varying proportions. Their biological antimicrobial activities may be significantly limited by several factors including temperature (destruction during heat treatment of milk), presence of several metallic ions (saturation of LF by iron or zinc), variations in thiocyanate content and limited sources of hydrogen peroxide, *ie* essential LP-s components (Reiter, 1985).

The antibacterial effects of LP-s are related to the chemical action of intermedi-

ate reactive products such as hypothiocyanite with enzymes and other compounds of the bacterial cytoplasmic membrane. LF only partly saturated with iron in its native state deprives some bacteria, such as *E coli*, of iron essential for their growth (Pruitt and Tenovuo, 1985; Reiter, 1985).

Reiter and Härnolv (1982) have confirmed the bactericidal effect of activated LP-s upon abomasal *E coli* in cannulated calves. The system has also been shown to improve weight gain in milk-fed calves (Reiter *et al*, 1981). LP-s was found to be effective in the treatment of experimental by induced enteric colibacillosis (ETEC strain 510) in calves (Prieels *et al*, 1989).

No *in vitro* studies in the calf on interactions between LP-s and/or LF and gastrointestinal virus such as rotavirus and coronavirus and protozoa (*Cryptosporidium*) were conducted. Only a non-significant ($P > 0.05$) reduction in duration of diarrhoea and improvement in weight gain was observed in mixed calf diarrhoea associated with these pathogens. This could be related to an insufficient activity of LP-s and LF against the microorganisms. The non-bacterial pathogens were detected only in a minority of calves from all 5 groups ($P > 0.05$) in the present study.

Mostly, they were detected during the last 2 days of the experimental period and after the diarrhoea associated with faecal ETEC. This suggests that in this study their role as pathogens is a minor one.

A low incidence of a mild diarrhoea in the 2 groups of non-infected animals suggests the lack of other hypothetical pathologies (not checked) which could have provoked gastrointestinal disorders in the animals.

The preparation containing the active LP-s and LF proved to significantly reduce ($P < 0.05$) the incidence of profuse watery diarrhoea lasting 2 days and more, duration of the watery diarrhoea and mortality

of the infected calves. A protective effect against clinical deterioration (dehydration, depression) was also shown ($P < 0.05$).

Significantly lower mortality ($P < 0.05$) was recorded in the infected calves treated with 10 g/l, but not in the 5 g/l treated animals, as compared to infected non-treated calves. These results were not apparently influenced by the insufficient immune state of calves, as the percentage of immunodeficient subjects was comparable ($P > 0.05$) in all 3 groups of infected animals (Table II).

These conclusions are applicable only to calves treated with a preparation of 10 g/l milk, but not for the 5 g/l group. These findings might be related to a lower intestinal availability of the product in the case of diarrhoea, *ie* 5 g/l *versus* 10 g/l. Dilution of the intestinal content due to hypersecretion caused by ETEC toxins can be significant (Bywater, 1977; Mornet and Espinasse, 1977; Tzipori, 1981) and could possibly contribute to reduction in the therapeutically required doses of the active principles of the product (Reiter, 1985). However, these premises should be further studied in *in vivo* models. *In vitro* studies of the LP-s and LF suggest that their bacteriostatic or bactericide effects are dose-related (Reiter, 1985).

Results in this study suggest that a preparation containing LP-s and LF, is effective in the treatment of ETEC neonatal calf diarrhoea. The treatment was successful without addition of other recommended drugs and rehydration. Milk nutrition was not interrupted during treatment. However, only a 10 g/l dose of milk was effective. The product is harmless to the calf digestive tract as evidenced by the positive results obtained in non-infected calves treated with 10 g/l of milk. The preparation is therefore recommended for treatment of ETEC diarrhoea.

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