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Comparative studies on Polyferm and Fermosorb, two oral (ferment + sorbent) – type preparations designed for therapy/prophylaxis of intestinal infections in animal neonates

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ABSTRACT

Polyferm and Fermosorb are oral acid resistant antimicrobial enzyme preparations designed specifically for therapy/prophylaxis of intestinal infections in animal neonates. Both are authorized for use throughout the former Soviet Union, but until now only Fermosorb is being applied on a large scale. The comparative studies on these two preparations, described in this paper, were carried out in order to find differences between the preparations. Characteristics that were compared included stability of the preparations in acidic environment as well as in storage (*in vitro* studies), and their efficacy for the treatment and prophylaxis of colibacillosis in newborn calves (*in vivo* studies). Results of *in vitro* studies revealed that proteolytic enzymes of Polyferm (as well as lytic enzymes of Fermosorb) were suitably (and in a very similar magnitude) protected from the influence of the acidic environment. The complete enzyme activity retention period in storage at room temperature of Polyferm and Fermosorb was equally high (5 years). *In vivo* studies performed on 2000 calves revealed that both preparations were highly effective and, although the efficacy of Polyferm was a bit lower than that of Fermosorb (93.6% vs. 95.0%, 94.6% vs. 95.8% for therapy and prophylaxis of colibacillosis, respectively), no statistically significant differences in the number of Polyferm vs. Fermosorb cured/protected animals were found. It is concluded that there were no reasons, other than the lack of supportive advertising materials, that might impede the utility of Polyferm.

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INTRODUCTION

Several enzyme preparations possessing antimicrobial activities have been proposed for the therapy/prophylaxis of gastrointestinal disorders in animal neonates (Bevz, 1974; Goryachev, 1974; Valaitis & Balchiunas, 1987; Biziulevichius & Kislukhina, 1988; Avizhienis *et al.*, 1989; Babenko, 1989; Bagniuk *et al.*, 1989; Kariniauskiene, 1989; Makaruk *et al.*, 1989; Abramov & Shevchenko, 1991; Biziulevichius & Arestov, 1997a,b), but application thereof was not always efficient because of partial inactivation of enzymes in the gastric region. In order to overcome the inactivation problem, enteric coated enzyme preparations were created and these have been shown to be effective measures in intestinal infections in animals (Mynott *et al.*, 1991, 1996; Chandler & Mynott, 1998). On the other hand, fabrication of oral enzyme preparations in the form of pellets or tablets coated with enteric polymers is laborious and time consuming (Mayer & Viernstein, 1994; Schulz & Schmidt, 1995).

Recently, we proposed a simplified approach in the design of oral preparations intended for intestinal delivery of enzymes, based on reversible immobilization of the latter onto a polymer matrix (Biziulevičius & Žukaitė, 1999, 2000). Fermosorb (an abbreviation of Ferment + sorbent) – type preparations, produced in such a novel way, are characterized as two-component delayed-release enzyme formulations being stable at acidic pHs and thus ensuring the protection of the active substance in the environment of the gastric region and liberating the active substance through dissociation of the enzyme–polymer complex at neutral pH values characteristic for the intestines. Two representatives, Fermosorb and Polyferm (an abbreviation of Polymer + ferment), the active substance of the former being lytic enzymes, while the active substance of the latter being proteolytic enzymes, have been shown to be highly effective for prophylaxis and therapy of gastrointestinal disorders, including colibacillosis, in newborn calves (Zotkin *et al*, 1987a,b; Sisyagin*et al*, 1988a,b). Both are authorized for use throughout the former Soviet Union. More than 150,000 newborn calves have already been treated with Fermosorb (Biziulevičius & Žukaitė, 1999), but for unknown reasons (most probably for the lack of information) a much smaller number of animals have been treated with Polyferm (Zotkin *et al*., 1987b; Sisyagin *et al*., 1988b). Moreover, comparative studies of the efficacy of Fermosorb and Polyferm for the treatment and prophylaxis of intestinal infections in newborn calves have never been performed.

This paper provides comparative results of the efficacy of Polyferm and Fermosorb for the treatment and prophylaxis of colibacillosis in newborn calves. The comparative results of *in vitro* studies with respect to stability of these preparations in acidic environment as well as in storage are also given.

MATERIALS AND METHODS

Fermosorb and Polyferm

Pilot-scale preparations of Fermosorb and Polyferm were produced as described in detail previously (Biziulevičius & Žukaitė, 1999, 2000). Briefly, enzymes were immobilized, using 250-L amounts of 1% lysosubtilin G10x solution or industrial *Bacillus subtilis* 103 submerged culture filtrate for the production of Fermosorb and Polyferm, respectively, onto a beaded cross-linked copolymer of metacrylic acid and triethylene glycol dimethacrylate, Biocarb L. Lysosubtilin G10x and *B. subtilis* 103 submerged culture filtrate were acquired from the State Joint-Stock Enterprise 'Biosinteze', Vilnius, Lithuania, while Biocarb L was purchased from the All-Union Research Institute of Chemical Technology, Moscow, Russia. The immobilization procedure was performed in a 0.6-m^3 closed metal vessel with an inert plastic-coated inner surface under constant mechanical agitation with a propeller stirrer (not less than 600 rpm). Procedures were carried out at room temperature and at optimal conditions for immobilization (1 h at pH 5.0 and 8 h at pH 4.6 for Fermosorb and Polyferm, respectively; v/w ratio of the liquid phase and Biocarb L equal to 10:1 in both cases). Then the procedures of vacuum-filtration, oven-drying and standardization of the products followed. The final products were small beads (colour: greyish or yellowish; size: up to 2 mm; moisture content: no more than 10%) with a lytic activity 5×10^4 U/g according to Kislukhina (1976) or a proteolytic activity 20 U/g according to modified Anson method (State Committee of the USSR for Standards, 1985) for Fermosorb and Polyferm, respectively.

Unless otherwise stated, activities of Fermosorb and Polyferm were measured after 2 h desorption of the appropriate enzymes with an approximately 10-fold amount of 50 mM phosphate buffer, pH 7.2. The enzyme activities were defined as follows: 1 U of lytic enzyme activity (1 LU) generates a decrease in turbidity of 0.001 optical density (OD)/min at 520–540 nm in a suspension of *Escherichia coli* K 12 dried cells (initial OD 0.6) in 1.0 mL of 4 mM phosphate buffer, pH 7.2 at 30 °C, while 1 U of proteolytic enzyme activity (1 PU) in 1 min at pH 7.2 and 30 °C converts into trichloracetic acid-nonprecipitatable state an amount of sodium caseinate corresponding to 1 μmol of tyrosine.

Experimental design

In vitro studies

Prior to determination of stability in acidic environment a sample of Polyferm was allowed to swell for 1 h in a 10-fold amount of 10 mM acetate buffer of pH 5.0. The sample was divided equally into six parts, which were adjusted, where appropriate, with 10% hydrochloric acid to pH values of 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0, respectively, and kept at 37 °C for 1 h under constant agitation using a magnetic stirrer. The residual activity of Polyferm was measured after 1 h desorption of enzymes with an approximately 10-fold amount of 50 mM phosphate buffer, pH 7.2 and the results were compared with the ones already described for Fermosorb (Biziulevičius & Žukaitė, 1999).

For determination of stability in storage, 11 identical samples of Polyferm were prepared in hermetically sealed plastic bags. Enzyme activity was measured at preparation of the samples (zero time) and later at 1-year intervals with the new bags being opened every test time. The results were compared with the ones already described for Fermosorb (Biziulevičius & Žukaitė, 1999).

In vivo studies

Experiments with newborn calves were approved by the former Soviet Union Chief Veterinary Medicine Board of the Ministry of Agriculture. The trials were performed in newborn calf colibacillosis problematic farms of three republics of the former Soviet Union (Byelarus, Lithuania, Russia) on 2000 animals up to 7 days of age. In all these farms Fermosorb was used (at least for six recent months) as a treatment of choice against newborn calf colibacillosis because of its high efficacy and no evidence of microbial adaptation to its lytic effects. The prevalence of colibacillosis among calves, confirmed by faecal microbiology and necropsy of acutely affected animals, of the different farms at the time when the experiments were performed varied considerably, but it was not lower than 50%, while the mortality

concentration of diseased animals if left untreated was not lower than 75%.

The calves were maintained under usual industrial breeding conditions. The calves were randomly allocated into groups with 100 animals in each. Clinical observation of the calves was performed at least every 8 h throughout the experiments.

The calves in the treatment groups were those in which the first signs of colibacillosis (Fraser *et al*., 1991) had been detected (generally on the second or third day of life). In each region one group of calves (control group) was treated with Fermosorb, while another one (study group) was treated with Polyferm. Calves were treated with Fermosorb in the manner proposed by us previously (Zotkin *et al*., 1987a), i.e. they were given the drug in the dose 10^4 LU/kg of weight immediately after calf initiation into the study and then later in the same dose three times daily (every 8 h) until recovery, for no longer than 3 days. Calves were treated with Polyferm by the previously described method (Zotkin *et al*., 1987b), i.e. they were given the drug in the dose 4 PU/kg of weight three times daily (every 8 h) until recovery, for no longer than 3 days. Fermosorb or Polyferm were administered in 50 mL of boiled water or weak tea using a nipple bottle. After the 3-day period, unsuccessfully treated calves of both groups were withdrawn from the experiment (unless the calves died) and were further treated in other ways or culled. The culled (as well as dead) animals were submitted for necropsy.

The calves of the prophylaxis groups were healthy ones. In each region one group of calves (control group) was administered Fermosorb, while another (study group) was administered Polyferm. The prophylaxis of the calves was performed as proposed by us previously for Fermosorb (Sisyagin *et al*., 1988a) or Polyferm (Sisyagin *et al*., 1988b), respectively, i.e. they were administered the drug in the same dose and by the same route as described above for the treatment group calves with the difference that the initial dose of Fermosorb or Polyferm was given 90 min after the first colostrum feed and then later twice daily (every 12 h) for 5 days. Unsuccessfully treated calves were withdrawn from the experiment and treated or, in case of sudden critically acute signs, were submitted for necropsy, as soon as the first signs of colibacillosis (Fraser *et al*., 1991) appeared.

Statistical analysis

The chi-squared test was used to evaluate the significant differences in the number of Polyferm vs. Fermosorb cured/protected animals as well as in the number of animals submitted for necropsy because of lack of effect. $P < 0.05$ was considered statistically significant.

RESULTS

In vitro studies

Retention of enzyme activities of Polyferm and Fermosorb in the acidic environment by the polymeric structures of Biocarb L is shown in Table 1. The results presented in Table 1 reveal that both proteolytic and lytic activities of Polyferm and Fermosorb, respectively, were protected to a very similar extent.

Table 1 . Residual enzyme activities of preswollen Polyferm and Fermosorb preparations after incubation for 1 h in acidic environments at 37°C

Results depicting stability in storage, one more important drug characteristic, of these two oral enzyme preparations are given in Table 2. No losses of enzyme activities were observed when stored at room temperature for 5 years and a further 2% yearly decrease was observed for five subsequent years, thus both Polyferm and Fermosorb possessed not only the identical high complete enzyme activity retention period, but their unavoidable activity diminishing patterns were identical as well.

Table 2 . Residual enzyme activities of dry Polyferm and Fermosorb preparations after different periods of storage at room temperature

In vivo studies

Comparative results of the efficacy of Polyferm and Fermosorb for the treatment and prophylaxis of colibacillosis in newborn calves are given in Table 3. Both preparations were highly effective and, although the efficacy of Polyferm was a bit lower than that of Fermosorb (93.6% vs. 95.0%, 94.6% vs. 95.8% for therapy and prophylaxis, respectively), no statistically significant differences in the number of Polyferm vs. Fermosorb cured/protected animals (in any region as well as total) were found. No statistically significant differences were found in the number of animals submitted for necropsy because of the lack of Polyferm or Fermosorb effect (20 calves in total, but no more than two animals in any

of the treatment or prophylaxis groups; full data not shown) as well.

Table 3 . Efficacy of Polyferm and Fermosorb for the treatment and prophylaxis of colibacillosis in newborn calves*

DISCUSSION

Antibiotic resistance is of current concern (Finch, 1998; Moellering, 1998; Mazel & Davies, 1999). In veterinary medicine, where group medication of affected animals as well as prophylactic use of antimicrobials (not to mention their use for growth promotion in food animals) are more common than in human medicine, the development of resistant microorganisms and their close relationship with human health become a very serious problem (Perez-Trallero & Zigorraga, 1995; Piddock, 1996; Aarestrup, 1999; Fidler, 1999; Shryock, 1999; Van den Bogaard & Stobberingh, 1999). Alternative therapies such as probiotics, immunomodulators, antimicrobial enzymes (Fuller, 1989, 1994; Kislukhina *et al.*, 1990; Barot-Ciorbaru, 1994; Abe *et al.*, 1995; Araneo *et al.*, 1996; Sakai *et al.*, 1996; Sava, 1996; Werner & Jollès, 1996; Kristiansen & Amaral, 1997) as well as antimicrobial resistance monitoring programmes (Aarestrup *et al.*, 1998a,b; Ministry of Health & Ministry of Food, Agriculture and Fisheries, 1999; Verhoef *et al.*, 1999) are therefore of interest.

To improve the arsenal of animal health care products we created lysosubtilin, a broad antimicrobial spectrum action preparation of lytic enzymes (Biziulevichius *et al.*, 1976, 1987, 1989, 1995; Biziulevichius & Kislukhina, 1987). When used as an alternative to antibiotics for the prophylaxis and/or therapy of gastrointestinal disorders in newborn calves as well as gynaecological diseases in cows, lysosubtilin has been shown to be more efficient than the commonly applied drugs (Biziulevichius & Arestov, 1997a; Biziulevichius & Lukauskas, 1998a,b). To make it even more suitable for the treatment/prophylaxis of intestinal diseases in animals we modified lysosubtilin by immobilization of lytic enzymes onto the polymer matrix Biocarb L resulting in its acid resistant derivative Fermosorb. *In vivo* evaluation studies performed on 1200 newborn calves revealed 95.2% therapeutic as well as 95.0% prophylactic efficacy of Fermosorb in respect to colibacillosis vs. 74.0 and 80.0% for lysosubtilin, respectively, the differences being statistically significant (Biziulevičius & Žukaitė, 1999). Consequently Fermosorb was authorized for use throughout the former Soviet Union and its application spread widely. Following the discovery that certain proteolytic enzymes, including *B. subtilis* neutral proteinase, possess antimicrobial properties that are characteristic for lytic enzymes and include the capability to lyse *E. coli* cell walls (Kislukhina & Shevchuk, 1976; Thorne *et al.*, 1976; Galich & Kolesnik, 1982; Dean & Ward, 1991; Selan *et al.*, 1993; Grenier, 1994), we created Polyferm, an enzyme-polymer complex designated for antimicrobial enzymotherapy/prophylaxis of intestinal infections in animal neonates. And although this preparation was also authorized for use, its application concentration was not as high as that of Fermosorb. This study was carried out in order to characterize differences between these two enzyme preparations and a causal relationship, if any, that might lead to an underutilization of Polyferm.

In vitro studies on Polyferm were performed to verify whether the preparation was of appropriate quality in respect to protection of the active substance (proteolytic enzymes) in the acidic environment as well as in storage. Our previous studies (Biziulevichius & Arestov, 1997a) have shown the abomasal acidity of newborn calves was for the most part pH 3.5–4.0 (pH range from 2.8 to 4.9 depending on the time of food intake). On the other hand, it is known that *B. subtilis* neutral proteinase is quickly and completely inactivated at pHs below 4.0 (Mosolov, 1971) and this is also true for the lytic enzyme complex of lysosubtilin (Biziulevichius *et al.*, 1987; Biziulevičius & Žukaitė, 1999). Thus results presented in Table 1 show that proteolytic enzymes (as well as lytic enzymes of Fermosorb) were suitably protected from acid. As for the active release of enzymes, including proteolytic ones, at neutral pH values characteristic for the intestines, from the enzyme–polymer complexes produced employing Biocarb L, this has already been shown previously (Biziulevičius & Žukaitė, 1999). The complete enzyme activity retention period of Polyferm (5 years in storage at room temperature) was equal to that of Fermosorb (Table 2) or 2.5 times higher when compared with lysosubtilin (Biziulevičius & Žukaitė, 1999).

In vivo studies on the efficacy of Polyferm and Fermosorb for the treatment and prophylaxis of colibacillosis in newborn calves, performed simultaneously and in analogous conditions, did not reveal significant differences between these two preparations (Table 3). Therapeutic and prophylactic efficacy of Polyferm was found to be 93.6 and 94.6%, respectively, and these values were among the efficacy range 93.4–100% and 93.5–100%, respectively, that have been declared on a basis of our preliminary results (Zotkin *et al.*, 1987b; Sisyagin *et al.*, 1988b). Therapeutic and prophylactic efficacy of Fermosorb (95.0 and 95.8%, respectively) was similar to that determined in field trials recently (95.2 and 95.0%, respectively) (Biziulevičius & Žukaitė, 1999). The results of both *in vitro* and *in vivo* comparative studies did not indicate reasons, other than lack of supportive advertising material, that might impede utilization of Polyferm. It is believed that the results presented in this communication will accelerate spread of this veterinary medicinal product. Introduction of simplified biotechnology of Polyferm production (Biziulevičius & Žukaitė, 2000) into industry may become of a decisive initiative.

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