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In vivo studies on lysosubtilin. 3. Efficacy for treatment of mastitis and superficial lesions of the udder and teats in cows

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Abstract – Lysosubtilin is a broad-spectrum preparation of lytic enzymes from *Bacillus subtilis* designed for veterinary medicine. This study demonstrates its efficacy for the treatment of reproductive system diseases (mastitis, superficial lesions of the udder and teats) in cows. Prior to determination of optimal therapeutic doses, samples taken from the milk and udder skin of sick animals were examined microbiologically. The examinations revealed a high incidence of polymicrobial infections (26.9 and 84.9 % for mastitis and superficial udder lesions, respectively) caused by various mixtures of bacteria (both Gram-positive and Gram-negative) and fungi/yeasts. Dose determination studies involved 115 cows with clinical signs of mastitis. The optimal dose for mastitis treatment was found to be 3.5×10^6 U lysosubtilin dissolved in 100 mL of distilled water, which was then administered into the mammary gland via the teat canal once daily until recovery. Such a dose yielded statistically significant decreases ($P < 0.05$) both in the length of time before clinical recovery (2 d versus 4 and 4.5 d with either of the two antibiotic-based traditional drugs) and in the percentage of animals who suffered relapses within a 2-month period following treatment (5 % versus 60 %, with one of the two drugs). A field experiment involving 106 cows was designed to compare the efficacy of 1 % lysosubtilin water–glycerin solution (1:9 v/v) and other traditional medications for the topical treatment of superficial lesions of the udder and teats as well as its potential for mastitis prevention. All drugs used yielded a 100 % cure rate, but lysosubtilin application made it possible to achieve a statistically significant decrease ($P < 0.05$) in the duration of the recovery period (2.5 d versus 4.5 to 5.5 d) when compared with any of the four other drugs tested. Its efficacy for mastitis prevention was at least 3.4 times higher than the efficacy of the other medications used (statistically significant, $P < 0.05$, with regards to two of the four drugs) as well. We therefore conclude that lytic enzyme preparations are prospective antimicrobial drugs and when used to combat animal diseases they may serve as a possible alternative to common antibiotics. © Inra/Elsevier, Paris

lysosubtilin / enzymotherapy / cow / mastitis / superficial udder lesion

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Résumé – Utilisation in vivo de la lysosubtiline. 3. Efficacité dans le traitement des mammites, des lésions superficielles du pis et des mamelles chez la vache. La lysosubtiline est une préparation à large spectre d'action, produite à partir d'enzymes lytiques de *Bacillus subtilis* et destinée à la médecine vétérinaire. Cette étude montre l'efficacité de la lysosubtiline dans le traitement des maladies du système reproducteur des vaches (mammite, lésion superficielle du pis et des mamelles). Avant de déterminer les doses optimales thérapeutiques, les échantillons pris dans le lait et sur la peau du pis ont été examinés pour leur qualité microbiologique. Les analyses ont montré un pourcentage élevé d'infections polymicrobiennes (26,9 % pour les mammites et 84,9 % des lésions superficielles du pis), provoquées par divers mélanges de bactéries (tant Gram-positifs que Gram-négatifs) et de champignons/levures. Cent quinze bovins ayant des signes cliniques de mammite, ont été examinés en vue de déterminer les doses efficaces. La dose optimale pour le traitement de mammite était de $3,5 \times 10^6$ U de lysosubtiline diluée dans 100 mL d'eau distillée. L'introduction de cette dose dans le pis par le canal de la mamelle (une fois par jour jusqu'à la guérison) a permis d'obtenir une diminution statistiquement significative ($p < 0,05$) de la durée d'atteinte de la guérison (2 j au lieu de 4 et 4,5 j avec deux médicaments traditionnels) et du pourcentage des rechutes pendant une période de 2 mois après la guérison (5 au lieu de 60 % avec l'un des deux médicaments). Des expériences sur le terrain (106 bovins) ont été entreprises pour comparer l'efficacité du traitement par la lysosubtiline (1 %) en solution dans un mélange eau-glycérine (v/v 1:9) avec d'autres médicaments traditionnels utilisés pour le traitement des lésions superficielles du pis et des mamelles et pour la prophylaxie des mammites. Tous les médicaments utilisés ont permis un raccourcissement statistiquement significatif ($p < 0,05$) de la durée d'atteinte de la guérison (2,5 au lieu de 4,5 à 5,5 j avec les quatre autres médicaments). L'efficacité de lysosubtiline pour la prévention des mammites était au moins 3,4 fois plus importante que celle obtenue avec les autres médicaments (différence statistiquement significative, $p < 0,05$, pour deux des quatre médicaments). Ceci permet de conclure que les préparations d'enzymes lytiques sont des médicaments antimicrobiens prometteurs qui pourraient être une alternative convenable aux antibiotiques traditionnels. © Inra/Elsevier, Paris

lysosubtiline / enzymothérapie / bovin / mammite / lésion superficielle du pis

1. INTRODUCTION

Mastitis is the major economically important disease in cattle, causing a substantial loss to the dairy industry [8, 19, 20, 43, 63, 75, 110, 113]. Superficial lesions of the udder and teats are a highly predisposing factor for the acquisition of mastitis [37, 54, 65, 67, 79, 96, 120].

A large number of bacteria (both Gram-positive and Gram-negative) are known to evoke inflammation in the udder [4, 25, 42, 45, 47, 55, 57, 109, 115]. *Listeria monocytogenes*, a pathogen that is dangerous for humans, is among the causative organisms [21, 36, 101, 111]. Fungi, most commonly yeasts, are also known to be involved in bovine mammary gland infections [1, 32, 35, 59, 61, 95, 104, 119]. Mixed (polymicrobial) infections are very common in a number

of cases where even representatives of various taxonomic groups are involved. These latter are especially difficult to treat.

Antibiotics are the preparations of choice in the treatment of cow intramammary infections [34, 38, 58, 72, 83–85, 93, 99, 102, 105, 108, 118, 121], despite the fact that there are undesirable consequences of their regular application [24, 29, 31, 53, 91, 112].

Bacteriolytic enzymes and some of proteolytic ones (those, that are able to degrade the proteinaceous components of microbial cell walls) are antimicrobial agents of great interest for veterinarians [9]. However, the number of publications on the enzymotherapy of bovine mammary gland diseases is limited.

A method of combined mastitis therapy including use of a single dose of

antibiotics dissolved in lysozyme-rich cow milk with a follow-up treatment using such milk alone has been described [86]. Different preparations of hen egg white lysozyme in conjunction with antibiotics were proposed for the treatment of bovine mastitis as well [64]. Some years later the efficacy of immosim, an immobilized proteolytic enzyme preparation, for the treatment of mastitis [124] and the efficacy of recombinant lysostaphin, a preparation of lytic enzymes of bacterial origin, for prophylaxis and/or treatment of this disease was shown [16, 22, 26, 82]. A recent publication on the latter enzyme preparation [117] is of special interest as it deals with the expression of the lysostaphin gene in a eukaryotic system and thus discusses a transgenic approach to controlling bovine staphylococcal mastitis. Lysostaphin also possesses elastolytic activity [90] and this characteristic may have possible uses for udder wound repair. In respect to the topical treatment of superficial lesions of the udder and teats in cows, immobilized proteolytic enzymes have interesting potential as well [5, 6, 39–41, 62, 73, 92]. Lytic enzyme preparations for treatment of local fungal infections in animals are also known [27, 28].

In addition to their positive features, the above-mentioned enzyme preparations have one essential shortcoming they are all narrow-spectrum acting antimicrobial agents. We have designed, and in 1983 started to produce on an industrial scale, a broad-spectrum action preparation of lytic enzymes for veterinary purposes, called lysosubtilin. Its properties, including its spectrum of antibacterial and antifungal action, have already been described [13–15]. *In vivo* the high efficacy and safety of lysosubtilin for prophylaxis and treatment of gastrointestinal disorders in newborn calves as well as for treatment of post-partum endometritis in cows have also been described [10–12, 122, 123].

In this paper we provide the results of our investigations on the efficacy of lysosubtilin when applied for treatment of mastitis and superficial lesions of the udder and teats in cows.

2. MATERIALS AND METHODS

2.1. Animals and experimental protocols

The cow experiments were allowed and supported by the Veterinary Medicine Board of Lithuania. The choice of control drugs, number of animals (total and per group), treatment procedures and cure evaluation parameters (clinical, microbiological, statistical) were coordinated with this organization.

2.1.1. Mastitis

The experiment was performed in the Birzhai regional veterinary station in Lithuania. One hundred and fifteen lactating Lithuanian Brown cows in which the clinical signs of mastitis had been detected (swelling, heat, pain and abnormal secretion in the gland accompanied in some cows by fever, anorexia and/or depression), were used for this experiment. The cows were divided randomly into five groups (three study and two control) and the treatment was initiated immediately after milk samples had been taken for culture. Clinical observation of cows was performed twice daily, before the morning and evening milking, throughout the experiment.

In all groups, the cows were treated until recovery, but no longer than for 5 days. A clinical cure was defined as the return of the secretion and quarter to normal appearance, as assessed by visual observation of foremilk stripped on the floor and by palpation at or before milking. If a quarter did not appear to be making satisfactory progress toward recovery during the observation period, the case was defined as a clinical failure. Milk samples for confirmation (or negation) of treatment success were taken for culture 5 days after the last therapeutic dose had been applied (the standard procedure in the Former Soviet

Union to assess the efficacy of intramammary therapy). The experimental period for the successfully treated cows continued for 2 months and any relapses were registered. A relapse was defined as the reappearance of clinical signs in the quarter previously infected followed by bacteriological examination results confirming that the same bacterial species is shed in milk.

2.1.2. Superficial lesions of the udder and teats

The experiment was performed in the farming area of the Voke branch of the Lithuanian Institute of Agriculture. One hundred and six cows of different breeds, predominantly the Lithuanian White and Black breed, in which visually observable signs of superficial lesions of the udder and teats (dermatitis, sunburn, chapping and cracking) had been detected, were used in the experiment. The animals were maintained under field conditions. The cows were divided randomly into five groups and the treatment was initiated immediately after samples from the lesions had been taken for culture. Clinical observation of cows was performed twice daily before the morning and evening milkings.

The experiment lasted for 4 months and if any new superficial lesions of the udder and teats appeared (of the same or different origin) the treatment procedures were repeated. Any cases of the intramammary infections in the experimental animals were registered throughout this period in order to determine the usefulness of the medications for mastitis prevention.

2.2. Sampling and microbiological procedures

Milk samples (5 mL) were aseptically collected from each quarter into sterile polystyrene snap-cap tubes immediately after pre-milk hand-stripping and cleaning of teat tips with cotton soaked with 70 % ethanol.

Samples from damaged areas of the udder and teats were taken using sterile cotton wool swabs, moistened in sterile distilled water, by introducing a swab for 5 s and rubbing it gen-

tly against the skin surface. Samples from pustules and papules of animals with pyoderma were taken using dry swabs. Swab tips were cut off into bijou bottles containing 2 mL of wash fluid (0.075 M phosphate-buffered saline containing 0.1 % v/v Triton X-100, pH 7.9) and shaken manually for 30 s as recommended by Lloyd [71].

The samples were kept on ice and sent to the regional veterinary laboratories within 2 h, where they were analysed by qualified microbiologists. The samples (0.01 mL) were inoculated on 5 % sheep blood agar as well as on Sabouraud dextrose agar (Difco Laboratories, Detroit, Michigan) plates and were incubated at 37 °C for 24 h and at 30 °C for 72 h, respectively. The isolates were identified using standard procedures [23].

2.3. Lysosubtilin, other drugs and treatment procedures

Lysosubtilin (more precisely Lysosubtilin G10X) is a commercial preparation of lytic enzymes from *Bacillus subtilis* SK-52 and was acquired from the State Joint-Stock Enterprise 'Biosinteze' formerly called the Vilnius Experimental-Industrial Plant of Enzyme Preparations (Vilnius, Lithuania). This product was a powder of light (greyish or yellowish) colour with a lytic activity 1 000 000 U/g according to Kislukhina [60]. A unit (U) is defined as follows: one unit generates a decrease in turbidity of 0.001 OD/min at 520–540 nm in a suspension of *Escherichia coli* K 12 dried cells (initial optical density 0.6) in 1.0 mL of 0.004 M phosphate buffer, pH 7.2 at 30 °C.

Five percent synthomycin liniment, 5 % sulfanilamide liniment, borasic vaseline, glycerin, hypochlorite and iodophor solutions were obtained from Sanitas (Kaunas, Lithuania). Mastisan, neomycin sulfate and sulfadimidine preparation (0.025 and 0.25 g, respectively, in 5 mL of oil base), and penicillin were obtained from Kharkov bioenterprise (Ukraine). The treatments were identified when administered.

The study groups of cows having mastitis were treated with different doses of lysosubtilin (2.5, 3.5 and 5 × 10⁶ U) dissolved in 100 mL

of distilled water. The number of animals per group were 32, 24 and 15, respectively. One control group (20 animals) was treated traditionally with mastisan, the other (24 animals) was treated according to Padegimas [86].

Treatment procedures were as follows: the mammary glands of the sick animals were emptied and antimicrobial drugs, warmed to body temperature, were infused intramammarily via the teat canal. Lysosubtilin solutions, prepared *ex tempore*, were infused once daily. Antibiotic based mastisan was used as recommended by the manufacturer, i.e. two treatment doses were mixed and infused once daily. The Padegimas method [86] was applied in its original version: 3×10^5 IU penicillin dissolved in 200 mL of lysozyme-rich milk was infused as a single dose and this was followed by infusions of the same sized doses of lysozyme-rich milk without the antibiotic in twice-daily intervals. The lysozyme richness of milk was determined according to Shipilov et al. [103].

One group of cows having superficial lesions of the udder and teats (33 cows) was treated with 1 % lysosubtilin solution in distilled water + glycerin (1:9 v/v). The other groups were treated with traditionally used medications: 5 % synthomycin liniment (19 cows), 5 % sulfanilamide liniment (20 cows), borasic vaseline (17 cows) and glycerin (17 cows). For the preparation of lysosubtilin solution, 10 g of lysosubtilin were dissolved in 100 mL of distilled water, then 900 mL of glycerin were added and all well mixed.

The cows were treated twice daily (after morning and evening milkings). The damaged places of the udder and/or teats were thoroughly cleaned by applying cheese-cloth napkins moistened in hypochlorite solution (4 % available chlorine), dried with disposable paper towels and then greased with one of the liniments/ointment mentioned. The medications were used until recovery. No teat dipping techniques were used except premilking teat dipping in an iodophor solution (0.5 % active iodine).

2.4. Statistical evaluation

Results are reported as mean \pm SEM. Mean values in the text and the tables were com-

pared using the Students *t*-test. Chi-square and exact Fisher tests as well as methodological references to biological statistics [98] were used to evaluate the significant differences between microbiological sample examination results as well as in the number of cured or relapsed animals in each treatment group. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Mastitis

The microbiological evaluation of milk samples (*table I*) revealed that 73.1 % of the infections were caused by a single microorganism. Among these *Staphylococcus aureus* and streptococci were most prevalent (31.3 and 29.1 % of all cases, respectively). Mixed infections involved representatives of no more than two species and associations of staphylococci with streptococci were most frequent. *Escherichia coli* as well as yeasts were only found in combinations with staphylococci. Coagulase-negative staphylococci and *Candida* spp. were found exclusively in mixed infections while *Streptococcus uberis* acted alone as the sole infectious agent. *Listeria monocytogenes* was not identified in any of the samples. Bacteriological and mycological investigations did not indicate any significant differences in distribution of microbial isolates from milk between the various experimental groups.

The results of lysosubtilin therapeutic dose determination studies are shown in *table II*. The optimal dose was found to be 3.5×10^6 U lysosubtilin and it yielded a statistically significant decrease ($P < 0.05$) in the duration of the clinical recovery period when compared with either of the two traditional drugs and a decrease in the percentage of animals relapsed within 2 months after treatment (statistically significant, $P < 0.05$, with regards

to one of the two drugs). Microorganisms present in the milk samples of the four cows that were unsuccessfully treated with this dose were *Actinomyces pyogenes* and non-speciated *Streptococcus* sp. (two cases of each). *Staphylococcus aureus* was isolated as the only microorganism from the milk sample of the sole cow that relapsed after the use of the optimal lysosubtilin dose.

3.2. Superficial lesions of the udder and teats

Microbiological examination of 106 swab samples obtained from udder and teat skin revealed the following facts. A single microorganism was isolated from 16 (15.1 %) samples and this was *Staphylococcus aureus*. Infectious agents belonging to two different species were

found in 44 (41.5 %) samples; 25 (23.6 %) samples contained representatives of three and 21 (19.8 %) of four species. The isolated microorganisms (number of microorganism-positive samples in decreasing order of frequency) were: *Staphylococcus aureus* (including single infections) 88 (83.0 %), *Streptococcus* spp. 47 (44.3 %), *Pseudomonas* spp. 30 (28.3 %), *Proteus vulgaris* 24 (22.6 %), fungi/yeasts (predominantly *Candida* spp.) 22 (20.8 %), *Escherichia coli* 20 (18.9 %), *Bacillus* spp. 15 (14.2 %), *Actinomyces pyogenes* 11 (10.4 %), others 6 (5.7 %). Bacteriological and mycological investigations did not indicate any significant differences in distribution of the microbial isolates from the udder and teat skin between the various experimental groups.

The results of the field experiment aimed at comparing the efficacy of differ-

Table I. Microorganisms isolated from quarter milk samples of cows with clinical mastitis.

Organism	Number of samples (%) containing infectious organism		
	alone	mixed ^a	Total
<i>Staphylococcus aureus</i>	42 (31.3)	11 (8.2)	53 (39.5)
CNS ^b	—	5 (3.7)	5 (3.7)
<i>Streptococcus uberis</i>	14 (10.4)	—	14 (10.4)
<i>Streptococcus agalactiae</i>	8 (6.0)	2 (1.5)	10 (7.5)
<i>Streptococcus dysgalactiae</i>	7 (5.2)	3 (2.2)	10 (7.5)
Other streptococci	10 (7.5)	3 (2.2)	13 (9.7)
<i>Escherichia coli</i>	7 (5.2)	5 (3.7)	12 (8.9)
<i>Pseudomonas aeruginosa</i>	6 (4.5)	2 (1.5)	8 (6.0)
<i>Actinomyces pyogenes</i>	4 (3.0)	2 (1.5)	6 (4.5)
<i>Candida</i> spp.	—	3 (2.2)	3 (2.2)
Total:	98 (73.1)	36 (26.9)	134 ^c (100)

^a Two species were isolated from the same sample.

^b CNS: coagulase-negative staphylococci.

^c 19 cows out of 115 had two quarters infected.

Table II. Efficacy of different lysosubtilin doses for the treatment of mastitis in cows.

Group of animals and dosage of lysosubtilin (U x 10 ⁶)	Clinical recovery (number of cured animals/total (%))	Duration of clinical recovery ^d (days)	Microbiological cure ^e (number of cured animals (%))	Relapses within 2 months (number of relapsed animals/total (%))
Control 1 ^a	15/20 (75)	4.5 ± 0.5	12 (60)	6/12 (50)
Control 2 ^b	14/24 (58.3)	4 ± 1	10 (41.7)	6/10 (60)
2.5 ^c	20/32 (62.5)	2 ± 1	15 (46.9)	5/15 (33.3)
3.5 ^c	23/24 (95.8)	2 ± 0.5* ^f	20 (83.3)	1/20 (5)* ^g
5 ^c	12/15 (80)	2 ± 1	11 (73.3)	2/11 (18.2)

Data are given as mean ± SEM. Cows were treated once daily (control 2 group was treated twice daily) until recovery, but no longer than for 5 days. ^a Cows were treated with mastisan. ^b Cows were treated according to Padegimas [86]. ^c The doses of lysosubtilin were dissolved in 100 mL of distilled water. ^d For recovered animals only. ^e This was assessed by culture 5 days after the last therapeutic dose had been applied. * Data are significantly different ($P < 0.05$) from: ^f any control group (t test); ^g control 2 group (exact Fisher test).

ent drugs for the treatment of superficial lesions of the udder and teats as well as their potential for mastitis prevention are shown in *table III*. All drugs yielded a 100 % cure rate, but the lysosubtilin application resulted in a statistically significant decrease ($P < 0.05$) in duration of the time period until recovery when compared with any of the four other drugs. Its efficacy for mastitis prevention was at least 3.4 times higher than the efficacy of the other medications used (statistically significant, $P < 0.05$, with regards to two of the four drugs).

4. DISCUSSION

Various local and national control programmes, strategies, schemes, monitoring systems, etc. have been developed worldwide with the aim of reducing the incidence of cow intramammary infections [30, 33, 50, 51, 56, 70, 76, 107]. In spite of all efforts, intramammary infections cause a substantial loss to the dairy industry. It is difficult to imagine that the ulti-

mate eradication of these disorders will be achieved; however, decreasing their negative impact remains a realistic possibility.

In this paper we provide results concerning the efficacy of lysosubtilin, a preparation of lytic enzymes we created, as an alternative to common antibiotics, for treatment of mastitis and superficial lesions of the udder and teats. The results supplemented our *in vitro* and *in vivo* observations [10–12, 14, 15, 123] and confirmed previous speculations concerning the great potential of this preparation for veterinary applications.

As the diseases of interest are of microbial etiology, identification of causative agents was a primary task. This was then followed by evaluation of the proposed drug formulations in comparison with traditionally used medications.

Staphylococci and streptococci were the most frequent microorganisms infecting the mammary gland (*table I*) and udder/teat skin (section 3.2.) and were found in 31.3 and 29.1 % of milk samples

Table III. Efficacy of different drugs for the treatment of superficial lesions of the udder and teats in cows ^a.

Drug	Disorder of the udder and/or teats						Incidence of mastitis within four months after treatment (number of cases/number of animals (%))
	Dermatitis		Sunburn		Chapping and cracking		
	Number of animals	Duration of recovery period (days)	Number of animals	Duration of recovery period (days)	Number of animals	Duration of recovery period (days)	
5 % synthomicin liniment	6	4.5 ± 0.5	3	3 ± 0.5	10	4 ± 0.5	4/19 (21.1)
5 % sulfamidamide liniment	6	4.5 ± 0.5	3	3 ± 0.5	11	4.5 ± 0.5	5/20 (25.0)
Borasic vaseline	4	5.5 ± 1.5	3	4 ± 1	10	5 ± 1	7/17 (41.2)
Glycerin	4	5.5 ± 1.5	3	4 ± 1	10	5 ± 1	8/17 (47.1)
1 % lysosubtilin solution ^b	12	2.5 ± 0.5* ^c	4	1.5 ± 0.5* ^c	17	2.5 ± 0.5* ^c	2/33 (6.1)* ^d
Total:	32		16		58		26/106 (24.5)

Data are given as mean ± SEM. Cows were treated twice daily until recovery. ^a All drugs yielded 100 % cure rate, the differences were found in duration of the recovery period and mastitis protection. ^b Lysosubtilin was dissolved in distilled water + glycerin (1:9 v/v). * Data within a column are significantly different ($P < 0.05$) from: ^c any other group (t -test); ^d borasic vaseline and glycerin treated groups (chi-square test).

as well as in 83.0 and 44.3 % of swab samples, respectively. These findings are to be discussed more thoroughly, because the microorganisms mentioned are historically and currently of special importance for veterinarians. During the last three or four decades *Staphylococcus aureus* replaced *Streptococcus agalactiae* as the most common mastitis pathogen. According to Myllys et al. [78] changes in bacterial ecology were associated with new antimicrobial treatments and introduction of modern milking machines. Antimicrobials influenced changes within *Staphylococcus aureus* strains, including the appearance of antibiotic resistant ones, during this period as well [2]. Moreover, new atypical *Staphylococcus aureus* infections have been observed recently [52, 66]. Thus, it is quite true that *Staphylococcus aureus* is currently a major bacterial pathogen causing intramammary infections as well as a most common inhabitant of udder skin [38]. Cases of mastitis caused by staphylococci other than *Staphylococcus aureus*, particularly coagulase-negative staphylococci, are also increasing [3, 48, 97]. Our studies did not indicate any staphylococci other than *Staphylococcus aureus*, except for some coagulase-negative staphylococci found in 3.7 % of the milk samples. Streptococci, both hemolytic and non-hemolytic, as well as other bacterial species were also commonly present in intramammary infections [38, 77, 81, 114].

The number of milk samples from which fungi/yeasts were isolated was relatively small (2.2 %), while among the swab samples it was appreciable (20.8 %). The causative agents were mainly yeasts (*Candida* spp.), but some individuals belonging to filamentous fungi (*Aspergillus* spp.) were isolated from the swab samples as well.

Veterinarians have developed different approaches for controlling and preventing

intramammary infections (some of these are discussed in the Introduction). The use of antibiotics remains the method of choice. Most antibiotics are effective against specific microbial communities. However, the number of complex polymicrobial infections (for example, the experimental results presented in this paper), including antibiotic resistant organisms, is tending to increase.

Our previous in vitro studies on lysosubtilin showed that the preparation possesses a very broad antimicrobial spectrum [14, 15]. It lyses both Gram-positive and Gram-negative bacteria (species *Bacillus*, *Corynebacterium*, *Escherichia*, *Staphylococcus*) as well as fungi (species *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, *Sclerotinia*, *Stemphylium*) and yeasts (species *Candida*, *Cryptococcus*, *Hansenula*, *Saccharomyces*). This feature of lysosubtilin allowed us to hope that the preparation would be successful in the treatment of polymicrobial infections.

Efficacy evaluation of lysosubtilin for treatment and prevention of bovine mastitis was performed in two stages. The first one was designed to find the optimal therapeutic dose when administered by direct infusion into the mammary gland via the teat canal and to estimate its impact on the course of the disease. The second one aimed to estimate the usefulness of lysosubtilin in diminishing the incidence of mastitis through its topical application for udder and teat superficial lesion healing enhancement.

Our preliminary in vivo studies showed that the doses of lysosubtilin lower than 2.5×10^6 U are ineffective for mastitis therapy, while the doses higher than 5×10^6 U are irritant for the mammary gland (unpublished). Thus, only three, it seems rather close, doses in the interval between 2.5×10^6 and 5×10^6 U, i.e. 2.5, 3.5 and 5×10^6 U have been chosen for this experiment. Here, 100 mL of

distilled water were found to be the optimal volume for dissolution of these doses. Moreover, safety for the mammary gland of all these doses dissolved in 100 mL of distilled water was ascertained prior to the dose determination studies.

In this study we found out that the therapeutic efficacy of the optimal dose (3.5×10^6 U) of intramammary lysosubtilin (clinical recovery as well as microbiological cure rates) was not only 20 % higher and the duration of the clinical recovery period was significantly shorter when compared with traditional mastisan, but it also yielded a decrease in the percentage of relapsed animals (*table II*). Moreover, the Padegimas method [86], which was successfully applied in Lithuania for many years, was found to be only slightly effective today (clinical recovery rate 97.2 % in 1968 [86] versus 58.3 % in this study), most probably due to the diminished susceptibility of pathogens to penicillin. These findings confirm once again that the re-evaluation of traditional antibiotics and the search for new antimicrobials replacing ineffective ones is a continuous process.

Our method of polymicrobial etiology bovine mastitis treatment with $3.5 \times 10^6 - 5 \times 10^6$ U lysosubtilin dissolved in 100 mL of distilled water and used once daily until recovery was submitted to the Former Soviet Union (FSU) Invention Committee in 1981, but an invention has not yet been granted. A similar method of enzymatic staphylococcal mastitis treatment with the help of lysostaphin was patented in Europe by Blackburn and Pollack [16] with priority dated 1988.

Various pre-milking and post-milking teat disinfection methods [7, 46, 68, 69, 74, 80, 87-89, 100, 106] are used for mastitis control, while the application of antimicrobial liniments, ointments or creams is not so wide spread. Prior to our experiments, pre-milking teat dipping, applying iodophor solutions and post-

milking disinfection with chlorine containing agents was used. Both approaches were shown by some authors [17, 18, 44, 49, 94, 116] to be quite effective, but the incidence of mastitis in the region remained very high (about 50 %). Additional application of 5 % synthomicin liniment or 5 % sulfanilamide liniment for the treatment of superficial lesions of the udder and teats allowed us to reduce this incidence up to 21.1 and 25.0 %, respectively (*table III*), while the efficacy of 1 % lysosubtilin water-glycerin solution was even several times higher (6.1 % incidence). In addition, the duration of the recovery period was approximately half as long for lysosubtilin when compared with the other medications used. These observations show that the topical application of lysosubtilin is highly prospective not only for the treatment of superficial lesions of the udder and teats, but for the diminishing of mastitis incidence as well. The close relationship between udder and teat lesions and acquisition of mastitis was discussed in the Introduction.

Some facts involved in the application of lysosubtilin for the treatment of superficial lesions of the udder and teats deserve comment. First, the concentration level of 1 % lysosubtilin, corresponding to 10 mg/mL, was chosen as a result of our preliminary in vitro antimicrobial property studies [14, 15]. Second, the beneficial effect of glycerin, used by us as a vehicle, for treating skin diseases has been known for a long time. Its use for prevention of teat chapping is recommended by Fraser [38] as well. Third, the lysosubtilin water-glycerin solution should not to be stored for more than 1 month in order to avoid inactivation of the antimicrobial enzymes. Fourth, an invention with priority dated 1980 was granted for us by the FSU Invention Committee for the proposed drug formulation, but because of the policy of the Committee its text was considered to be

unpublishable as in the case of post-partum endometritis treatment method inventions [12]. Fifth, in independent Lithuania, the Veterinary Preparations Commission of Pharmacological Committee in 1993 approved the lysosubtilin application for treatment of superficial lesions of the udder and teats, which also implies the permission to use it in Estonia and Latvia. Sixth, a method for preventing bovine staphylococcal mastitis including dipping teats in a lysostaphin solution, the dipping being carried out before and after each milking, was patented recently by Blackburn and Pollack [16], confirming, in general, the effectiveness of our methodological approach not only to enzymatic mastitis treatment, but to its prevention as well.

In the first part of this research report [10] we mentioned that about 1 500 kg of lysosubtilin had already been used for the treatment of reproductive system diseases in cows. Approximately 1 200 kg of this amount (the exact number cannot be checked) were used throughout the FSU for the treatment of post-partum endometritis [12] and the rest (300 kg) was used for the treatment of superficial lesions of the udder and teats.

For the treatment of superficial lesions of the udder and teats, an approximate dose of 10 g of 1 % lysosubtilin water-glycerin solution needs to be used twice daily for 2.5 days (*table III*). Thus, 50 g of the solution corresponding to 0.5 g of lysosubtilin are needed to treat a single cow. By dividing the above-mentioned amount (300 kg) by 0.5 g we obtain the number of cows with superficial lesions of the udder and teats having been treated with lysosubtilin, namely 600 000. If we add to this the 675 000 calves having been protected or treated for gastrointestinal disorders [10] as well as the 200 000 cows having been treated for post-partum endometritis [12], we find that about 1.5 million animals have

received lysosubtilin in order to reduce their morbidity and mortality.

The selling price of the preparation in January 1997 was USD 20 per kg (subject to change), thus the cost of the treatment of superficial lesions of the udder and teats is USD 0.10 per animal (the cost of lysosubtilin is USD 0.01, while the rest is for the cost of glycerin). $3.5 \times 10^6 \text{ U} \times 2 = 7 \times 10^6 \text{ U}$ or 7 g of lysosubtilin needed for mastitis treatment (*table II*) will cost USD 0.14. The reader can judge for himself whether these costs are high or low.

In conclusion, lytic enzyme preparations are perspective antimicrobial drugs and when used to combat animal diseases they may serve as a good alternative to common antibiotics. One can only speculate that in conjunction with probiotics immunomodulators or modern treatment techniques, such as electromagnetic fields and laser therapies, their efficacy may be even higher, but this remains to be demonstrated experimentally.

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