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F O R E W O R D

The University of Veterinary Medicine in Košice celebrates its 60th anniversary this year. It was established by the Slovak National Council Act No. 1/1950 Coll. of December 16, 1949. It was initially known as the Veterinary College in Košice beginning from the first full academic year in 1949/1950. The change in its name to the University of Veterinary Medicine in Košice was laid out by the Slovak National Council Act No. 137/1992 Coll.

The University of Veterinary Medicine in Košice fulfils its mission in the system of higher education in accordance with the Act. No. 131/2002 Coll.

The University of Veterinary Medicine in Košice is a one-faculty university and offers training in accredited study programmes at all three levels of higher education. It is the only institution of its kind in the Slovak Republic providing pre-graduate and post-graduate veterinary education.

During its 60-year history the university has gone through many complicated constructional, organisational and personnel changes, from a one-faculty college to the current one-faculty university. During these changes it did not relent, even for one day, in its efforts to provide a high standard of quality education. However, the most significant progress in the field of veterinary education has been realized in the past decade.

The most recent complex accreditation process was completed on October 14, 2008, at the session of the Accreditation Commission of the Slovak Republic in Nový Smokovec. The deputy chairman of the government and Minister of education of SR, Prof. Ing. Ján Mikolaj, CSc. awarded the university the right to bestow academic titles to its graduates in 23 study programmes at all three levels of higher education. Our university was also accredited to carry out habilitation procedures and to appoint professors in 12 study branches.

The University of Veterinary Medicine in Košice provides higher education of the first level in the study programmes of Cynology and Safety of food and feed.

The study programme of Cynology was accredited within the study branch 6.3.8. Cynology. It has been taught at our university since the academic year 2004/2005 in full-time and part-time forms, with the standard length of study of 3 years (6 semesters). After successful completion of this study programme, the graduate is awarded the academic title of Bachelor (Bc.).

The study programme for Safety of food and feed was accredited within the study branch 6.3.2 Food hygiene and has been taught at our university since the academic year 2007/2008 in full-time and part-time forms with the standard length of study of 3 years (6 semesters). After successful completion of this study programme the graduate is awarded the academic title of Bachelor (Bc.).

The higher education of the second level is provided as doctoral study programmes in General veterinary medicine and Food hygiene. A magister programme in Pharmacy was also started from the academic year 2006/2007.

The study programme of General veterinary medicine was accredited within the study branch 6.3.1 General veterinary medicine and has been taught at our university in full-time form with the standard length of study of 6 years (12 semesters). After successful completion of this study programme, the graduates are awarded the academic title of Doctor of veterinary medicine (DVM/MVDr.).

Since 1991 the study programme General veterinary medicine is taught also in the English language.

The study programme of Food hygiene was accredited within the study branch 6.3.2 Food hygiene and is taught at our university in full-time form with the standard length of study of 6 years (12 semesters). After successful completion of this study programme the graduates are awarded the academic title of Doctor of veterinary medicine (DVM/MVDr.).

After the § 53 Art. 3 of the Act No. 131/2002 Coll. on higher education (which involved changes and supplements to some acts), the study programmes of General veterinary medicine and Food hygiene (the first and second levels of higher education) is provided as one unit.

In 2006 the University of Veterinary Medicine in Košice was awarded the right to bestow the academic title of Magister (Mgr.) on the graduates of the full-time form of the five year magister study programme in Pharmacy taught within the study branch 7.3.1 Pharmacy. On the basis of this, starting from the academic year 2006/2007, our university provides the relevant education within this study programme.

Veterinary medicine is one of five regulated professions, the study of which or the curricula, must comply with the European Directive 2005/36/ES (former 78/1026/EEC and 78/1027/EEC).

This is one of the reasons why the evaluation on the international level is so very important to our university.

Veterinary education provided by the University of Veterinary Medicine in Košice in the study programmes of General veterinary medicine and Food hygiene was evaluated by the Joint Education Committee of EAEVE and FVE in 1996 and re-evaluated in 2006.

The outcome of the latest re-evaluation of our university was entitled the "Report of the visit to the University of Veterinary Medicine in Košice". This Evaluation Report was published after the meeting of the session of the Joint Education Committee of EAEVE and FVE on July 4, 2006. The principal aims of the recommendations outlined in the Evaluation Report, was to increase the quality of the provided education, scientific research and other related activities of the university. We started with the implementation of the suggested areas of improvement in 2007 after the new university representatives had been elected and took up their offices.

On April 16, 2007, the Rector's suggested a new organizational structure for our university. This reorganization involved the establishment of 11 departments, 5 clinics and three self-sustained specialised establishments. This was approved by the Academic Senate, effective of September 1, 2007.

The Scientific Board of the University accepted this reorganization at its session on December 18, 2007. The new curricula for the study programmes of General veterinary medicine and Food hygiene are now based on the credit system, with the orientation on animal species in the clinical education.

These organizational changes involved particularly the re-organization of the clinical units based on animal species as the clinical education is an essentially part of veterinary education. In relation to this, five clinics were established; i.e. Clinics for small animals, Clinics for horses, Clinics for ruminants, Clinics for pigs and Clinics for birds and exotic and free living animals.

The aim of this new organizational structure was to improve the education and research activities within the individual target species or groups of animals; but also to secure better conditions for complex preventive-therapeutic actions taken for the benefit of the animals and the animal owners and breeders. The changes also brought improvement in the use of the financial means from EU structural funds for reconstruction, building of new premises and obtaining new equipment for the clinics and other units of our university.

Today the University of Veterinary Medicine in Košice is a part of the European higher education and

research areas. With its erudite teachers, scientists and other qualified personnel, it offers at all three levels of higher education within its departments, institutes and clinics, a productive and creative environment for students from Slovakia and from abroad to enjoy their studies of accredited study programmes.

At the beginning of this new decade the school will face new challenges. The results obtained in the past few years will certainly help to deal with them. Accreditation of the new study programmes, the right to carry out habilitation procedures and appoint professors within the approved system of study branches may present some of these new challenges. As the results of the repeated evaluation of our university by international evaluation commissions and independent agencies involved in the last complex accreditation attest, we shall continue to make significant improvements in our programmes.

Generations of graduates of our university contributed, and I believe they will continue doing so, to the good name of our university. I would like to express my appreciation and thanks for their everyday and frequently difficult work in the field emergency room or state service at home and abroad. I am happy that many of them I can meet personally either at our "golden graduations" or at work meetings or social gatherings with representatives of the state Veterinary and Food Administration and Chamber of Veterinarians of SR. The latter bodies are our closest partners that helped us to prepare the celebrations of the 60th anniversary of the establishment of our university in this commemorative year of 2009.

Finally, I am pleased to announce that our university enters the new decade under a new name indicating its expansion. On December 1st, 2009, the National Council of the Slovak Republic approved the change in the name of our university to the University of Veterinary Medicine and Pharmacy in Košice, effective of January 15, 2010.

Prof. MVDr. Emil Pilipčinec, PhD.
The Rector

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QUINOLONES FROM THE POINT OF VIEW OF PHARMACOLOGY AND VETERINARY INDICATIONS (A Review)

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ABSTRACT

Quinolone therapeutics is an important group of antimicrobial drugs used in animals for various bacterial infections. Particularly important are fluoroquinolones. The first of them, enrofloxacin, was already introduced to veterinary practices in the nineteen eighties. This paper gives a brief review of: mechanism of action and antimicrobial spectrum of quinolones; development of antimicrobial resistance; the most important pharmacokinetic data and principal indications of these drugs (particularly of fluoroquinolones); their adverse side effects; contraindications and interaction with other drugs. Within the scope of the classification of quinolones, we also provide brief characteristics of the most important representatives of individual generations which are used in the treatment of infectious diseases of animals.

Key words: animals; fluoroquinolones; pharmacology; therapeutic indications

INTRODUCTION

Quinolones, particularly fluoroquinolones, play an important role in the therapy of infectious diseases of animals. While the drugs of the first generation were indicated for therapy of urinary tract infections, the newly developed molecules of subsequent generations extended the possibilities of the use of these drugs for other organ systems, e.g. respiratory tract, gastrointestinal tract (GIT), skin and soft tissues (61, 98, 46, 68). In clinical practice these drugs (ciprofloxacin, danofloxacin, enrofloxacin, marbofloxacin, norfloxacin and sarafloxacin) were used not only for specific veterinary purpose, but also many fluoroquinolones were applied to human medicine conditions (16). Discussed in this review will be important characteristics of these pharmaceutical agents, including their: spectrum of effectiveness; pharmacokinetic properties; principal indications; dosage and adverse side effects.

HISTORY OF DISCOVERY AND DEVELOPMENT OF QUINOLONES

Discovery of the first quinolones (the pharmaceuticals included among antimicrobial chemotherapeutics), dates back to

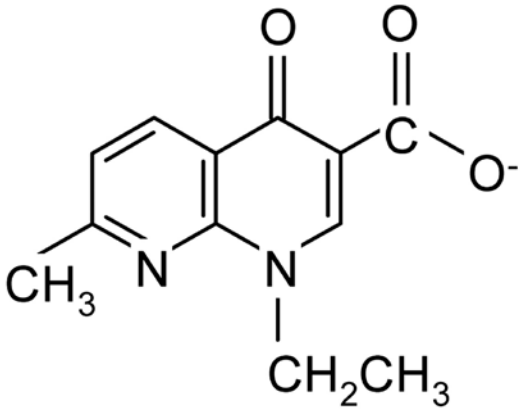

Fig. 1. Nalidixic acid (www.123bio.net)

1962 to G. T. Leshler, who was a staff member of the Sterling Winthrop pharmaceutical company. During the synthesis of an antimalaric drug, chloroquinone, he obtained a nalidixic acid (8-aza-4-quinolone, Fig. 1) as a side product. It was observed that this naphthyridine derivative (quinolone azaanalogue) had an *in vitro* bactericidal effect on many Gram-negative organisms. Orally administered clinical tests showed high concentrations of this compound in the urine. However, its levels in the blood and tissues following systemic therapy were not sufficient, despite the high doses used. Pharmacokinetic and pharmacodynamic properties limited the indications of this acid derivative as well as of its other analogues [oxolinic acid (synthesized in 1967), pipemidic acid (used since 1975), quinoxacin and rosoxacin] for therapy of urinary tract infections (42, 41).

In 1974 an enzyme, DNA gyrase, was isolated from *Escherichia coli,* which contributed to the identification of the mechanism of action of this group of antibacterial chemotherapeutics. A break occurred in the nineteen eighties when substitution of fluorine to the carbon at the 6th position of the chemical structure of quinolone brought about a new generation of quinolones, i. e. fluoroquinolones (Fig. 2). By adding a piperazine substituent at the C7 position, the preparation acquired hydrophilic properties which increased its concentration in the blood, tissues and body fluids and decreased its elimination in the urine and bile (11, 7). Another discovery in 1990 revealed bacterial topoisomerase IV, which lead to the identification of the site of the cytotoxic effect of quinolones, particularly in Gram-positive bacteria. The turn of the millennia brought about many new very effective molecular manipulated quinolones but also the detection of toxicity of the newly introduced quinolones. The latest developmental line in the group of quinolones is represented by "non-fluorinated" quinolones, with wide antibacterial effects against resistant Gram-positive organisms (41).

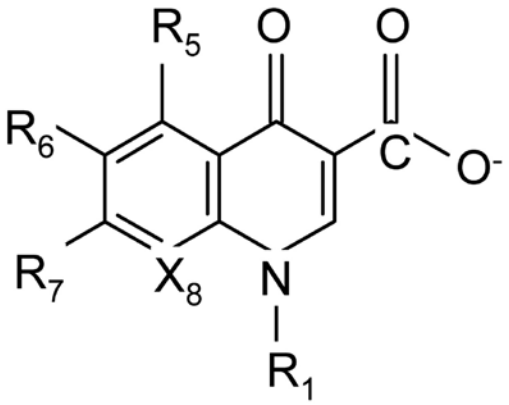

Fig. 2. General structure of fluoroquinolone (www.123bio.net). As a rule an alkyl group is substituted at the R_1 position, the R_5 position is occupied most frequently by CH_3, $-NH_2$, R_6 by F, R_7 is frequently substituted by piperazine derivative and a methoxyl group occupies position X_8

MECHANISM OF QUINOLONE ACTION

The action of antibacterial quinolones is based on selective blocking of topoisomerase II (the so-called DNA gyrase) and topoisomerase IV. These topoisomerases are bacterial prokaryotic enzymes which are essentially important for the synthesis of bacterial DNA. Topoisomerases play an important role in replication, transcription and "repair" of bacterial DNA. Topoisomerase IV affects the division of bacterial chromosomes.

DNA gyrase consists of A and B subunits. The most common target site for fluoroquinolones is the A subunit of DNA gyrase, coded by the gene *gyrA*. Under suitable conditions, DNA gyrase is capable of producing the so-called negative (laevogyrate) tertiary structure of DNA (super coils), from the originally relaxed circular DNA, catalysing the coiling of negative super helical structures in a way which enables it to form covalently closed, circular chromosomal and plasmid molecules of DNA of a size that can fit inside bacterial cell. Its inhibition results in prolongation of chromosomes and failure to close up the bacterial DNA complex. Another target of certain antibacterial quinolones is the topoisomerase IV enzyme composed of subunits *parC* and *parE* (54, 78, 14).

With some limitations, one can say that the antimicrobial activity of quinolones against Gram-negative bacteria is mediated by their influence on DNA gyrase, while with Gram-positive bacteria its main action is predominately on topoisomerase IV. The new quinolones exhibit dual activity against both DNA gyrase and topoisomerase IV which increases their antibacterial effectiveness and decreases the risk of the development of resistance. The bactericidal effect of quinolones increases with concentrations up to 30-fold minimal inhibitory concentration (MIC). Higher concentrations can inhibit ribonucleic acid (RNA) synthesis which, paradoxically, decreases their antimicrobial activity (16, 6, 14).

ANTIMICROBIAL ACTIVITY

Quinolones of the Ist generation (nalidixic acid and oxilinic acid) exhibit a medium-wide spectrum of activity. They act against Gram-negative bacteria (*E. coli, Salmonella* spp., *Shigella* spp., *Enterobacter* spp., *Haemophilus* spp., *Proteus* spp., *Campylobacter* spp. and *Neisseria* spp.) with the exception of pseudomonas.

Fluoroquinolones of the IInd generation (enrofloxacin) are effective against a wide spectrum of Gram-negative pathogens including *Pseudomonas aeruginosa*. They also act on some Gram-positive aerobes (*Staphylococcus aureus*) with the exception of *Streptococcus pneumoniae*.

Fluoroquinolones of the IIIrd generation (orbifloxacin) act against the spectrum mentioned for the IInd generation but extend to *Streptococcus pneumoniae* and the most frequent intracellular pathogens (*Chlamydia pneumoniae, Mycoplasma pneumoniae, Legionella pneumophila, Brucella* spp. and *Mycoplasma* spp.).

The **IVth generation** (moxifloxacin) is more effective against Gram-positive bacteria and acts also on anaerobic micro-organisms while retaining its effect on Gram-negative bacteria (68, 92).

Table 1. Pharmacokinetic data on important fluoroquinolones in veterinary medicine (www.vetpharm.unizh.ch)

Active ingredient	Animal	Dose ($mg.kg^{-1}$)	Way of administration	t1/2[1] (h)	C_{max}[2] ($\mu g.ml^{-1}$)	Bioavailability (%)	Plasma protein-binding (%)	References
Enrofloxacin	dog	5	p. o.	2.4	1.16	53		Kung and Wanner, 1994 (55)
	horse	5	i. m.	9.9 ± 1.0	1.28 ± 0.07	72.8 ± 7.7	-	Kaartinen *et al.*, 1997 (49)
	pig	2.5	i. m.		1.17 ± 0.23	74.53 ± 5.2	-	Anadón *et al.*, 1999 (5)
	cattle	5	i. m.	5.9 ± 1.44	0.73	82 ± 14	36–45	Kaartinen *et al.*, 1995 (48)
Ciprofloxacin	calf	2.8	p. o.	8	-	53	70.0 ± 4.2	Nouws *et al.*, 1988 (71); Spoo, 1995 (89)
	pig	3.3	p. o.	3.1	-	37.3	23.6 ± 1.7	Nouws *et al.*, 1988 (71)
	dog	5	p. o.	2.16	0.291 ± 0.18	-	-	Kung and Wanner, 1994 (55); Abadía *et al.*, 1994 (1)
	sheep	2.5	i. m.	9.98 ± 2.33	0.14 ± 0.02	-	-	Mengozzi *et al.*, 1996 (65)
Danofloxacin	pig	10	p. o.	9.8	2.4	89	53	Lindecrona *et al.*, 2000 (57)
		5	i. m.	6.8	0.82	76	-	Nakamura, 1995 (69); Mann and Frame, 1992 (59)
	cattle	5	i. m.	2.9	0.8	78	-	Nakamura, 1995 (69); Mann and Frame,1992 (59)
	goat	1.25	i. m.	2.37	0.33	65.7	13.55	Atef, 2001 (8)
	broiler	5	p. o.	6.62	0.47	99.2	-	Knoll *et al.*, 1999 (53)
Difloxacin	pig	5	p. o.	11.8 ± 0.88	3.61 ± 0.58	93.7	-	Inui *et al.*, 1998 (47)
		5	i. m.	-	-	92	-	EMEA, 2000 (28)
	cattle	5	i. m.	-	-	88	-	EMEA, 2000 (28)
	goat	5	i. m.	6.6	4.1	95.4	13.79	Atef *et al.*, 2002 (9)
	hen	5	p. o.	7.35 ± 1.58	0.96 ± 0.23	86.9	-	Inui, 1998 (47)
Marbofloxacin	dog	2	p. o.	14.0 ± 4.9	0.3	105.3 ± 29.7	-	Schneider, 1996 (85)
	cat	2	p. o.	10	-	85	-	Dossin *et al.*, 1998 (24)
Sarafloxacin	pig	5	i. m.	4.66 ± 1.34		81.8 ± 9.8		Ding *et al.*, 2001 (23)
		5	p. o.	7.2 ± 1.92		42.6 ± 8.2		Ding *et al.*, 2001 (23)
	broiler	10	p.o.	3.89 ± 1.19		59.6 ± 13.8		Ding *et al.*, 2001 (23)
	eel	15	p.o.	30.13	2.64 ± 0.42			Ho, 1999 (45)

1 – elimination half-life; 2 – maximum plasma concentration

BACTERIAL RESISTANCE

Resistance to quinolones has developed in some Gram-positive and Gram-negative organisms and the following mechanisms may be associated with its development (52, 12):

- Alteration of quinolone enzymatic targets due to mutation of genes coding for individual subunits of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*),
- Decrease in permeability of hydrophilic pores in the outer membrane of the bacterial cells,
- Active efflux of the chemotherapeutic agents out of the micro-organism (particularly in Gram-positive microbes) mediated by multidrug-resistance (MDR) transmembrane transport proteins. In Gram-negative pathogens the MDR is combined with a decreased number of porines (canals for passage of low molecular weight substances into the cell) in the outer membrane,
- Mediated by plasmids which code for products of *gyr* genes and decrease binding of DNA gyrase to the DNA (95, 70).

PHARMACOKINETICS

With regard to their lipophilic properties, quinolones accumulate intracellularly in phagocytic cells (macrophages, polymorphic leukocytes, and neutrophilic granulocytes). Their binding to plasma proteins is variable. They are well resorbed from the site of their administration and are rapidly distributed to tissues and tissue fluids in which they reach levels 5–10 times higher than in the plasma. Particularly high levels are found in the kidneys, liver, lungs and bile. Lower levels are detected in the cerebrospinal fluid and bones. They pass through the placenta and are eliminated in milk (73). After oral administration of quinolones their peak levels in the plasma are reached within 1–4 hours. Bioavailability to chickens, turkeys and pigs after oral administration reaches 80–90 %, but it is lower in ruminants and horses (75 %). Parenteral administration of fluoroquinolones ensures almost 100 % bioavailability. The half life of quinolones ranges between 2 and 7 hours depending on their properties (16 and 62). Intake of food has no effect on the quantity of their absorption, but can affect the time needed for reaching the maximum concentration in the plasma. Multivalent ions (Al, Mg, Ca, Fe, Cu and Zn) form chelated bonds with quinolones and reduce their bioavailability markedly (73).

Quinolones are metabolised in the liver and are eliminated mostly by renal excretion, with 15–50 % of the molecules passing in the urine in an unchanged form by tubular and glomerular filtration. When administered to animals with impaired renal function, the dosage must be reduced. Small quantity of quinolones are eliminated with the bile into the intestine (62).

Pharmacokinetic data for the most important fluoroquinolones used in veterinary medicine are presented in Table 1.

INDICATIONS

Quinolones are indicated for the therapy of local and systemic infections caused by quinolone-susceptible micro-organisms. The therapy has been successful when treating urogenital infections, infections of joints and bones, GIT, respiratory tract, septic conditions and febrile neutropenia. In dogs and cats quinolones were used to treat bacterial infections of skin (pyodermia), some less serious tissue infections (prostatitis, pyelonephritis, and endometritis), respiratory infections and chronic cystitis of dogs. Fluoroquinolones have been administered to cattle to treat respiratory and GIT infections (colibacillosis). They have also been used for the treatment of enterotoxaemia and Mastitis-Metritis-Agalactia (MMA) syndrome in pigs. Quinolones have been administered to chicken to prevent mortality associated with *E. coli* and *Salmonella* spp. and to turkeys to decrease mortality induced by *E. coli* and *Pasteurella multocida* (16, 95, 62).

ADVERSE SIDE EFFECTS

While quinolones of the Ist generation exhibited undesirable side effects, the relatively newer and novel fluorinated quinolones are tolerated very well.

Preparations based on quinolones are not recommended for the treatment of young animals in which they may induce erosive changes in cartilage. Also, they are not indicated in gravid or lactating animals. All quinolones have toxic effect on immature cartilage. An irreversible chondrotoxic effect (disruption and erosion) has been described for all types of quinolones (52, 13, 63, 99, 56). They should not be administered to dairy cows or laying hens where the eggs are intended for human consumption. In approximately 4–6 % of recipients, undesirable effects on GIT, skin or central nervous system (CNS) have been observed. The most frequent manifestations include nausea, vomiting and diarrhoea. Quinolones have a neurotoxic effect manifested by ataxia, spasms, tremor and convulsions and therefore are not recommended for the treatment of animals with impaired CNS. Rare cases of interstitial nephritis have been described. Additional side effects include thrombocytopenia, leucopoenia and anaemia (90, 62). Studies were conducted to observe the cardiotoxic effect on animals of quinolones administered parenterally (i. v.) which showed moderate changes in the electrocardiographic QT interval and because of that, it is not recommended to combine them with arrhythmogenic drugs (84, and 76). The skin reactions included erythema, pruritus or urticaria. Some risk of a photochemical skin reaction is associated with all quinolones. All quinolones form chelate complexes with bi- and trivalent cations (50, 91).

INTERACTIONS

Absorption of orally administered quinolones is decreased by parallel administration of drugs containing Al, Mg, Ca, Fe and Zn because of the formation of insoluble cationic chelate complexes in GIT (91, and 73). Parallel administration of quinolones increases plasma concentration of theophyline, warfarin and some non-steroidal antiphlogistics drugs – NSAID (15, 97). The antibacterial effect of quinolones is reduced by nitrofurane

antibiotics (43). Antagonism may be observed with parallel oral administration of amphenicols, tetracyclines and macrolides. Injection forms are incompatible with all beta-lactam antibiotics and therefore these should be administered separately. Parallel treatment with neuroleptics and phenothiazines should be applied with caution (78, 73).

CONTRAINDICATIONS

Quinolones should not be administered to animals younger than 12 months or to gravid or lactating animals. They are contraindicated also in animals with hypersensitivity to quinolones. Quinolones can stimulate the CNS in subjects suffering from CNS diseases (16).

These pharmaceuticals should not be administered to animals or human patients with renal insufficiency. One should also reconsider carefully their use when treating risky patients, such as older animals (94).

CLASSIFICATION OF QUINOLONES

According to their spectrum of efficacy and pharmacological properties, quinolones are divided to 4 groups – generations (62, 72):

The Ist generation

The Ist generation includes the obsolete preparations which were used in the past for the treatment of urinary tract infections induced by *E. coli* and other Gram-negative intestinal bacteria (enterobacteria). This group of drugs are represented by nalidixic acid, oxolinic acid, pipemidic acid and flumequin.

Nalidixic acid, as the first drug of this pharmacological group, was used for the first time in 1960. Together with **oxolinic acid,** it belongs to the oldest representatives of quinolones. Unfortunately both of these acid derivatives show insufficient antimicrobial activity.

From the point of view of development, pipemidic acid and flumequin are interesting, because of the appearance of some substituents (piperazine, fluor, substitution on the N-atom), which indicate typical characteristics of IInd generation (41).

Flumequin is structurally close to nalidixic and oxolinic acid. It exhibits a wide spectrum of antibacterial activity. It acts particularly against enterobacteria (*E. coli, Salmonella* and *Pasteurella*). Flumequin is especially effective against bacteria resistant to other antibiotics and sulphonamides (114, 67). The MIC is in the range of 0.125–1 $mg.l^{-1}$. After rapid absorption it is distributed throughout the organism. The maximum plasma concentration (1–10 $\mu g.ml^{-1}$) is reached as soon as 0.5 h after its administration. It is hydrolysed and glucuronized in the liver. Up to 60 % of the dose is eliminated by the kidneys. In the urine it is present as conjugated flumequin and 7-hydroxyflumequin. Its bioavailability is in the range of 55.6–85.0 %. Flumequin is indicated for the therapy of GIT, respiratory and urinary tract infections and septicaemia. Its indications are considerably limited, based on the diagnosis of the disease agent and determination of its susceptibility and resistance to other antibiotics (114, 22). It is contraindicated in salmonellosis and tuberculosis of poultry and in young, gravid or lactating animals. Dosage: cattle – 5 $mg.kg^{-1}$ i.m. and i.v., 12 $mg.kg^{-1}$ p.o.; pigs – 12 $mg.kg^{-1}$ i.m., i.v. and p.o.; sheep – 6 $mg.kg^{-1}$ i.m. and i.v., 12 $mg.kg^{-1}$ p.o.; poultry – 12 $mg.kg^{-1}$ p.o. (67, 22, 94).

The IInd generation

The IInd generation exhibits higher antimicrobial activity, wider spectrum of activity and reliable systemic distribution. The representatives of this group used in veterinary medicine are enrofloxacin, danofloxacin and difloxacin. Other well known pharmaceuticals included in this group are ciprofloxacin, marbofloxacin, norfloxacin, sarafloxacin, pefloxacin, ofloxacin, ibafloxacin and enoxacin.

Enrofloxacin has been used in veterinary medicine since 1983 for the treatment of various infectious diseases of all domesticated animal species. It has a wide spectrum of antimicrobial activity. Enrofloxacin acts against Gram-negative and Gram-positive bacteria, mycoplasma and chlamydia. Its bactericidal action affects both the stationary and growth phase of bacterial replication. Together with its metabolite ciprofloxacin, it reaches the lowest level of minimum concentration in blood ranging between 0.008 $\mu g.ml^{-1}$ (*Pasteurella multocida*) and 0.75 $\mu g.ml^{-1}$ (*C. pyogenes*) (110). The highest level in the blood serum is reached after 2 h and is maintained for 24 h. It is absorbed well and distributed rapidly to all tissues. The liver is the primary organ of its metabolism. It is eliminated in the urine and bile. The protracted post-antimicrobial effect of this drug ensures its prolonged antimicrobial activity. It should not be used to treat cats and dogs younger than 1 year, lactating animals or laying hens. In sporadic cases, digestive disorders were observed in the course of treatment. When administered to pigs, tissue necrosis may occur locally at the injection site (94). It must not be used in combination with macrolides, tetracyclines or chloramphenicol. It should not be administered in parallel with non-steroidal antiphlogistics (88).

Enrofloxacin can be administered orally, intramuscularly and subcutaneously. The general dosage of enrofloxacin is 2.5–5 $mg.kg^{-1}$ b.w. and in poultry it ranges between 10 and 20 $mg.kg^{-1}$ b.w. (89, 93, 38, 83).

Danofloxacin is a fluoroquinolone developed especially for veterinary use. It shows affinity to pulmonary tissue and because of that it is used for treatment of respiratory diseases of cattle, sheep and poultry. It is preferred particularly in the treatment of mycoplasmoses because of its high activity against *Mycoplasma* spp. It shows considerable activity against another two pathogens of poultry, *E. coli* and *Pasteurella multocida* (37, 18 and 35). After parenteral, i.m. or s.c. administration the drug, it is absorbed rapidly and 100 % or 94 % of the administered dose is utilised (31). At oral administration of a dose of 5 $mg.kg^{-1}$ b.w. the bioavailability reaches 90 % (96). After i.m. administration of 5 $mg.kg^{-1}$, the maximum plasma concentration (approximately 0.8 $\mu g.ml^{-1}$) is reached after 0.8 h in pigs and 0.7 h in cattle (69). Danofloxacin is metabolised to a N-desmetylene form (26). Regardless of the method of administration, about 40 % of the dose is eliminated by the kidneys and the rest in the bile.

Penetration of danofloxacin into milk after i.m. and i.v. administration of a single dose to lactating cows was observed

after 90–120 min. Its presence in milk can be detected even after 24 h (87).

Dosage: pigs – i.m. 1–5 mg.kg^{-1} for max. 3–5 days, s.c. 5 mg.kg^{-1}, p.o. 10 mg.kg^{-1} for 3–5 days; cattle – i.m. 5 mg.kg^{-1} for max. 3–5 days (69), birds – p.o. 5 mg.kg^{-1} every 6–8 h for 3 days (26).

From the chemical aspect, **difloxacin** it is an aryl fluoroquinolone derivative with a p-fluorophenyl group at the position 1 and a methyl group in a piperazine ring. It shows activity against Gram-positive and Gram-negative bacteria, cocci and mycoplasma, particularly against *Klebsiella* spp., *Staphylococcus* spp., *E. coli, Campylobacter, Shigella, Proteus* and *Pasteurella* spp. (18).

It is resorbed relatively rapidly even after i.m. administration. The maximum plasma concentration (4.1 μg.ml^{-1}) in goats is reached in 1 h after i.m. administration of 5 mg difloxacin.kg^{-1} b.w. Bioavailability after oral administration of 5 mg.kg^{-1} is 86–93 %, depending upon the animal species. It is metabolised to N-desmethyldifloxacin and N-oxid-difloxacin and eliminated in the faeces and urine (28).

The principal indications are infections of the urinary, respiratory and gastrointestinal tract. Good results have been attained in dogs, pigs, cattle, goats, sheep and birds. The general dose ranges between 2.5 and 5 mg.kg^{-1}. It is administered usually for 5 days. Mycoplasmosis induced by *Mycoplasma galisepticum* is treated by administering 10 mg.kg^{-1} for 5 days (51, 47).

Ciprofloxacin is a fluoroquinolone with bactericidal effect on both the stationary and growth phase, capable of devitalising the target bacterial cells within 20–30 min. It acts against Gram-negative and Gram-positive bacteria, mycoplasma, chlamydia and rickettsia. However, its effect on anaerobic micro-organisms is very weak (21, 109). It was introduced in the nineteen eighties. Usually, it is not used as the first line of treatment but it is more suitable for the treatment of mixed infections. It is a metabolite of enrofloxacin. Antibacterial activity of this pharmaceutical is increased by a N atom at the position 1, a hydroxyl group at the position 3 and a ketone at the position 4. A piperazine group at the position 7 determines its effectiveness against pseudomonas and a F atom at the position 6 increases its activity against Gram-negative bacteria (25). Due to its good solubility in fat and weak binding to proteins this pharmaceutical penetrates rapidly into all organs. It is absorbed rapidly and efficiently as are its metabolites oxyciprofloxacin and desetylenciprofloxacin. It is eliminated by tubular and glomerular filtration and 15–50 % of ciprofloxacin is found in the urine and faeces in an unchanged form (17, 4). The bioavailability of ciprofloxacin ranges between 37 and 80 %. Its maximum plasma concentration, which is dependent on animal species, dosage and way of administration, is in the range of 0.13–0.14 mg.ml^{-1} and is reached within 0.2–15 hours. The oral dosage is 2.5–5 mg.kg^{-1}, 2.5–10 mg.kg^{-1} i.v. and 2.5–7.5 mg.kg^{-1} i.m. (71, 1, 10, 17, 75).

Marbofloxacin is one of the more recent fluoroquinolones. It is used to treat infections of the: skin and mucous membranes of dogs and cats; urogenital infections of dogs; respiratory infections of cattle and pigs; and MMA-syndrome of pigs. It shows very good antibacterial activity against *E. coli*, salmonellae, pathogens involved in the development of mastitis (*Staphylococcus aureus*, *Streptococcus uberis, S. agalactiae* and *S. dysgalactiae*), and agents of respiratory infections (*Mannheimia haemolytica, Pasteurella multocida* and *Haemophilus somnus*). The MIC90 values for *E.coli* are 1.275–5.098 μg.ml^{-1} and for salmonellae 0.073 μg.ml^{-1} (66). Marbofloxacin is never used as a first choice antibiotic but it is prescribed only on the basis of an antibiogram. With regard to its lipophilic properties, higher concentrations of this pharmaceutical were found in organs (lungs, liver, and kidneys) than in the plasma after both oral and parenteral administration. In the liver, it is transformed to two inactive forms (N-desmetylmarbofloxacin and N-oxid-marbofloxacin). It is eliminated by the kidneys (27). After oral administration, 35–40 % of marbofloxacin is eliminated in the urine in an unchanged form. The bioavailability of this pharmaceutical ranges between 70 and 85 %. After 20–60 min, it is measurable in the plasma. The general dosage for animals is 2 mg.kg^{-1} b.w. when administered i.v., i.m. or orally. It is usually administered for 3–5 days (respiratory infections, MMA-syndrome, and pyoderma). The urinary tract is treated with a dosage of 4 mg.kg^{-1} s.c., during 3 days (85, 86, 24).

With fluorine at the 6th position and a piperazine or pyrolidine group at the 7th position, **norfloxacin** belongs to the IInd generation of quinolones – fluoroquinolones (32). With regard to its amphoteric properties, orally administered norfloxacin is absorbed rapidly but incompletely. High concentrations in the serum were observed for the agent itself (56.85 %) and also for its metabolites N-desetylennorfloxacin (34.69 %) and oxonorfloxacin (7.15 %). It passes across the placenta. Concentrations of norfloxacin in the milk are considerably higher than in the serum. The highest plasma concentration is achieved after 50–60 min and reaches 0.95–3.47 μg.ml^{-1} (36, 33, 79). The dosage used for cattle at i.v. and i.m. administrations, ranges between 7.5 and 15 mg.kg^{-1} (32). The following dosages are recommended for oral administration to dogs: 5–20 mg.kg^{-1}, pigs: 400–800 mg.kg^{-1} twice a day, sheep: 60 mg.kg^{-1}, poultry: 8 mg.kg^{-1} (4, 32, 36).

Sarafloxacin is a metabolite of difloxacin. It is the first fluoroquinolone introduced to veterinary practice for mass oral administration via the water to broiler chickens infected with *E. coli*, and in some countries also to treat turkeys. It can also be administered for infectious diseases of eels. The maximum plasma concentration (2.6 μg.l^{-1}) is reached after 12 h following the treatment with water with a temperature of 24 °C. Its biological half-life is 30.13 h. In pigs and chickens after i.m., i.v. and p.o. administration of 5 or 10 mg.kg^{-1} sarafloxacin, the elimination half-time (t1/2) reaches 3.37–7.2 and 2.5–6.8 h, respectively. Its bioavailability in pigs after i.m. and p.o. administration is 81.8 % and 42.6 %, resp., and 72.1 % and 59.6 %, resp., in broilers.

Recommended dosages: pigs – i.m. 10 mg.kg^{-1} every 12 h (23); broilers – p.o. 20–40 mg.l^{-1} drinking water for 5 days; turkeys – p.o. 30–50 mg.l^{-1} drinking water for 5 days (16); eel – p.o. at the dosage of 15 mg.kg^{-1} (45).

Pefloxacin belongs among the antibacterial fluoroquinolones with very good activity against Gram-positive and Gram-negative bacteria and staphylococci including methicillin-resistant strains. It is absorbed rapidly after oral administration and the maximum serum concentrations are reached after 90 min. Its

biological half-life is approximately 8 h. Pefloxacin penetrates well into tissues (heart, bronchial mucosa, CNS and bones). Approximately 50 % is eliminated in an unchanged form. The rest is released in metabolised forms as demethylpefloxacin and pefloxacin-N-oxid. Its side effects are similar to those observed with other quinolones. Photosensitivity may also occur (105). Dosage: goats – i. v. 10 mg.kg^{-1}, p. o. 20 mg.kg^{-1}, chickens – p. o. 10 mg.kg^{-1} (58, 77).

With regard to its wide spectrum of activity, **ofloxacin** is indicated for acute, chronic and recurrent infections of the respiratory tract caused by *Haemophilus influenzae* or other Gram-negative and multi-resistant pathogens or by *Staphylococcus aureus*. It is effective in the treatment of pneumonia caused by *E. coli, Klebsiella, Enterobacter, Proteus, Pseudomonas* and *Staphylococcus.* Oxoflacin is also used for the treatment of soft tissues and skin infections, bacterial diarrhoea, infections of the kidneys, urinary tract and genitals. It is absorbed rapidly after oral administration. Its absorption half-time is 5–7 h. Plasma proteins bind approximately 25 % of ofloxacin. Less than 5 % is subjected to biotransformation to desmethyl and oxid forms and 80–90 % is eliminated in the urine in an unchanged form. Glucurone form of ofloxacin is found in the bile (106). Dosage: dogs – p. o. 20 mg.kg^{-1} daily for 8 days (100).

Properties and use of **temafloxacin** are similar as those of ciprofloxacin. It is effective particularly on staphylococci and streptococci. The MIC ranges between 0.12 and 1 μg.ml^{-1}. The activity of temafloxacin against *Staphylococcus pneumoniae* is 8-times higher than that of ofloxacin or ciprofloxacin. It is used to treat the respiratory tract infections of mice and guinea pigs. It is administered s.c. at a dosage of 25 mg.kg^{-1}. One hour after its administration the pulmonary tissue contains 14.3 μg.g^{-1} temafloxacin (11).

Ibafloxacin is a recent fluoroquinolone developed exclusively for veterinary use. It has a wide spectrum of activity which includes Gram-positive and Gram-negative bacteria, particularly *Pasteurella* spp., *E. coli, Klebsiella* spp., *Proteus* spp. and *Staphylococcus* spp. (MIC < 0.5 μg.ml^{-1}). Ibafloxacin is ineffective against enterobacteria, pseudomonas and streptococci (104). After oral administration it is rapidly almost completely absorbed. The maximum plasma concentration 3.8–5.9 μg.ml^{-1} is reached after 1–2 h. Its bioavailability ranges between 69 and 81 % and biological half-life reaches 4–5 h. Ibafloxacin is used for the treatment of the respiratory, urinary and skin infections of dogs and cats. It is contraindicated in young animals in the growth phase, i. e. younger than 8 months, and in dogs of large breeds up to 18 months of age. It must not be administered to dogs weighing less than 3 kg. It may be administered only on the basis of the results of an antibiogram or resistance test. It is administered orally at a dosage of 15 mg.kg^{-1} once a day (46, 19, 60).

Enoxacin is a wide-spectrum azafluoroquinolone with antibacterial activity, intended for oral administration. The maximum plasma concentration (0.93 μg.g^{-1} and 2.0 μg.g^{-1} after doses of 200 or 400 mg *pro toto*, resp.), is reached after 1–3 h. It diffuses into the cervix and myometrium. Plasma proteins bind 40 % of enoxacin. Enoxacin is primary eliminated by the kidneys and its bioavailability reaches approximately 90 %. It is not administered to the young because of the development of arthropathies. It is indicated for the treatment of infections of the urinary tract induced by *E. coli, Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Enterobacter cloacae* (44, 108).

The IIIrd generation

This generation extends the spectrum of activity to *Streptococcus pneumoniae* and some other Gram-positive cocci. These preparations exhibit better pharmacological properties. From the preparations used in veterinary practice, orbifloxacin belongs to this class. Other IIIrd generation representatives are: levofloxacin, sparfloxacin and grepafloxacin.

Orbifloxacin is a fluoroquinolone derivative of carboxylic acid developed especially for veterinary use. It has a wide spectrum of activity. Orbifloxacin exhibits bactericidal activity against both Gram-positive and Gram-negative bacteria. It is active particularly against *E. coli, Proteus mirabilis* and *Pseudomonas* spp. (40). After oral administration it is absorbed almost completely within 46 min (103). In calves it is metabolised to the glucuronide and oxidative forms, while in pigs there is only the glucuronide metabolite. It is eliminated in the urine and faeces. Its binding to plasma proteins is difficult (5–15 %). Similar to other quinolones, it is used to treat respiratory and urogenital infections and also diseases of the skin in dogs and cats. Its indications are limited. Orbifloxacin is prescribed only for the treatment of very serious infections and on the basis of antibiotic resistance determination of the respective infectious agent. The dose used for the treatment of pigs and calves is 5 mg.kg^{-1} i. m. When administered to dogs and cats, the recommended p. o. dose is 2.5–7.5 mg.kg^{-1} and s. c. dose 5 mg.kg^{-1}. It is contraindicated in cats younger than 12 months, dogs of small and medium breeds up to 8 months and large and giant breeds up to 12 and 18 months, resp. (102, 64).

Levofloxacin belongs to the more recent fluoroquinolones. It is indicated only for serious infections caused by micro-organisms susceptible to levofloxacin: i. e. pneumonia; complicated infections of the urinary tract; pyelonephritis; and skin and soft tissues infections. When administered orally, it is absorbed rapidly and reaches peak plasma concentrations within 1 h. Its bioavailability reaches almost 100 %. Approximately 30–40 % of levofloxacin binds to serum proteins. It is metabolised to a small degree to desmethyllevofloxacin and levofloxacin N-oxid. Levofloxacin is eliminated by the kidneys (85 %). It is contraindicated in young and gravid animals and animals with impaired kidneys. It is administered i. m. and p. o. at a dosage of 10 mg.kg^{-1} (61, 2).

Sparfloxacin is characterised as a fluoroquinolone known for its activity against a wide range of Gram-negative and Gram-positive organisms including *Streptoccocus pneumoniae*. It is also effective on *Haemophillus influenzae, Moraxella catarrhalis, Mycoplasma pneumoniae* and *Chlamydia pneumoniae* (34, 61). With regard to its long half-time (t1/2), which reaches 7–8 h, it is administered once a day. It inhibits 99–100 % of isolates of streptococci strains (β-haemolytic, virilising group and *Streptoccocus pneumoniae*) at concentrations lower or equal to 1 μg. ml^{-1} and its potency is two to eightfold higher in comparison with levofloxacin, ofloxacin and ciprofloxacin (81).

The IVth generation

This generation exhibits potentiated activity against Gram-

positive cocci and some anaerobes. It includes the following substances: trovafloxacin, moxifloxacin and gemifloxacin.

Trovafloxacin belongs to the latest fluoroquinolones known for increased activity against Gram-positive and anaerobic micro-organisms. Its bioavailability after oral administration is approximately 88 %. The elimination half-life t1/2 is approximately 10 h with less than 10 % trovafloxacin eliminated in the urine in an unchanged form. The efficiency of treatment of respiratory diseases with this pharmaceutical reaches 90 %. An advantage is its administration once a day (80, 3).

Observation of antibacterial activity of this substance showed that its potency against penicillin-susceptible and penicillin-resistant species of *Streptoccocus pneumoniae* is 16-fold higher than that of ciprofloxacin or ofloxacin. It provides two to eightfold higher activity against Gram-positive bacteria (staphylococci and streptococci) than ciprofloxacin (39). Parallel administration of antacids or cation-containing food decreases its absorption (29).

Moxifloxacin is a recent fluoroquinolone favoured in human medicine for the treatment of acute exacerbation of chronic bronchitis, pneumonia and acute sinusitis. When tested i*n vitro,* it is active against Gram-positive (*Enterococcus faecalis, Staphylococcus aureus* and *Streptococcus pneumoniae*), Gram-negative (*Enterobacter cloacae, Haemophilus influenzae, Klebsiella pneumoniae, Moraxella catarrhalis* and *Proteus mirabilis*) and anaerobic (*Bacterioides fragilis)* organisms. The MIC for the penicillin-resistant strain of *Streptococcus pneumoniae* is 0.125 $\mu g.ml^{-1}$. When administered orally, it is well absorbed in the gastrointestinal tract. It passes to the cerebrospinal fluid and because of this, it can be used for the treatment of meningitis. Its bioavailability is as high as 90 %. The maximum plasma concentration (of 400 mg moxacilin *pro toto*) is 3.1 $\mu g.ml^{-1}$. Moxifloxacin is metabolised by glucuronidation and sulphate conjunction. Approximately 45 % of the dose is eliminated in an unchanged form in the urine and faeces. The dose of 30 $mg.kg^{-1}$ per day, administered orally to young dogs, causes arthropathies. No local intolerance was observed at i. v. administration to adult animals (74, 107). Moxifloxacin causes hearing loss when used with a non-intact tympanic membrane in chinchilla (20). The dose of 5 $mg.kg^{-1}$, administered i. m. or p. o., was effective for the treatment of staphylococci infections in rabbits (30).

Gemifloxacin is bactericidal against a wide spectrum of Gram-positive and Gram-negative organisms. *In vitro* MIC of gemifloxacin reaches 0.25 $\mu g.ml^{-1}$ for *Enterococcus faecalis* and 0.03 $\mu g.ml^{-1}$ for *Streptococcus pneumoniae*. It is also efficient against methicillin-resistant staphylococci, many Gram-negative respiratory pathogens (*Haemophilus influenzae* and *Moraxella catarrhalis*) and atypical strains such as *Legionella pneumophila, Chlamydia* spp. and *Mycoplasma* spp. Because of this, it is particularly useful in the treatment of respiratory infections. After oral administration, it is absorbed rapidly and its bioavailability reaches 71 %. Maximum plasma concentrations are reached within 30–120 min and reach values of 0.7–2.62 $\mu g.ml^{-1}$. It is metabolised in the liver to E-isomer (4–6 %), acyl-glucuronid (2–6 %) and N-acetyl-gemifloxacin (2–5 %). It is eliminated in the urine and faeces with approximately 20–30 % of the drug in an unchanged form. The t1/2 is 6–8 h. Experiments showed that similar to other quinolones, gemifloxacin tends to crystallize in the alkaline urine of rodents and induce nephropathies. Dosage: dogs and rodents – p. o. 10–30 $mg.kg^{-1}$ daily, i. v. 10 $mg.kg^{-1}$ daily (82, 101, 98).

CONCLUSION

Quinolones are efficacious antibacterial chemotherapeutics agents. They form an attractive pharmacological group, with a multitude of beneficial uses in veterinary medicine. Studies of their pharmacological properties and information about their individual variations are of extreme importance to practising veterinarians. One of the most important considerations of pharmacotherapy safety and effectiveness is to pay close attention to the instructions provided by the drug manufacturers. Additionally, a thorough updated knowledge and awareness of the specific characterizations of the relevant animal infections are invaluable.

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EVALUATION OF SOME URINARY PARAMETERS IN BEEF CATTLE FED WITH DIETS DIFFERENT FOR CA/P RATIO AND MOISTURE CONTENT

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ABSTRACT

Urolithiasis is a multifactorial disease, predisposed by high concentrate, low roughage, low Ca:P ratio, high magnesium diets and alkaline urine. Recognition of the possible alterations of these parameters is useful in the early suspicion of possible renal dysfunctions. In order to determine such dietary electrolytes modifications, the electrolytes and their fractional excretions were analyzed. Sixty-eight calves, divided into two groups, were kept under the same feeding condition during the first two periods of experimental observations (1 and 2). During period 3 two different kind of food, with different moisture percentage and Ca:P ratio, were offered to the animals. Urine samples were collected and the fractional excretions were calculated for; urea (% Cr/Urea), Ca (% Cr/Ca), P (% Cr/P), Mg (% Cr/Mg), K (% Cr/K), and Cl (% Cr/Cl). The statistical analysis (ANOVA) showed a significant effect of the period on the following parameters: for the "Group A"; specific gravity, pH, % Cr/P, and % Cr/Ca; and for the "Group B"; specific gravity, pH; % Cr/P, % Cr/Cl, and %Cr/Mg. A significant effect of the food was present only on % Cr/Mg (this is an important predictive factor for the pathogenesis of urolithiasis, because an overload of Mg intake could results in an overflow in the urine). The fractional excretion of P showed a constant decrease during the periods 2 and 3 in the D diet. Of particular importance in our study was the fact that the variations in % Cr/Mg and % Cr/P were indicative of an alteration in the renal excretion.

Keywords: calves; electrolytes; fractional excretion; urinary parameters; urolithiasis

INTRODUCTION

Many studies aimed at advancing the knowledge of animal nutrition, especially in ruminant feeding, have had very important repercussions in economical terms (9, 13). One of the most frequent pathological conditions associated with dietary imbalance is urolithiasis (9, 11, 13). Various theories consider this problem as a multifactorial disease which is present both in humans and in animals (2, 8). Stress can result in an increase excretion of Calcium and, together with urinary pH, represents an important contribution towards the crystallization of the initial nuclei of stones (13). Most of the calculi are composed of minerals and when these are at a high level, they tend to

crystallize, aggregate and develop calculi in the urine (16). The stones constantly irritate the pelvis and the mucosa of the bladder. This develops into a chronic inflammation that can contribute to a complete blockage of the urethra (9, 10). The formation of phosphate calculi is encouraged by; high concentrate, low roughage, low Ca/P ratio, high magnesium diets and alkaline urine (10). Normally, phosphorus is recycled through the saliva and excreted via the feces in ruminants (2, 9). Diets with high grain concentrations and low roughage concentration decrease the formation of the saliva and increase the amount of phosphorus excreted in the urine. Many authors have investigated the incidence of urolithiasis in cattle and the relationship between dietary Ca/P ratio and the onset of pathology (2, 5, 9, 10, 11, 13). Other researchers have demonstrated that in lambs the level of P should not exceed 0.57 % and Mg 0.28 %, while the Ca/P ratio should be 2/1 (10).

In fact, the excessive intake of Mg, over 1.4 %, or a Mg/P ratio more than 1.6 %, lead cattle to urolithiasis (4). High risk situations are represented by Ca/P relationship of equality (1 : 1) and by imbalanced ratio as Ca/P 1 : 2 (10, 11). The formation of urinary calculi results when inorganic or organic urinary solutes are precipitated out of solution as crystals or amorphous deposits (9). Few studies have analyzed the urinary minerals involved in urolithiasis of ruminants and, fractional excretion measurements in veterinary medicine are rarely used (3, 7, 12, 13, 14, 15). The fractional excretion is the fraction of the electrolyte filtered by the glomerulus, and excreted into the urine (7). It is determined and expressed as the ratio of the clearance of a given electrolyte compared with the creatinine clearance (7). The urinary load of solutes depends on the type of diet and the time of feeding (8). Other researchers have determined that in cattle, after a supplementation of minerals in the diet, the means of % Cr/Ca and % Cr/P significantly increased after two days (4).

Alterations of some of these parameters could be used as reference values for the early recognition of a dysfunction, such as urolithiasis (13).

The aim of this study was to verify how modifications of electrolytes and the moisture content of a diet can influence the threshold level over which urolithiasis may arise. For this purpose we analyzed the electrolytes and their fractional excretion as predictive parameters of renal failure.

MATERIALS AND METHODS

Sixty-eight beef calves, cross breeds and of different gender, from a farm with a high incidence of urolithiasis were monitored from 60 kg to 250 kg of body weight in 3 different periods: milk feeding (period 1); weaning (period 2); and final diet (period 3). Calves were assigned to 2 groups (Group A and Group B) on the basis of their age: in particular calves that were forty days old were assigned to Group A and one year old calves were assigned to Group B. The two groups, formed by 34 subjects each, were kept under the same management and feeding condition in the first two period while during the third period, animals were fed with two different kind of food. The "D diet", was a dry diet that had a C/P ratio of 1 : 3 (moisture 13.4 %) and consisted of grain maize and concentrate; the "W diet", composed of maize ensiled and chard pulp, was a wet food with an elevate moisture percentage (28.71 %), but with a Ca/P ratio of 1 : 1. In table 1 and 2, the results of the analysis of the composition of the food administered as D diet and W diet are shown. In both groups, during the three periods in the morning at the same hour (9:00 a. m.) clinical conditions were recorded and urine samples were collected by spontaneous urination in plastic tubes. Immediately after collection, the urine specific gravity was measured with a refractometer (Clinical Refractometer Cosmo R-308®) and pH with a portable pH-meter (Piccolo, Hanna Instruments, Leighton Buzzard, Bedfordshire, UK). Urine samples were centrifuged and analyzed for a range of clinical chemistry parameters (urea, creatinine, calcium (Ca), phosphorus (P), magnesium (Mg), potassium (K) and chloride (Cl)) using an automated analyzer (BM Hitachi 911, ROCHE, Basel, Switzer-

Table 1. Chemical composition of the diet characterized by a low water content, expressed in percentage, with the Ca/P ratio (D)

Parameters	D diet
DDM	87.40 %
Ash	9.80 %
Ca	1.43 %
Mg	0.32 %
P	0.62 %
K	1.22 %
Ca/P ratio	1 : 3

Table 2. Chemical composition of the diet characterized by a high water content, expressed in percentage, with the Ca/P ratio (W)

Parameters	W diet
DDM	71.20 %
Ash	6.80 %
Ca	0.59 %
Mg	0.22 %
P	0.38 %
K	1.20 %
Ca/P ratio	1:1

Table 3. Mean values ± SE of the parameters considered, expressed in conventional units of measurement, and statistical significance in group A, fed low water content diet (D)

Parameters	Samples			
	Period 1	Period 2	Period 3	Reference values°
Specific gravity	1009.90 ± 1.86*•□	1020.60 ± 3.15	1016.80 ± 2.35	1025–1045
pH	6.98 ± 0.23•	7.33 ± 0.15	7.82 ± 0.02	7.40–8.40
% Cr/Urea	42.98 ± 2.13	46.09 ± 1.18◇	41.46 ± 1.53	39.78 ± 3.50
% Cr/Ca	0.13 ± 0.02*	0.36 ± 0.75	0.05 ± 0.01	0.25 ± 0.03
% Cr/P	11.36 ± 1.37	8.12 ± 3.10	3.39 ± 0.4•	0.15 ± 0.01
% Cr/Mg	3.37 ± 0.91	4.40 ± 1.28•	1.84 ± 0.74•	4.20 ± 0.40
% Cr/K	36.87 ± 5.38□	32.08 ± 3.25	33.55 ± 2.55	126.70 ± 7.20
% Cr/Cl	1.20 ± 0.26□	1.25 ± 0.24	0.16 ± 0.03	1.39 ± 0.18

Significances after ANOVA application: *–Vs 80 days ($P < 0.0001$); •–Vs 120 days ($P < 0.001$)
Significances after Student's *t*-test application: □–Period 1 ($P < 0.0001$); ◇–Period 2 ($P < 0.002$); •–Period 3 ($P < 0.02$)
°–Kaneko *et al.* (6)

Table 4. Mean values ± SE of the parameters considered, expressed in conventional units of measurement, and statistical significance observed in group A, fed high water content diet (W)

Parameters	Samples			
	Period 1	Period 2	Period 3	Reference values°
Specific gravity	1017.80 ± 2.60	1020.70 ± 3.60	1018.90 ± 2.76△	1025–1045
pH	7.07 ± 0.16*•	7.09 ± 0.13•	8.07 ± 0.08△	7.40–8.40
% Creat/Urea	47.19 ± 3.02	45.90 ± 2.10	43.90 ± 1.98	39.78 ± 3.50
% Creat/Ca	0.06 ± 0.01*•■	0.33 ± 0.07	0.55 ± 0.19	0.25 ± 0.03
% Creat/P	14.17 ± 1.62•	6.96 ± 0.94	1.95 ± 1.17	0.15 ± 0.01
% Creat/Mg	2.36 ± 0.56•■	6.32 ± 1.72•	10.47 ± 2.11	4.20 ± 0.40
% Creat/K	35.40 ± 2.60■	27.50 ± 2.10	34.90 ± 3.62△	126.70 ± 7.20
% Creat/Cl	1.41 ± 0.21	1.32 ± 0.16▲	1.18 ± 0.17△	1.39 ± 0.18

Significances after ANOVA application: *–Vs 80 days ($P < 0.0001$); •–Vs 120 days ($P < 0.001$)
Significances after Student's *t*-test application: ■–Period 1 ($P < 0.01$); ▲–Period 2 ($P = 0.02$); △–Period 3 ($P < 0.001$)
°–Kaneko *et al.* (6)

land). Ca/Mg and Ca/P urine ratios were calculated. Eventually, the fractional excretions were calculated for urea (% Cr/Urea), Ca (% Cr/Ca), P (% Cr/P), Mg (% Cr/Mg), K (% Cr/Mg), and Cl (% Cr/Cl), according to the following formula:

$$X_u/X_s \times Cr_u/Cr_s \times 100 = \% \text{ CrX}$$

where X_u is the urinary electrolyte value and X_s the serum value of the same electrolyte. Cr_u and Cr_S are respectively the urinary and serum creatinine level.

Two way repeated measures of the analysis of variance (ANOVA) was applied on all parameters to determine significant differences. The factors were period and food. $P < 0.05$ was considered statistically significant. Bonferroni's test was

Table 5. Mean values ± SE of the parameters considered, expressed in conventional units of measurement, and statistical significance observed in group B, fed low water content diet (D)

Parameters	Samples			
	Period 1	Period 2	Period 3	Reference values°
Specific gravity	1025.80 ± 3.22□	1028.60 ± 2.21	1025.00 ± 1.26	1025–1045
pH	7.46 ± 0.28*	7.21 ± 0.08˙	7.72 ± 0.11	7.40–8.40
% Creat/Urea	45.30 ± 1.88	46.37 ± 1.61◊	42.20 ± 2.44	39.70 ± 3.50
% Creat/Ca	0.22 ± 0.06	0.10 ± 0.02	0.28 ± 0.06	0.25 ± 0.03
% Creat/P	11.40 ± 4.43•	8.38 ± 1.12	2.75 ± 1.00•	0.15 ± 0.01
% Creat/Mg	4.42 ± 1.65˙	4.06 ± 0.67˙	10.40 ± 2.30•	4.20 ± 0.40
% Creat/K	51.70 ± 4.98□	35.80 ± 4.09	36.60 ± 4.22	126.00 ± 7.20
% Creat/Cl	1.81 ± 0.23□	1.47 ± 1.18	1.18 ± 0.26	1.39 ± 0.18

Significances after ANOVA application: *–Vs 80 days ($P < 0.01$); ˙–Vs 120 days ($P < 0.02$)
Significances after Student's *t*-test application: □–Period 1 ($P < 0.0001$); ◊–Period 2 ($P < 0.002$); •–Period 3 ($P < 0.02$)
°–Kaneko *et al.* (6)

Table 6. Mean values ± SE of the parameters considered, expressed in conventional units of measurement, and statistical significance observed in group B, fed high water content diet (W)

Parameters	Samples			
	Period 1	Period 2	Period 3	Reference values°
Specific gravity	1019.90 ± 4.80*˙	1022.60 ± 0.23	1024.50 ± 1.90△	1025.00–1045.00
pH	7.29 ± 0.11	7.78 ± 0.06	7.68 ± 0.12△	7.40–8.40
% Creat/Urea	45.30 ± 2.50	46.60 ± 1.93	44.28 ± 1.13	39.78 ± 3.50
% Creat/Ca	0.75 ± 0.27■	0.25 ± 0.05	0.27 ± 0.10	0.25 ± 0.03
% Creat/P	12.39 ± 4.15*˙	0.95 ± 0.28	1.94 ± 0.80	0.15 ± 0.01
% Creat/Mg	10.93 ± 1.46■	9.18 ± 1.75	8.39 ± 1.46	4.20 ± 0.40
% Creat/K	48.60 ± 2.64■	78.40 ± 30.41	51.42 ± 4.89△	126.70 ± 7.20
% Creat/Cl	1.11 ± 0.08*˙	2.61 ± 0.42▲	2.31 ± 0.14△	1.39 ± 0.18

Significances after ANOVA application: *–Vs 80 days ($P < 0.01$); ˙–Vs 120 days ($P < 0.02$)
Significances after Student's *t*-test application: ■–Period 1 ($P < 0.01$); ▲–Period 2 ($P = 0.02$); △–Period 3 ($P < 0.001$)
°–Kaneko *et al.* (6)

applied for post-hoc comparison and then the *t*-test for unpaired values was performed to compare the two groups. All data were analyzed using Graph Pad Prism software, version 5.

RESULTS

Tables 3, 4, 5 and 6, show the mean values ± SE of the urinary parameters analyzed, expressed in their conventional units of measurement.

Two way ANOVA showed a significant effect of "period" on the following parameters: for the Group A specific gravity ($F_{(2,64)} = 0.86$; $P < 0.0001$); pH ($F_{(2,64)} = 35.66$; $P < 0.0001$); % Cr/P ($F_{(2,64)} = 5.54$; $P = 0.0061$) and % Cr/Ca ($F_{(2,64)} = 8.02$; $P = 0.0008$). For the Group B specific gravity ($F_{(2,64)} = 3.71$; $P = 0.0298$); pH ($F_{(2,64)} =$

3.28; P = 0.0440); % Cr/P ($F_{(2,64)}$ = 13.66; P < 0.0001); % Cr/Cl ($F_{(2,64)}$ = 4.72; P = 0.0122) and % Cr/Mg ($F_{(2,64)}$ = 3.82; P = 0.0272).

No statistical significant effect of period was observed on % Cr/Urea, % Cr/K, % Cr/Cl in animals of the Group A. In Group B no statistical significance was observed on the following parameters: % Cr/Urea, % Cr/K, % Cr/Ca.

A statistically significant effect of food was observed only on % Cr/Mg of Group B ($F_{(1,64)}$ = 7.90; P = 0.0084).

Moreover, the Student's t test application showed a significant variation inside the "D diet" during "period 1" for specific gravity (P < 0.0001; t = 4.525; DF = 32); % Cr/K (P = 0.0065; t = 2.913; DF = 32); % Cr/Cl (P = 0.0027; t = 3.253; DF = 32); during "period 2" only for % Cr/Urea (P = 0.0026; t = 3.268; DF = 32); during "period 3" for % Cr/P (P = 0.0483; t = 4.146; DF = 32) and % Cr/Mg (P = 0.0002; t = 4.146; DF = 32).

For the "W diet" a significant variation during "period 1" was demonstrated for % Cr/Ca (P = 0.0112; t = 2.692; DF = 32); % Cr/Mg (P < 0.0001; t = 5.964; DF = 32) and % Cr/K (P < 0.0001; t = 4.701; DF = 32); at "period 2" % Cr/Cl (P = 0.0268; t = 2.322; DF = 32); during the "period 3" specific gravity (P = 0.004; t = 3.099; DF = 32); pH (P = 0.0015; t = 3.463; DF = 32); % Cr/K (P = 0.0029; t = 3223; DF = 32) and % Cr/Cl (P = 0.0001; t = 4.401; DF = 32).

DISCUSSION

In the present study, all of the parameters considered, except % Cr/Urea and % Cr/K, showed a significant effect of time. Especially, specific gravity, pH and % Cr/Cl showed a significant effect of time. This can be seen in the variation of values during the period 3. The specific gravity value had a significant decrease during the period 3, when the dry diet was administered. The significance of this variation was confirmed by the *t*-test. Also, at the end of the period 3, the pH had a statistically significant increase of its value, when calves were fed the dry diet. This can be explained by the low content of water present in the dry food. However, while % Cr/K showed no significant effect for time or for food, the *t*-test demonstrated a significant variation with the wet food in the periods 2 and 3. During these two periods, this parameter had a swinging trend, with an increase followed by a decrease in the Group B and an opposite trend in Group A. Furthermore % Cr/Cl showed a significant effect of time confirmed by the *t*-test results: during the period 3, a significant reduction was observed in Groups A and B during the administration of wet food. Only % Cr/Mg showed a significant effect of food (with a statistically significant increase of this parameter in the period 3 for Group A). This parameter is an important predictive factor for the pathogenesis of urolithiasis. An overload of Mg intake can result in an overflow in the urine which increases the concentration of urinary electrolytes responsible for urolithiasis (1). This parameter in our research showed a significant variation in the period 3 when D diet was administered. A different consideration might be done for fractional excretion of P (% Cr/P). P electrolyte is one of the most important factors responsible for urolith formation, because the excessive dietary intake (detrimental to Ca), leads to a reduced salivary excretion. This occurs when the food is poor in fibre and cannot stimulate the salivary utilization of this electrolyte (2, 3). So the surplus of this mineral is eliminated via renal excretion. This is promoted by a low Ca/P ratio that makes possible the higher urinary P content (17). The Ca/P ratio in our D diet was low (1:3); nevertheless, most authors attributed the high urinary excretion of P to the deficiency of dietary Ca (17). In the present study, the fractional excretion of P had a constant and significant decrease in both the periods 2 and 3 with the D diet. We could explain these unexpected values by considering that the % Cr/Ca did not showed any significant variation during the experiment, and there was not the right condition for calculi formation. The results obtained from other authors (2) demonstrated that it is very difficult to observe uroliths, if the Mg level is lower than 0.23 % and P level is lower than 0.46 % Digestible Dry Matter (DDM). The food composition analysis of our D diet gave a value of 0.32 % for Mg and 0.62 % for P. In conclusion we can affirm that the fractional clearances analysis contributes to renal damage evaluation. Particularly in our research, the variations observed for % Cr/Mg and % Cr/P is indicative of an alteration in the renal excretion.

Surely further investigation seems to be necessary to evaluate the effect of a dry diet with higher amounts of these electrolytes.

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DIETARY *ROSMARINUS OFFICINALIS* EXTRACT CAN MODULATE CALCIUM, BILIRUBIN AND LIPID METABOLISM IN BROILER CHICKENS

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ABSTRACT

A study was conducted to investigate the effects on the blood chemistry of broiler chickens after being fed diets supplemented with graded doses of rosemary essential oil (EO) during an 11 week period. Rosemary EO (Rosmarini aetheroleum of *Rosmarinus officinalis* L.) was dissolved in sunflower oil before the diet supplementation. Forty, one-day-old broiler chicks were divided into 5 groups: two controls (1 – basal diet, 2 – basal diet + sunflower oil) and three experimentals, in which the basal diet + sunflower oil were supplemented with rosemary EO (3 – 0.1%, 4 – 0.05% and 5 – 0.025%). Plasma biochemical parameters were determined by the colorimetric methods using spectrophotometric kits and a Reflotron spectrophotometer autoanalyzer at the end of the experimental feeding period. The presence of rosemary EO in the diets caused a dose-dependent decrease in the calcium plasma concentration. The alkaline phosphatase activity of all chicks fed the diet supplemented with 0.025% rosemary EO was significantly reduced compared to the other diets. There was a significant increase in the total bilirubin in birds fed the diet supplemented with 0.1% rosemary EO compared to the basal diet + sunflower oil. Dietary intake of 0.1% and 0.05% rosemary EO increased the plasma total lipids. It was concluded that the feeding of diets supplemented with rosemary essential oil could affect the calcium, bilirubin and lipid metabolism in broiler chickens.

Key words: blood biochemistry; essential oil; poultry; rosemary

INTRODUCTION

Essential oils are volatile, simple and complex compounds synthesized as secondary metabolites by all organs of aromatic plants. They can contain about 20–60 components at quite different concentrations. Essential oils are characterized by two or three major components at fairly high concentrations compared to the other components present in trace amounts. Generally, these major components determine the biological properties of the essential oil. They can act in a synergistic manner or operate to regulate one another (3). Greater attention on rosemary is being focused by those with special interest in herbs and spices as a source of biologically active compounds.

The results of published studies have demonstrated that biologically active compounds (mostly oxygenated monoterpenes) in rosemary essential oil exhibit cytotoxic properties. Fu *et al.* (8) reported that essential oils from clove and rosemary alone or in combination provided a significant antimicrobial effect against *Staphylococcus epidermidis, Escherichia coli* and *Candica albicans*. Similar results were reported by Agaoglu *et al.* (1). Bozin *et al.* (4) reported that the essential oil of rosemary exhibited significant antifungal activity. The antioxidant activity of the polar extracts of rosemary is related to their content of phenolic compounds (i.e., carnosol, carnosic acid). Some constituents in rosemary have shown a high degree of antioxidant activity as well as pharmacological activities in cancer chemoprevention and therapy in both *in vitro* and *in vivo* models (1, 7 and 18). A mechanism of action that may be responsible for the impairment of the carcinogenesis initiation by rosemary extracts, may be related to the metabolism of

chemical carcinogens which may generate inactive metabolites. Ethanolic extract of the leaves of *Rosmarinus officinalis* has been shown to have antidiabetic properties in rabbits (2).

Some cultural traditions have advocated the use of several herbal species in the treatment of certain cognitive deficits, such as those associated with ageing. Sanders *et al.* (14) reported that rosemary essential oil has the main action of stimulating the nervous system under sympathetic control, leading to increases in memorizing and concentrating abilities. It was determined that Rosemary essential oil was capable of affecting rumen microbial fermentation and this fact may allow for the manipulation of rumen fermentation to improve animal performance (5).

There is a dearth of published reports regarding the effects of dietary rosemary essential oil on the blood biochemistry of broiler chickens. Therefore, the objective of this current study is to investigate the effects of different doses of *Rosmarinus officinalis* essential oil in the diet on the blood biochemistry of broiler chickens.

MATERIALS AND METHODS

Forty, one-day-old female broiler chicks of a commercial strain Ross 308 (Párovské háje, Slovakia) were randomly distributed into five groups of 8 chicks each. The birds were placed in large pens with wood shaving. Rearing of the chickens started with a lighting regimen of 23 h of light to 1 h dark and lasted for 4 weeks. The initial room temperature of 32–33 °C was reduced weekly by 1 °C to a final temperature of 28 °C.

All birds had free access to water and feed. All birds received the basal diet for chicks HYD-04/a for 6 weeks and then they were fed diet HYD-04/b for 5 following weeks (BIOFER, Prešov, Slovakia). The composition of the basal diets formulated by the manufacturer is given in Table 1. The first group was fed a basal diet. The sunflower oil was added to the basal diet in a 1% concentration for the second group. The third, fourth and fifth groups consumed the same diets as the second group with different concentrations of rosemary. The essential oil was diluted with sunflower oil before preparing the diet supplementations. The final concentration of sunflower oil was 1 % of the diet and the final concentrations of rosemary essential oil were 0.1 %, 0.05 % and 0.025 %, respectively. The composition of the essential oil from rosemary leaves (Rosmarini aetheroleum of *Rosmarinus officinalis* L.) of Spanish type reported by the producer Calendula a.s., Nová Ľubovňa, Slovakia, was as follows: α-pinene (18–26 %), camphene (8–12 %), β-pinene (2–6 %), β-myrcene (1.5–5 %), limonene (2.5–3 %), cineole (16–25 %), p-cymene (1–2.2 %), camphor (13–21 %), bornyl acetate (0.5–2.5 %), α-terpeneol (1–3.5 %), borneol (2–4.5 %), and verbenone (0.7–2.5 %). The animals of both controls and experimental groups were fed their diets for 11 weeks.

At the age of 11 weeks all chickens were anaesthetized with intraperitoneal injection of xylazine (Rometar 2, Spofa, Czech Republic) and ketamine (Narkamon 5, Czech Republic) at a dose of 0.6 and 0.7 ml.kg^{-1} of body weight, respectively. After laparotomy, blood was collected by intracardial puncture into heparinized vials and centrifuged for plasma specimens at 1 180 g for 15 min. Samples of plasma for analysis were frozen and stored at –65 °C. The plasma levels of potassium, uric acid, glucose, cholesterol, and activity of alkaline phosphatase were determined using a REFLOTRON spectrophotometer autoanalyzer (Boehring Manheim, Germany). The concentrations of bilirubin, total protein and calcium were determined using the commercial kits BIOLA-test (PLIVA-Lachema, Brno, Czech Republic) and spectrophotometer GENESYS 10 UV (Thermo Electronic Corporation, USA). The concentrations of magnesium, phosphorus, triglycerides and total lipids were measured using the commercial kits purchased from RANDOX Lab. (Crumlin, UK) and using a spectrophotometer GENESYS 10 UV (Thermo Electronic Corporation, USA).

The results are expressed as the mean ± SEM. The statistical significance was performed by a one-way analysis of variance (ANOVA) with the *post hoc* Tukey multiple comparison test.

The experiments were carried out in accordance with the established standards for the use of experimental animals. The protocol was approved by the local ethic and scientific authorities.

Table 1. Composition of basal diets for chickens

Ingredient	HYD–04/a (0–6 weeks)	HYD–04/b (7–11 weeks)
Calculated values		
Metabolizable energy (MJ)	11.9	11.5
Crude nitrogen (g)	195.0	175.0
Lysine (g)	10.5	8.0
Methionine and Cystine (g)	7.5	7.0
Methionine (g)	4.0	3.5
Calcium (g)	8.0	8.0
Phosphorus (g)	5.0	5.0
Natrium (g)	1.2–2.5	1.2–2.5
Linoleic acid (g)	8.0	8.0

Vitamin-mineral premix provided per kilogram of diets HYD–04/a and HYD–04/b: vitamin A, 10 000 and 8 000 IU, respectively; cholecalciferol, 2 000 and 1 500 IU, respectively; vitamin E (α-tocopherol) 15 and 12 mg, respectively; vitamin K 2 mg; thiamin 4 mg; riboflavin 4 mg; pyridoxine 4 mg; cobalamine 10 mg; biotin 0.2 mg; folic acid 1 mg, niacin 40 mg; betain 200 mg; iodine 0.8 mg; panthotenic acid 15 mg; selenium 0.1 mg; cobalt 0.2 mg; manganese 70 and 50 mg, respectively; iron 60 mg; copper 6 mg; zinc 50 mg

RESULTS

As it is shown in Table 2, the feeding of diets supplemented with rosemary essential oil decreased plasma calcium concentration and its effects on calcium levels appeared to be dose-dependent. Animals fed diets supplemented with 0.025 % rosemary essential oil had increased plasma phosphorus levels compared to chicks fed the diet supplemented with 0.1 % rosemary essential oil and the group fed the basal diet. Plasma alkaline phosphatase activity of chicks fed the diet supplemented with 0.025 % rosemary essential oil was lower than those fed the diets supplemented with 0.1 % or 0.05 % rosemary essential oil and the second group with sunflower oil. The chicks fed the diet with sunflower oil had increased plasma alkaline phosphatase activity compared to the basal diet. Plasma potassium and magnesium were not affected by the different diets.

The effect of *Rosmarinus officinalis* on blood plasma biochemical parameters are presented in Table 3. Plasma uric acid was not altered by the different diets. Chicks fed diets supplemented with 0.1 % and 0.05 % rosemary essential oil and sunflower oil had increased plasma total lipid concentrations compared to the basal diet. Plasma cholesterol and triglycerides concentrations were not affected by the different diets. The supplementation of rosemary essential oil to the diets also had no effect on plasma glucose concentrations. Plasma total bilirubin concentration of chicks fed the diet supplemented with 0.1 % rosemary essential oil was higher than those fed the basal diet with sunflower oil. Chicks fed the diet supplemented with 0.05 % rosemary essential oil had increased plasma total protein concentrations compared to the basal diet and the basal diet with sunflower oil.

DISCUSSION

Dietary addition of rosemary essential oil to broiler chickens for 11 weeks caused a significantly lower plasma calcium level. Its effect on plasma calcium levels tended to be dose-dependent. Chicks fed the diet supplemented with 0.025 % rosemary essential oil had decreased alkaline phosphatase activity compared to other diets (except that of the basal diet).

The results of several studies demonstrate that essential oils and their monoterpene components affect bone metabolism when added to the food of animals. Mühlbauer *et al.* (12) tested the effects of some common herbs (sage, rosemary and thyme) and their constituent essential oils and monoterpenes on bone resorption in ovariectomized rats. Bone resorption was inhibited by the addition of 1g of powdered leaves of each herb. The essential oil extracted from sage and rosemary also inhibited bone resorption. Pure components from the essential oils were also studied. A mixture of the four major monoterpenes which occur in sage oil (thujone, eucalyptol, camphor and borneol) in proportion similar to that found in the natural oil was shown to inhibit bone resorption in a similar manner to unmodified sage oil extract. It is thought that these monoterpenes act directly on bone cells (not via the stimulation of calcinotropic hormones) and the mode of action is thought to be via the inhibition of mevalonate pathway and the prenylation of small G-proteins such as Ras, Rho and Rac (12). The same research group previously showed that herbs such as dill, Italian parsley and common parsley inhibited bone resorption in rats at a dose of 1 $g.day^{-1}$ (11).

Dolder *et al.* (6) studied the effects of monoterpenes present in aromatic plants on activity and formation of

Table 2. The effect of feeding diets supplemented with rosemary essential oil on blood plasma mineral indices and ALP activity in broiler chickens

Group	1	2	3	4	5
	Basal diet	Basal diet + 1 % Sunflower oil	Basal diet + 0.1 % Ros. ess. oil	Basal diet + 0.05 % Ros. ess. oil	Basal diet + 0.025 % Ros. ess. oil
Potassium ($mmol.l^{-1}$)	7.1 ± 0.13	6.9 ± 0.05	7.2 ±.11	7.10 ± 0.16	7.20 ± 0.16
Calcium ($mmol.l^{-1}$)	2.2 ± 0.05^{c}	2.4 ± 0.07^{abd}	1.7 ± 0.07^{cdef}	2.0 ± 0.04^{bf}	2.1 ± 0.05^{ae}
Magnesium ($mmol.l^{-1}$)	0.96 ± 0.01	0.87 ± 0.04	1.1 ± 0.08	1.1 ± 0.08	1.1 ± 0.1
Phosphorus ($mmol.l^{-1}$)	1 ± 0.12^{a}	1.3 ± 0.04	1.1 ± 0.08^{b}	1.1 ± 0.08	1.4 ± 0.08^{ab}
Alkaline phosphatase ($\mu kat.l^{-1}$)	14 ± 0.04^{ace}	22 ± 1.2^{ab}	25 ± 1.7^{ef}	28 ± 1.8^{cd}	16 ± 0.79^{bdf}

Significant differences within a row are indicated by the same superscript letter, $P < 0.05$; mean ± SEM, n = 8

Table 3. The effect of feeding diets supplemented with rosemary essential oil on blood biochemistry in broiler chickens

Group	1	2	3	4	5
	Basal diet	Basal diet + 1 % Sunflower oil	Basal diet + 0.1 % Ros. ess. oil	Basal diet + 0.05 % Ros. ess. oil	Basal diet + 0.025 % Ros. ess. oil
Total protein ($g.l^{-1}$)	70 ± 0.98^{ad}	72 ± 0.75^{b}	76 ± 1.5^{d}	80 ± 1.0^{abc}	74 ± 1.2^{c}
Uric acid ($\mu mol.l^{-1}$)	210 ± 7.9	220 ± 9.5	240 ± 14	210 ± 18	210 ± 8.6
Cholesterol ($mmol.l^{-1}$)	3 ± 0.09	3.2 ± 0.11	3.2 ± 0.09	3.1 ± 0.08	3 ± 0.05
Triglycerides ($mmol.l^{-1}$)	2.6 ± 0.11	2.7 ± 0.11	2.5 ± 0.04	2.5 ± 0.06	2.5 ± 0.03
Total lipids ($g.l^{-1}$)	3.58 ± 0.82^{bdf}	4.32 ± 0.17^{af}	4.48 ± 0.02^{de}	4.83 ± 0.01^{bc}	3.57 ± 0.0^{ace}
Glucose ($mmol.l^{-1}$)	18 ± 0.38	18 ± 0.23	17 ± 0.69	17 ± 0.22	17 ± 0.53
Bilirubin ($\mu mol.l^{-1}$)	4.4 ± 0.36	3.8 ± 0.17^{a}	5.4 ± 0.32^{a}	4.4 ± 0.2	4.8 ± 0.12

Significant differences within a row are indicated by the same superscript letter, $P < 0.05$; mean ± SEM, n = 8

osteoclasts. They reported that monoterpenes inhibited the activation of osteoclasts at high concentrations but inhibited their formation at much lower concentration.

Recently Jeong *et al.* (9) studied the anabolic activities of 89 natural compounds by measuring the amount of newly incorporated calcium in the differentiation process of mouse osteoblastic MC3T3-E1 subclone 4 cells. A low concentration of 3-carene, which is bicyclic monoterpene in essential oil extracted from pine trees, was shown to stimulate significantly the activity and expression of alkaline phosphatase, an early phase marker of osteoblastic differentiation, on differentiation day 9. On day 15, it dramatically promoted the induction of calcium in a dose-dependent manner. Authors suggested that the use of natural additions to the diet including essential oils should have a beneficial effect on bone health.

Although these studies report apparently contradictory data, they should not be considered to be mutually exclusive, since bone metabolism is a complex multifactorial process and it is known that some stimuli can give rise to anabolic or catabolic effects depending on the dosage, timing, and site of application.

In the present experiments, there was a significant increase in plasma total bilirubin in birds fed the diet supplemented with 0.1 % rosemary essential oil compared to the basal diet with sunflower oil. There are no published reports of the effects of feeding diets supplemented with rosemary essential oil on bilirubin level in the blood. An increase in the total bilirubin level in plasma in the current study might be explained by an abnormality of uridine diphosphate glucuronosyltransferase enzyme (UDP-glucuronosyltransferase). This liver enzyme is essential to the disposal of many exogenous and endogenous substances including bilirubin.

There are many reports concerning the alteration of hepatic UDP glucuronosyltransferase by various xenobiotics. Watkins and Klaassen (19) examined the effects of xenobiotics on UDP-glucuronosyltransferase activity in rats. They reported that monoterpene borneol reduced UDP-glucuronic acid (UDPGA) by 85–90 %. Further evidence supporting these results can be seen in the results of Siraki *et al.* (15), who investigated the physiochemical requirements of 21 nonsteroidal anti-inflammatory drugs (NSAIDs) for glucuronidation and toxicity in isolated rat hepatocytes. They determined that borneol inhibited glucuronidation of NSAIDs which resulted in the increased NSAID cytotoxicity. This suggests that acyl-glucuronide metabolites are acutely less cytotoxic.

Glucuronidation of carboxylic-acid-containing drugs, however, can yield reactive acyl (ester-linked) glucuronide metabolites that are able to modify endogenous molecules. Recent research has shown that several carboxylic acid drugs are genotoxic in isolated mouse hepatocytes, and that DNA damage is prevented by the glucuronidation inhibitor, borneol. Similar results were reported in human cells by Southwood *et al.* (17).

Rosemary (*Rosmarinus officinalis L.*) is used in traditional Turkish folk medicine for the treatment of hyperglycaemia. Bakirel *et al.* (2) investigated the possible effect of ethanolic extract of the leaves of *Rosmarinus*

officinalis on glucose homeostasis in rabbits. The results of their studies showed that ethanolic extracts of the leaves of *Rosmarinus officinalis* (L.) lowered blood glucose level in both normoglycaemic and glucose-hyperglycaemic rabbits. The repeated administration of rosemary extract to alloxan-diabetic rabbits led to a decrease in the blood glucose level and a significant increase in serum insulin levels. Unlike the results of Bakirel *et al.* (2), our data showed that the supplementation of rosemary essential oil to the diets of chickens had no effect on plasma glucose concentration. The reasons for these discrepancies might be due to differences in species in glucose metabolism, duration of the experiments or to concentrations and compositions of the rosemary essential oil.

In the present study, the dietary intake of 0.1 % and 0.05 % rosemary essential oil increased plasma total lipids while cholesterol and triglycerides were not affected by the experimental diets.

To the best of our knowledge, no other studies to date have reported the effects of feeding diets supplemented with rosemary essential oil on blood lipids in broiler chicks. However, there are many reports with contradicting results concerning the effects of diets supplemented with natural products on lipid metabolism in different species. Lee *et al.* (10) demonstrated that feeding thymol, cinnamaldehyde and commercial preparations of essential oil components (CRINA® Poultry) to female broilers had no effect on the plasma lipid concentrations. In an earlier report by Sodimu *et al.* (16), they had shown that garlic oil prevented an increase of cholesterol, triglycerides and total lipids both in serum and in other tissues. A mechanism of action suggested was inactivation of thiol group enzymes HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase) and CoA-SH (coenzyme A), the rate limiting enzyme for cholesterol biosynthesis and multi-enzyme complex for fatty acid biosynthesis. In agreement with this study, Mükherjee *et al.* (13) reported a reduction in increased serum level of cholesterol when ovariectomized rats were fed oil extract of garlic.

CONCLUSION

In conclusion, our results demonstrate that feeding diets supplemented with rosemary essential oil to broiler chickens could be an important means of reducing the incidence of metabolic bone diseases, or at least reduce the extent of mineral loss from bones, as exhibited by the reduced plasma calcium level. Additionally, the rosemary extract may alter the detoxification of the metabolites through conjugation and metabolism of total lipids by broiler chickens.

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IMMUNOHISTOCHEMICAL DEMONSTRATION OF NERVE FIBRES IN THE VESICULAR GLAND OF THE BULL

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ABSTRACT

The nerve fibres in the vesicular gland of the bull were studied and localized by immunohistochemical methods. The S-100 protein and acetylated α-tubulin (AT) antibodies were used to detection and localize the nerve fibres. Both antibodies demonstrated thick nerve bundles in the adventitia and in close association with larger blood vessels. The circular muscle layer contained rich accumulations of the nerve fibres among the smooth muscle cells. A few thick bundles of the nerve fibres were observed to enter the propria of the gland via the connective tissue septa. Accumulations of fine nerve fibres were seen around the glandular secretory tubules and alveoles as well as around the intralobular ducts. A tightly woven subepithelial nerve plexus formed a layer below the epithelial cells. Short branches of the nerve fibres penetrated into the basal zone of the secretory epithelial cells. Positive immunoreactions to S-100 protein and acetylated α-tubulin were observed among the basal cells of secretory alveoles.

Key words: bull; immunohistochemistry; nerve fibres; vesicular gland

INTRODUCTION

In the bull (*Bos taurus*), anatomically, the vesicular gland (syn. seminal vesicle) is a compact, lobulated organ and histologically it is characterized as a paired compound tubuloalveolar gland. The vesicular gland is surrounded by a capsule of dense connective tissue containing a few smooth muscle cells. The capsule is covered by either a tunica serosa or tunica adventitia. A tunica muscularis of varying width and arrangement surrounds the organ proper and encloses the propria-submucosa. The interlobular septa are predominantly muscular and divide the parenchyma into lobes and lobules. Intralobular secretory ducts drain the slightly coiled tubular portions of the gland. The basal cells have large lipid droplets and glycogen (6).

A combination of light microscopic, neurohistochemical, immunohistochemical and electron microscopic techniques has been used to study the distribution of autonomic nerves in the vesicular gland. The innervation of the vesicular gland has been studied in the human (14, 17, 18) and various laboratory animal species (5, 7, 8, 11, 13, 15, 16). In large domestic animals, the innervation of the vesicular gland has not been studied very often. Arrighi and Domeneghini (1, 3, 4) studied the localization of regulatory peptides in the male urogenital apparatus of cattle and horses.

Compared with the distribution of adrenergic and cholinergic nerves in the prostate, marked differences in the density of the innervation were detected in human seminal vesicles (18). Lange and Unger (12) in their immunohistochemical study demonstrated the presence of neuropeptide Y and vasoactive intestinal peptide (VIP) immunoreactivity in nerve fibres in human prostate gland and seminal vesicle. Abou-Elmagd *et al.* (1) studied the ultrastructure and innervation of Water Buffalo (*Bubalus bubalis*) seminal vesicles and found dense networks of unmyelinated nerve fibres in the muscular layer as well as in the subepithelial layer of secretory tubules and ducts. Observations of the Kaleczyc *et al.* (10) in the boar showed that their seminal vesicles are the most abundantly innervated organ in comparison with other accessory genital glands. Falck *et al.* (8) and Al-Zuhair *et al.* (2) in the guinea-pig seminal vesicles demonstrated peripherally located

adrenergic neurons innervating the vas deferens and the seminal vesicle. These authors noted differences in the density of the innervation compared with the distribution of adrenergic and cholinergic nerves in the prostate.

The aim of this paper was to study the distribution of the nerve fibres in the vesicular gland of the bull by immunohistochemical methods.

MATERIAL AND METHODS

Tissue samples of five adult bulls (*Bos taurus*) were used in this study. The vesicular gland was dissected out at the local slaughterhouse immediately after death. Samples of the tissue were fixed in 10 % neutral formalin in 0.2 mol phosphate buffer for 24 h and thereafter routinely embedded in paraffin. Sections of 5 μm thickness were cut and mounted on 3-aminopropyltriethoxysilane (APES)-coated slides. Consecutive sections were used for histological, control and immunohistochemical procedures.

Immunohistochemistry

For immunostaining procedures, histological sections were deparafinized, rehydrated, and pretreated with 3 % H_2O_2 in methanol to block endogenous peroxidase activity. They were then preincubated with 2 % goat serum to mask unspecific binding sites. Washed sections were incubated overnight with monoclonal anti-acetylated α-tubulin antibody (mouse IgG2b, clone 611 B-1, Sigma) and polyclonal anti-S-100 protein (rabbit, IgG, Sigma). The sections were washed in phosphate-balanced salt solution (PBS) and incubated with biotinylated secondary antibody for 30 min. The sections were then washed in PBS and incubated with avidin-biotin-peroxidase complex according to Hsu *et al.* (9) (Vectastain ABC kit; Vector, Burlingame, USA). After washing with PBS, peroxidase activity was visualized with diaminobenzidine (DAB) and H_2O_2 in TRIS buffer within 5 min at room temperature. Sections were counterstained with Mayer's hematoxylin. For negative controls, the first antibody was substituted by PBS.

RESULTS

In light microscopic sections the capsule consisted of dense irregular connective tissue. The muscular coat consisted of largely circularly orientated smooth muscle cells with an additional longitudinal layer. The interlobular septa were predominantly muscular, derived from the muscular layer. The highly vascularized loose connective tissue of the propria-submucosa was continuous with dense connective tissue trabeculae, which subdivided the organ into lobes and lobules.

After immunostaining, with both S-100 protein and AT, thick nerve bundles were observed in the tunica adventitia, whereas fine nerve fibres occurred in close association with blood vessels present in this layer. In the muscular layer a dense network of myelinated nerve fibres was seen to be distributed among the smooth muscle cells (Fig. 1). More nerve fibres were seen in the area where smooth muscle cells formed bands. Thicker nerve bundles traversed the muscular layer to reach the mucosa-submucosa layer (Fig. 2).

Unmyelinated and myelinated fine nerve fibres were seen traversing the connective tissue of the mucosa layer. Accumulations of nerve fibres were seen next to small blood vessels (Fig. 3). The nerve fibres accumulated around glandular secretory alveoles and tubules and around

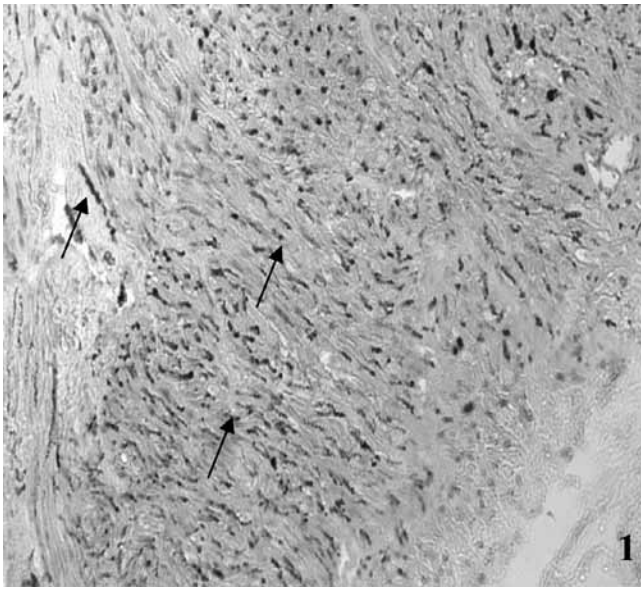

Fig. 1. Muscular layer of the vesicular gland. Numerous acetylated α-tubulin-positive nerve fibres are seen inside the muscle layer (arrows). Magn. × 100

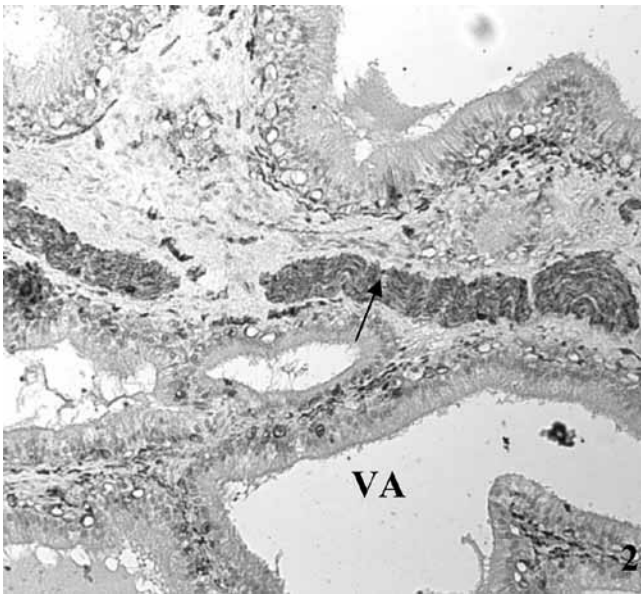

Fig. 2. Connective tissue septa with vesicular alveoles (VA). S-100 protein-positive thick nerve bundle (arrow). Magn. × 100

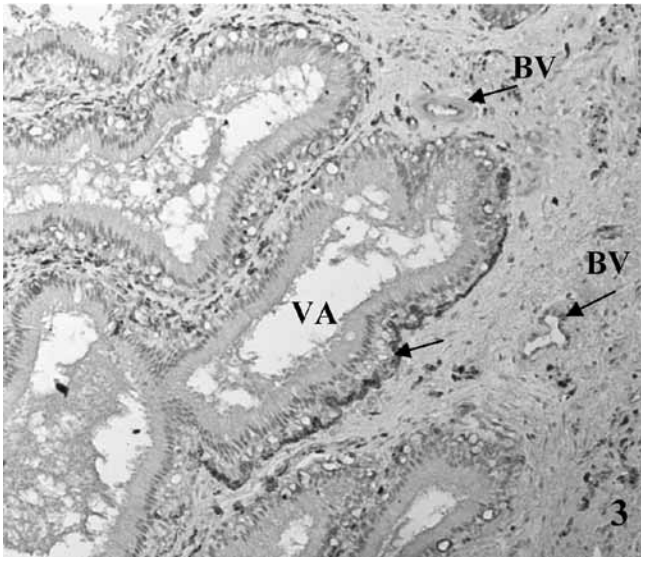

Fig. 3. Vesicular alveoli (VA) and the connective tissue septa of the gland. S-100 protein-positive fine nerve fibres are present in vicinity of blood vessels (BV) and between neighbouring secretory alveoles (arrows). A continuous layer of the nerve fibres is seen on the basal side of the secretory alveoles. Magn. × 100

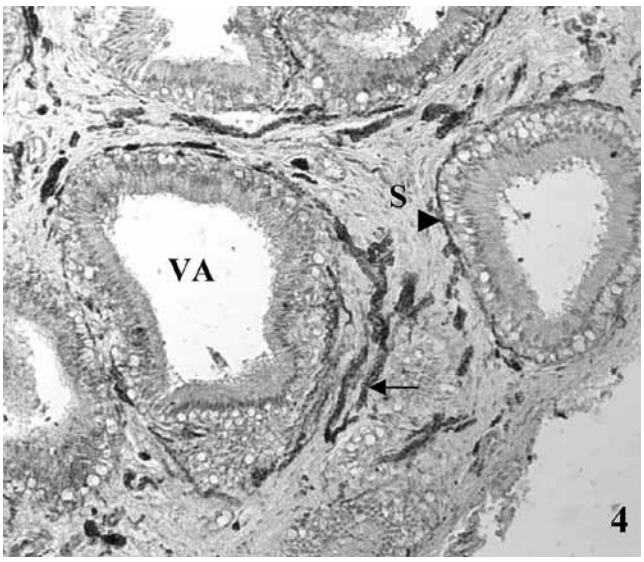

Fig. 4. Vesicular alveoles (VA) and the connective tissue septa (S). Numerous acetylated a-tubulin-positive bundles of the nerve fibres run among the secretory alveoles (arrows). Fine nerve fibres are seen in close vicinity to secretory cells (arrowhead). Magn. × 100

intralobular ducts. These fibres formed the nerve plexus in the space between neighbouring tubules and alveoles. Below the secretory epithelial lining of the alveoles and ducts a tightly woven subepithelial layer of nerve fibres was observed (Fig. 4). From this layer short branches of acetylated α-tubulin positive nerve fibres were seen to penetrate into the basal zone of the secretory epithelial cells which contained numerous lipid droplets. Besides the endothelium of the blood and lymphatic vessels, some smooth muscle cells present in the muscle layer were markedly positive for S 100 protein.

DISCUSSION

In the present study a dense concentration of the nerve fibres was found in the muscle layer and in the muscosal layer of the bull seminal vesicles. This rich nerve plexus which we observed corresponds to the observations of Abou-Elmagd *et al.* (1) in Water Buffalo (*Bubalus bubalis*) seminal vesicles. They found dense networks of unmyelinated nerve fibres in the muscular layer as well as in the subepithelial layer of the secretory tubules and ducts. Dense plexus of noradrenergic nerves throughout the muscle coat of the seminal vesicles was found also in guinea pigs by Al-Zuhair *et al.* (2). These authors described numerous terminal nerve regions. We also observed numerous terminal nerve regions very close to adjacent smooth muscle cells in the bull. Additionally, we observed a rich plexus of positive nerves in the circular muscle layer. On the contrary, only a few nerves were found in the outer longitudinal layer. According to Abou-Elmagd *et al.* (1) the muscular contraction in the seminal vesicle is predominantly under the influence of the sympathetic nervous system.

Very few noradrenergic nerves were found in the mucosa-submucosa layer of the seminal vesicle of the water buffalo; those which were observed usually accompanied the vascular supply. In contrast, a rich AchE-positive nerve network was observed in the submucosa of both the seminal vesicles and the ductus deferens (1). Electron microscopic studies revealed numerous groups of submucosal axons. In the bull vesicular gland a few thick nerves were observed in the submucosa. On the other hand, numerous thin bundles of nerve fibres as well as individual fibres were seen closely associated with the blood vessels, which corresponds to the noradrenergic nerves described by Abou-Elmagd *et al.* (1).

Around the glandular secretory tubules and below the epithelial lining of the glandular duct a tightly woven subepithelial nerve plexus with short branches to the basal zone of the epithelium was observed. In the seminal vesicles of the Water Buffalo a dense network of unmyelinated nerve fibres in the subepithelial layer of secretory tubules and ducts has been described (1). The epithelial nerves of this network were devoid of Schwann cells and basal lamina (naked axons) and were situated within the intercellular spaces between the principal and basal cells. In our study, the axons marked by acetylated α-tubulin were occasionally seen to enter in between the basal cells of the vesicular gland of the bull.

Lange and Unger (12) in their study of the human seminal vesicles, observed the peptidergic innervation to be generally moderate to low. Although neuropeptide Y (NPY) and VIP-immunoreactive fibres with prominent peptidergic fibre networks were localized in the subepithelial

connective tissue, most NPY-immunoreactive fibres were observed in the musculature of the seminal vesicles. In addition, NPY-and VIP-immunoreactive fibres were demonstrated in the walls of the blood vessels. Also, Tainio (17) who investigated by immunohistochemical methods the innervation of the human prostate, seminal vesicle and vas deferens, described that neuropeptidergic innervation of all organs studied was very dense. The author also found that neuropeptide Y-and tyrosine hydroxylase-positive nerve fibres were most abundant and localized mainly in the smooth muscle layer. On the contrary, vasoactive intestinal polypeptide-positive nerve fibres were mainly found beneath the epithelium.

In the human seminal vesicles and prostate, the VIP nerve fibres were equally distributed in both mucosal and muscular layers, whereas the catecholaminergic nerve fibres were mainly distributed in the smooth muscle layer. These results suggest that the VIP nerves play important roles in vascular reaction and epithelial activities, while the catecholaminergic nerves are involved in smooth muscle actions. The observations of Park *et al.* (14) suggest that the innervation of the seminal vesicle by various neuroactive peptides may be involved in the autonomic regulation of this organ. The presented data suggest that adrenergic innervation may be deeply involved in the control of blood flow and motor function of the organs studied.

Several studies have indicated that cholinergic and adrenergic innervation of the seminal vesicle is predominantly localized in the submucosal parts of this organs, whereas comparatively few fibres supply the smooth muscle cells. Recent studies in several mammals also demonstrated a peptidergic innervation of seminal vesicles. Pharmacological studies have indicated that peptides may play a role, together with adrenergic and cholinergic innervation, in the physiological regulation of secretory activity, muscle contractility and blood supply of the urogenital tract.

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THE USE OF DEXMEDETOMIDINE WITH BUTORPHANOL FOR SEDATION DURING HIP AND ELBOW DYSPLASIA RADIOLOGICAL EXAMINATION OF DOGS

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ABSTRACT

Some diagnostic radiological projections require appropriate myorelaxation and sedation of the patients for various durations. Based on these requirements we used short-term sedation induced with a combination of xylazine and butorphanol. In relation to the application of new radiological techniques, enabling more detailed, complex examination of the investigated area or bigger number of projections (e. g. early diagnosis of hip joint dysplasia), we have increasingly used new preparations and their combinations to attain high quality and prolonged sedation. This was the motivation to evaluate the practical use of various combinations of preparations, such as dexmedetomidine and butorphanol. This study was carried out on 18 dogs which were administered dexmedetomidine and butorphanol intravenously (i.v.). The dogs were observed for the rapidity of sedation onset, heart and respiration rate, presence of palpebral eye reflex, capillary filling time and colour of the mucous membranes. We also evaluated subjectively the level of myorelaxation and the responses to painful or acoustic stimuli. The mean onset of clinically important sedation following i.v. administration of the combination was approx. 4.1 min and the mean period of arising after atipamezol antagonisation reached 17.3 min. We recorded a decrease in respiration rate within 10 min after administration, ranging between 9.35% and 21.5% and a decrease in the heart rate ranging between 53.6% and 59.2% (in comparison to that immediately before administration). After administration of atipamezol, both the heart rate and the respiratory rate increased approximately after 30–35 minutes. The combination used ensured very good myorelaxation and sedation of the patients.

Key words: canine hip dysplasia; dexmedetomidine; dog sedation

INTRODUCTION

Medetomidine is one of the α_2 adrenoreceptor agonists, a racemic molecule having dextrorotatory (dexmedetomidine) and levorotatory (levomedetomidine) enantiomers. The dextrorotatory enantiomer is responsible for the pharmacological effect of this molecule. In clinical doses levomedetomidine is an inactive component having neither sedative nor analgetic effects in dogs (19). No influence of this enantiomer on adrenergic neuronal activity or blood pressure of rats was reported in previous studies(15).

Dexmedetomidine as a purer molecule exerts a lower load on metabolism than the racemic form and, at the same time, exhibits lower potential for pharmacological interactions with other substances. In contrast, levomedetomidine has a higher inhibitory effect on ketamine metabolism than dexmedetomidine (18) and lengthens the effect of dexmedetomidine in combination with ketamine.

Physiology of α_2 receptors

Adrenergic receptors are divided to α and β receptors on the basis of categorisation into a sequence according to the effectiveness of various natural and synthetic catecholamines in various preparations. There was a belief that the activation of one of the α or β adrenergic receptors had an excitatory effect in some tissues and inhibitory effect in other tissues (3, 20). Later on, it was observed that a subclass of α recep-

tors controlled the release of neurotransmitters. This allowed scientists to deduce that the location of the receptor is pre-synaptic (20). Classification of receptors on the basis of anatomic localisation is problematic as the α_2 receptors were found in both post-synaptic and extra-synaptic loci (10). One can assume the highest clinical importance of pre-synaptic α_2 receptors as they affect the release of epinephrine and ATP through a negative feedback.

The effect of α_2 receptors is mediated by activation of guanine-nucleotide regulatory binding proteins (so-called G proteins). The activated G proteins modulate cellular activity by signalling through second messengers or by modulation of ion canal activity. The activation through the second messenger system results in the inhibition of adenylate cyclase which, subsequently, causes decreased production of 3,5-cAMP. Specific, cAMP-dependent kinases modify the activity of target proteins by affecting their phosphorylation (9). Modulation of ion canal activity results in the hyperpolarisation of the cellular membrane. Potassium reflux through the activated canal causes hyperpolarisation of the membrane and acts as an effective means of suppression of nerve stimuli. Stimulation of α_2 receptor also suppresses the entry of calcium into the nerve endings which can result in its inhibitory effect on secretion of neurotransmitters. From the anaesthesiological point of view, neuronal hyperpolarisation is the key element in the mechanism of action of α_2 adrenoreceptor agonists (5).

Action of dexmedetomidine

Dexmedetomidine is an α_2 adrenergic agonist (complete on α_2 adrenergic receptors α_2A, α_2B, α_2C) exhibiting the highest adrenoroceptor selectivity. This selectivity is highly dependent on the administered dose (25). High selectivity to α_2 adrenoreceptors was observed in animals administered low to medium doses (10 to 300 $\mu g.kg^{-1}$) by slow infusion (25). Dexmedetomidine differs from other α_2 agonists by its selective affinity to receptors of α_2A subtype which results in much more effective sedation and analgesia (Tables 1 and 2).

Stimulation of pre-synaptic adrenoreceptors results in a decreased release of norepinephrine and thus results in arresting the spreading of pain. Stimulation of post-synaptic adrenoreceptors inhibits sympathetic activity and may lead to decreased blood pressure and heart rate.

Dexdomitor binds to pre-synaptic α_2 adrenoreceptors which prevail in the central nervous system. Inhibition of the release of noradrenalin and neutrotransmisssion induces sedation, analgesia and muscular relaxation. Regarding the intensity of action, dexmedetomidine is 1–2-fold stronger than medetomidine (19).

The α_2 receptors are present in the peripheral and central nervous systems, blood platelets and other organs, such as the liver, pancreas, kidneys and eyes. Stimulation of these receptors in the brain and the spinal cord inhibits transfer of stimuli among neurons, causing hypotension, bradycardia, sedation and analgesia. Other organs respond to such stimulation by decreased salivation, decreased intestinal secretion and motility, inhibition of renin release, increased glomerular filtration, increased secretion of sodium and water in the kidneys, decreased intraocular pressure and decreased pancreatic release of insulin (12).

Table 1. The effect of dexmedetomidine on individual receptor types (8; 23)

Receptor subtype	Effect
α_2A	Central effect: analgesia, sedation, mild anaesthetic response Neuroprotective effect Spinal antinociception and synergy with opiates Arterial vasoconstriction
α_2B	Arterial vasoconstriction
α_2C	Venous vasoconstriction

Table 2. Selection coefficient for α_2 agonists (1)

Component	Selection coefficient (α_2 to α_1)
Dexmedetomidine	1300
Medetomidine	1200
L-medetomidine	23
Xylazine	160

Pharmacology

Dexdomitor is lipophilic and absorbs very well after intramuscular administration. It binds strongly to plasma proteins (> 90 %), penetrates rapidly through haemoencephalic barrier and its concentration in the central nervous system is several folds higher than that in plasma (experiment on rats). It is metabolised in the liver (in dogs by hydroxylation, conjugation with glucuronic acid and N-methylation, in cats by hydroxylation) and all its metabolites are inactive, primarily eliminated in the urine (95 % in humans, 82 % in dogs, 51 % in cats) and less in the faeces. The half-time of elimination is approximately 2 hours.

Pre-synaptic α_2 receptors are also stimulated by decreasing release of norepinephrine resulting in the decline of the blood pressure and heart rate (1, 2). Such effects may be observed in the post-operative period and may be very easily affected by administration of atropine, ephedrine or by infusions (17) which can prove harmful to patients with hypovolaemia. The extent of respiratory depression caused by dexmedetomidine is smaller compared to that of other sedatives (4).

The side effects include hypotension, hypertension, nausea, bradycardia, atrial fibrillation, hypoxia (11) and various atrioventricular blocs. The majority of these side effects occur during or immediately after administration of a medicinal bolus. Because of that, administration of dexmedetomidine to patients with cardiovascular or serious system diseases or to gravid or suckling animals and puppies up to 6 months and kittens up to 5 months of age is not recommended. Atipamezol may be used as an antagonist of dexmedetomidine.

Overdose may result in atrioventricular blockage of the first or second degree. When used in combination with isofluran or halothane, dexmedetomidine reduces the blood supply to the brain in the dogs by 30–45 % but without symptoms of cerebral ischaemia (14).

MATERIAL AND METHODS

Our study investigated the possibilities of the use of the combination of dexmedetomidine and butorphanol for the purpose of radiological examination of dogs for hip and elbow dysplasia.

Dexmedetomidine and butorphanol was administered to 18 dogs of 7 breeds (Slovakian hound, Bavarian bloodhound, Hungarian vizsla, Hovawart, Cairn terrier, American Staffordshire terrier and mongrel). There were 12 females and 6 males (66.6 %: 33.3 %). The mean age of the animals was 21.2 months (min. 7.5 months, max. 40 months) and the mean weight 19.6 kg (min. 8.5 kg, max. 32 kg). All experimental dogs were put into a deep sedation, induced by a combination of dexmedetomidine and butorphanol, and examined radiologically to diagnose hip joint dysplasia (HJD) and in some cases also elbow joint dysplasia.

After the i. v. cannulisation of the patients, immediately before X-ray examination, the dogs were administered dexmedetomidine i. v. at a dose of 300 $\mu g.m^{-2}$ body surface and subsequently butorphanol at a dose of 0.2 $mg.kg^{-1}$ body weight.

We observed: the onset of sedation; heart and respiratory rate; presence of palpebral eye reflex; CRT and the coloration of the mucous membranes. We also evaluated subjectively the degree of myorelaxation and response to pain and acoustic stimuli. During sedation the dogs received either Ringer's lactate or 0.9 % NaCl at a dose of 20 $ml.h^{-1}.kg^{-1}$body weight by infusion. After the onset of sedation (with the possibility of handling the patient including the sufficient myorelaxation of the pelvic limbs) we performed X-ray examinations lasting 25 min on average (min. 22 min, max. 35 min). After the examination, atipamezol at a dose of 0.05 $mg.kg^{-1}$ was administered as a dexmedetomidine antagonist.

Statistical evaluation was carried out using Student paired *t*-test (compared with the results obtained with the combination of xylazine and butorphanol, n = 20) or non-paired *t*-test. $P < 0.001$ was considered the level of very high significance, $P < 0.01$ level of high significance and $P > 0.05$ was considered an insignificant level.

RESULTS

The time of onset of the sedation after i. v. administration varied around 4.00 ± 1.56 min with the range from 90 sec to 7 min ($P < 0.0001$, statistical significance ***). When using the combination of xylazine and butorphanol (n = 20) the time from administration up to the onset

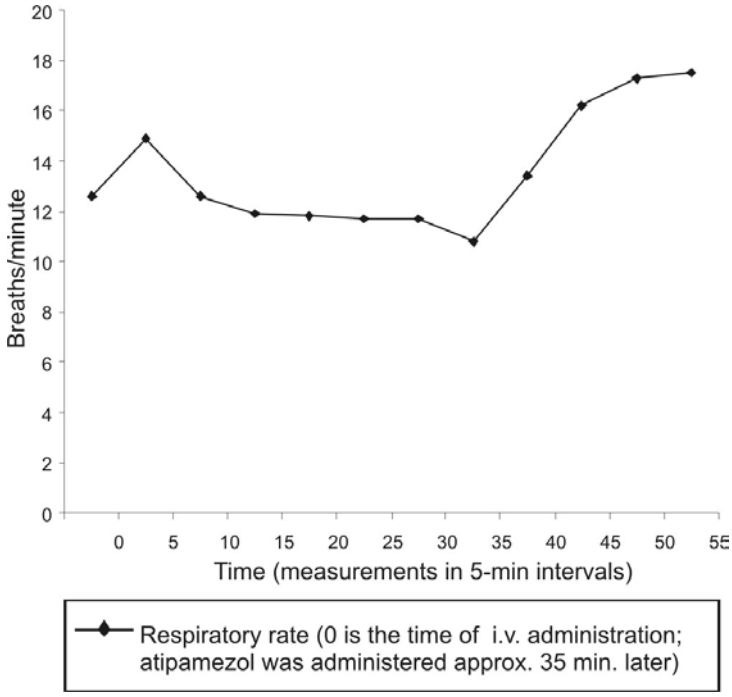

Fig. 1. Changes in respiratory rate after i.v. administration of dexmedetomidine and butorphanol

The first respiratory rate value was recorded immediately before i.v. administration of anaesthetics or during pre-anaesthesiological examination of the patient. The figure clearly shows a decrease in respiratory rate up to 10 min after administration which persisted almost in all patients (5.62 to 10.58 %) throughout the sedation. After administration of atipamezol, approx. at 30–35 min, the respiratory rate increased (mean respiratory rate during sedation was 12.25 ± 1.17).

P = 0.0008, very high significance (***) in comparison with change in respiratory rate in patients (n = 16) administered combination of xylazine and butorphanol (18.06 ± 5.03)

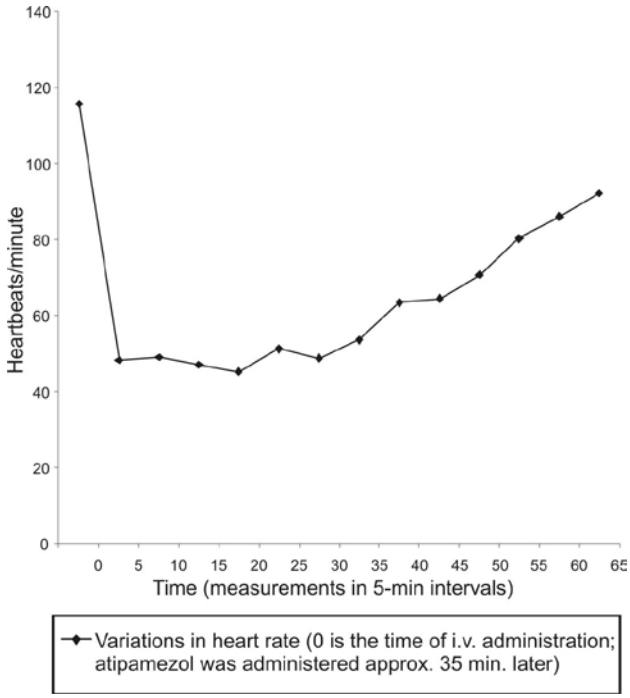

Fig. 2. Changes in heart rate after i.v. administration of dexmedetomidine and butorphanol
The first heart rate value was recorded immediately before i.v. administration of the respective compounds or during pre-anaesthesiological examination of the patient. A decrease in heart rate occurred 5 min after administration and persisted throughout the sedation (53.63 to 60.71 %). After administration of atipamezol, approx. at 30–35 min, the heart rate increased (mean heart rate during sedation was 61.60 ± 20.26). P = 0.0002, very high significance (***) in comparison with change in heart rate in patients (n = 12) administered combination of xylazine and butorphanol (95.08 ± 7.13)

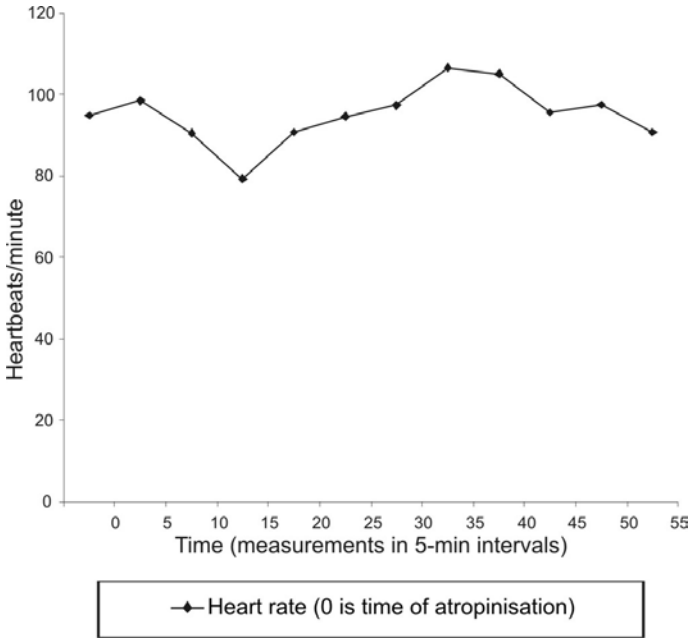

Fig. 3. Changes in heart rate after i. m. administration of xylazine and butorphanol (premedication of the patient with atropine at a dose of 0.03 mg.kg^{-1} body weight of the patient approx. 10 min before administration of anaesthetics

of sedation was 12.00 ± 4.22. Changes in the heart and respiratory rates during sedation are shown in Figures 1 and 2. For comparison, we present also changes in the heart and respiratory rates after i. m. administration of the combination of xylazine and butorphanol (Fig. 3).

The CRT value in all patients was in the range of 1–15 sec. The palpebral reflex was present in all patients throughout the sedation. Although within the period 5–15 min after administration we registered insufficient relaxation of masseters and increased resistance to opening of the mouth (6 patients – 33.33 %), myorelaxation of the patients, evaluated as the possibility to manipulate the pelvic limbs at positioning, was very good. No response of patients to manipulation or acoustic stimuli was observed (Tab. 3).

The following side effects were observed in patients under anaesthesia: mucosal anaemia (2 patients – 11.11 %); tongue cyanosis (3 patients – 16.67 %); accentuation of respiratory arrhythmia with apnoeic pauses lasting ap-

Table 3. Parameters observed in the duration of the effect of dexmedetomidine and butorphanol

Parameter	Value
Time of sedation onset	4.00 ± 1.56 min.
Heart rate	Decrease by 57.53 ± 1.664 % (period of 5 min after administration up to antagonisation)
Respiratory rate	Decrease by 8.10 ± 2.48 % (period of 10 min after administration up to antagonisation)
Palpebral reflex	Present
CRT	1–1.5 sec (no change during sedation)
Mucosa colouration	Pale pink to anaemic
Myorelaxation	Very good
Reaction to manipulation	No reaction
Reaction to acoustic stimuli	No reaction
Wake-up time after administration of atipamezol	17.28 ± 4.24 min
Time from application until the patient is able to leave	44.38 ± 6.88 (relatively)

prox. 20–30 sec (13 patients – 72.22 %); and bradycardia (17 patients – 94.44 %). The peripheral pulse at *a. femoralis* was in all cases easily palpable and strong.

The wake-up period after antagonisation with atipamezol (from s. c. administration until being able to leave) was on average 17.28 ± 4.24 min ranging between 10 min and 25 min (P = 0.0956, statistically insignificant (ns)).

In one case (5.56 %), we recorded waking up after 35 min following the administration of the antagonist and in another case (5.56 %) repeated onset of the sedation effect of dexmedetomidine was reported after 110 min when the patient returned home.

DISCUSSION

The first α_2 adrenoreceptor agonist was synthesized in early sixties of the 20th century and was used to decrease the hyperaemia of the nasal mucosa. The first administration of the new substance (clonidin) produced unexpected side effects, namely sedation lasting 24 hours and symptoms of serious depression of the cardiovascular system in patients (24). It should be stressed that the use of α_2 adrenoreceptor agonists as anaesthetics was not new because preparations based on xylazine or medetomidine had been used in veterinary medicine for a long time (7).

Some authors reported that the administration of dexmedetomidine caused more pronounced changes in the level of some parameter affecting the vital functions (19). A biphasic cardiovascular reaction was described after administration of dexmedetomidine (13). Administration of the initial dose of dexmedetomidine (1 $\mu g.kg^{-1}$) induces a short-term increase in blood pressure and reflexive decline in heart rate, particularly in young, healthy patients (6). Stimulation of the receptors in the smooth muscles of vessels causes this transient increase which could be alleviated by slow infusion. Even slower infusion of the solution resulted in an increase in the mean arterial pressure during the first ten minutes by up to 7 % and decrease in heart rate at the level of 16–18 % (13). The primary response of an organism lasts approx. 5 to 10 min and is followed by a slight decrease in blood pressure. Due to peripheral vasoconstriction and venous desaturation, the mucous membranes may appear pale or with a blue tinge. Such effects may be observed in the post-operative period and can be influenced by administration of atropine, ephedrine or infusion (17). However, these effects may be harmful to patients with hypovolaemia.

Rauš er *et al.* (22) failed to record deviations in the level of these parameters (e. g. decreased heart rate) and observed only insignificantly increased level of medium arterial pressure. Also other authors reported that dexmedetomidine had no direct effect on the heart function (16). The extent of respiratory depression caused by dexmedetomidine is smaller than that induced by other sedatives (4).

Our study showed a decreased respiratory and heart rate after the onset of the effects of the administered substances (dexmedetomidine and butorphanol). Because some authors reported that butorphanol, as a synthetic opioid, may induce in dogs, respiratory depression and a decline in blood pressure with reduced heart rate through vagus mechanism (13), it was impossible to determine whether it was dexmedetomidine or butorphanol which caused the observed changes. We did not form any control group of patients administered solely

dexmedetomidine.

Other studies also described effective and safe use of combination of dexmedetomidine with butorphanol or diazepam at X-ray examination (21).

CONCLUSIONS

The combination of dexmedetomidine as an α_2 agonist and butorphanol as a synthetic opioid appeared suitable for inducing deep sedations in patients subjected to X-ray examination for the purpose of the diagnosis of dysplasia of hip and/or elbow joints. The sedation of patients was very good, with excellent myorelaxation and without reactions to external stimuli (manipulation, sounds). Compared to the combination used in the past: xylazine, butorphanol administered intramuscularly, the new combination is advantageous due to the elimination of the waiting period between administration and onset of action (the total period from atropinisation until being able to leave was 64.25 ± 10.75 min) and also because of the possibility of using atipamezol as the dexmedetomidine antagonist which allows for a more rapid waking up.

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THE EFFECTS OF MILK INDICATORS OF SHEEP MAMMARY GLAND HEALTH STATE ON SOME MILK COMPOSITION AND PROPERTIES

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ABSTRACT

The milk quality and curds recovery depends on the health state of the mammary gland in all ruminants. Therefore, it is important to improve upon the knowledge about these various health parameters. The bulk milk samples (BMSs) of 60 sheep (Tsigai) were investigated. BMSs were obtained from the first two thirds of lactation during the winter, spring and summer seasons. The log somatic cell count (SCC) was 560 (geometric mean) and 949 ± 1,393 ths.ml^{-1}. It was higher ($P \leq 0.05$) and lower as compared to similar cow and goat milk data respectively. Log SCCs were correlated ($P \leq 0.05$; as linear or non linear regressions) to: fat 0.64; lactose –0.58; crude, true, whey protein fractions and casein from 0.38 to 0.57; total solids 0.70; fat/crude protein ratio 0.45; urea 0.52; citric acid –0.31; electrical conductivity 0.39; milk freezing point –0.62; pH acidity –0.51; titration acidity 0.26; Ca –0.46; Na 0.26; and Mg 0.40. Insignificant ($P > 0.05$) relationships of log SCC existed to casein number, specific mass, acetone, alcohol stability or cheeseability. The less desirable udder health (higher SCC) does not adversely affect the milk protein recovery and protein and fat values in such an essential way in sheep milk as compared to cow's milk. According to the results presented in this report and by noting the relevant relationship between the various parameters, it may be possible to improve the practical rules for better monitoring and prevention of milk secretion disorders, as well as to increase the protein recovery in sheep milk.

Key words: less favourable area; macroelement; microelement; milk; protein fraction; somatic cell count; sheep; technological properties

INTRODUCTION

With the Czech reduction of agriculture, there has been problems with exploitation of less favourable areas during the last twenty years. One of the ways of how to utilize the less favourable areas for economic productivity is to keep small ruminants in the most ecological way possible. Despite some known environmental risks, such as soil surface erosion, the keeping of small ruminants is an important part of organic agriculture and the wise use of livestock in less favourable areas. The importance of the alternative use of goat and sheep milk is increasing in the field of human nutrition. This is especially true in the groups of people with more susceptible health situations in terms of their specific nutrition and metabolic demands.

Sheep milk product consumption is not as traditional in the Czech Republic as compared to some countries like France, Spain and others. The sheep population has gone through important changes in the past twenty years. The total sheep population (sheep and rams) was reduced from 429,714 hs. (heads) in 1990 to 84,108 hs. in 2000 (a reduction of 80.4 %). The dairy sheep population includes 0.7 % of livestock breeding. Only recently (2006) there has been an increase in the livestock population to the extent of 148,412 hs. (3, 14). The keeping of small ruminants is found mainly in the less favourable areas. This is where 96 % of sheep population is kept.

All sheep milk is processed and virtually none is used for direct milk consumption. Sheep udder health is very important for milk processing and the over all health of the animal. Some production disorders such as mastitis can adversely influence milk quality which is important for wholesome milk products.

Table 1. Investigated milk indicators with relevant units

Abbrev.	Milk indicator	Unit	Note
DMY	daily milk yield	Kg of milk per day	
F	fat	$g.100g^{-1}$; %	
L	lactose	monohydrate; $g.100g^{-1}$; %	
SNF	solids non fat	$g.100g^{-1}$; %	
DM	dry matter (total solids)	$g.100g^{-1}$; %	
SCC	somatic cell count	$ths.ml^{-1}$	
F/CP	ratio between fat and crude protein		
U	urea	$mg.100ml^{-1}$	Photometrically
A	acetone	$mg.l^{-1}$	Photometrically
CA	citric acid	$mmol.l^{-1}$	Photometrically
AS	alcohol stability	ml	consumption of 96% ethanol to protein coagulation in 5 ml of milk
TA	titratable acidity according to Soxhlet-Henkel	ml 0.25 $mol.l^{-1}$ NaOH solution	used for the titration of 100 ml of milk
pH	actual acidity		log H ion concentration
MFP	milk freezing point	°C	
EC	electrical conductivity	$mS.cm^{-1}$	
RCT	rennet coagulation time	second (s)	bacterial enzyme Renilase was used for determination
CQ	curds cake quality	from 1st (excellent) to 4th (poor) class	subjective estimation of CQ by aspection and touch
CF	cheese curds firmness	cm	depth of penetration of corpuscle into curds cake after its fall under the standard conditions, measured value is in the opposite relationship to firmness
WV	whey volume	ml	obtained from curds cake during the process of enzymatic cheese coagulation (1 hour)
CP	crude protein (total nitrogen (N)×6.38)	$g.100g^{-1}$; %	
TP	true protein (protein N×6.38)	$g.100g^{-1}$; %	
CAS	casein (casein N×6.38)	$g.100g^{-1}$; %	
WP	whey protein (difference TP-CAS)	$g.100g^{-1}$; %	
NPNM	non protein nitrogen matters (CP-TP nitrogen ×6.38)	$g.100g^{-1}$; %	
UNR	ratio of urea N in non protein N	%	
CN-CP and CN-TP	casein numbers for CP and TP	%	ratio of casein in protein fraction
FAM-T	fermentation ability of milk	TA of yoghourt in ml of 0.25 $mol.l^{-1}$ $NaOH.100ml^{-1}$	yoghurt test with noble microbial culture
FAM-pH	actual acidity of yoghourt pH		
FAM-TCM	total count of the fermenting noble microorganisms	$CFU.ml^{-1}$	plate count
FAM-CL	FAM lactobacilli	$CFU.ml^{-1}$	plate count
FAM-CS	FAM streptococci	$CFU.ml^{-1}$	plate count
FAM-RSL	streptococci and lactobacilli ratio		all previous FAM indicators were measured after the yoghurt test fermentation
RIS	residues of inhibitory substances (mostly antibiotic drugs)	+ or – finding	by microbiological pH test with *Bacillus stearothermophillus* var. *Calidolactis*
Ca, P, Na, Mg, K	macroelements	$mg.kg^{-1}$	AAS
Mn, Fe, Cu, Zn, Ni	Microelements	$mg.kg^{-1}$	AAS
I	Microelement	$\mu g.l^{-1}$	Photometrically

Abbrev. – abbreviation, it is valid for all following tables; CFU – colony forming unit; AAS – atom absorption spectrophotometry

Also mastitis affects curds recovery as well. The previously cited sheep population increase is why it is important to improve the knowledge about possible effects of mammary gland health parameters (such as somatic cell count, lactose, electrical conductivity and natrium content) on milk composition and properties. The aim of this paper was to carry out the evaluation of the impacts of various milk indicators of udder health and on other milk quality factors in sheep.

MATERIALS AND METHODS

Breed and flock of animals, their keeping conditions, nutrition and milk samples

Bulk milk samples were collected monthly in one sheep flock (C, Tsigai; n = 60 bulk milk samples) for three years (2005, 2006 and 2007) in the winter, spring and summer seasons. The flock was kept at an altitude of 572 m above sea level with a total precipitation of 1,200 mm and a mean air temperature of 3.7 °C, which means under typical climate conditions in the submountain areas in the Czech Republic. The flock was located in the less favourable areas (18). The nutrition of the animals was carried out in a typical way under the Czech Republic conditions. Sheep were fed by the natural grass and herb pasture and by the grain supplement with a daily ration 0.3 kg per head (mixture of barley, maize, wheat and rape seed oil and mineral components). All sampled animals were in the first two thirds of their lactation and had typical milk yields for this breed under country conditions (0.36 kg milk per day). All animals were milked twice a day by machine milking (milking parlour, side by side milking equipment, Alfa Laval). Sixty sheep were kept on deep straw bedding in a free stable. The same investigation was carried out in a goat herd (White short-haired), which were kept in the same stable under completely identical conditions (6, 7). Similar investigation in terms of sampling was carried out in three cow herds (Czech Fleckvieh; 11, 13) for the possibility of mutual comparisons of milk indicator relationships between the various species.

Chemical-compositional, physical, health and technological milk indicators

Milk analyses were performed in the institute's accredited testing laboratory in Rapotin. Various analytical methods were used according to valid standards and standard operation manuals (8). Used milk indicators with relevant abbreviations are shown in Tab. 1. The following instruments were used for milk analyses: MilkoScan 133B (Foss Electric, Denmark) was regularly calibrated according to the results of reference methods for fat (F), lactose (L) and solids non fat (SNF); protein fractions such as crude protein (CP), true protein (TP) and casein (CAS) were determined by the reference Kjeldahl's method on the instrument line Tecator with Kjeltec Auto Distillation unit 2 200 (Foss-Tecator AB, Sweden); the somatic cell count (SCC) was determined by Fossomatic 90 instrument (Foss Electric, Denmark), which was checked quarterly by relevant proficiency testing; regularly calibrated Cryo-Star automatic Funke-Gerber (Germany) was used for milk freezing point (MFP) determination; the Spekol 11 instrument (Carl Zeiss Jena, Germany) was used for acetone, urea, citric acid and iodine investigation by the photometric method; the electrical conductivity (EC) was measured by the OK 102/1 (Radelkis, Hungary) conductometer; active acidity was measured by the pH-meter CyberScan 510 (Eutech Instruments); the macro- and microelement milk contents were investigated by atom absorption spectrophotometry with the equipment Spectrometer SOLAAR S4 and 6F S97 Thermo Elemental (England).

Statistical procedures

The processing of the results included the calculations of the basic statistical parameters, regression analysis and correlation coefficients using Excel PC softwareprogramme. Because of the abnormal data frequency distribution existence, the somatic cell count and acetone values were logarithmically transformed (decimal basis) and after that, the geometrical means were used as well.

RESULTS AND DISCUSSION

The main statistical parameters of sheep milk indicators are shown in Tab. 2. All residues of inhibitory substances (RIS) findings were negative. Sheep milk somatic cell count (SCC) as the main indicator of udder health was 560 as geometric mean and 949 ± 1,393 ths.ml^{-1} as arithmetic mean (vx = 146.9 %; Tab. 2). It was significantly ($P \leq 0.05$) higher and lower as compared to similar data sets for cow and goat milk (131 and 141 ± 57 ths.ml^{-1} in Holstein and 159 and 230 ± 222 ths.ml^{-1} in Czech Fleckvieh cows and 3,646 and 4,267 ± 2,279 ths.ml^{-1} in White short-haired goats) as compared to other previous works (6, 7, 11, 13). In other work (2), the mean somatic cell count values varied from 1,570 to 260 ths.ml^{-1} from beginning to the end of lactation. However, Paape *et al.* (24) mentioned clearly lower sheep mean somatic cell counts in USA, only the goat SCCs were similar to our results. Romeo *et al.* (28) reported lower mean SCC for uninfected (185 ths. ml^{-1} and never exceeded 477 ths.ml^{-1}) and higher (1,500 ths. ml^{-1} and never fell below 1,000 ths.ml^{-1}) for infected sheep udder halves.

The following parameters were chosen as important milk indicators of udder health (4, 5, 9) for expression of correlations: log SCC; electrical conductivity; and lactose (Tab. 3, 4 and 5). The arithmetic mean of electrical conductivity was 4.36 ± 0.35 mS.cm^{-1} (vx = 7.9 %). The arithmetical mean and standard deviation of lactose content were 4.44 ± 0.38 % (vx = 8.5 %; Tab. 2). In recent times (1, 2, 15, 17, 21, 22, 23, 24, 25, 26, 27, 29) the sheep milk quality has been investigated more intensively and by more indicators (4, 5, 8, 9, 19, 20, 28, 30) than in the previous time.

Log SCC was significantly (Tab. 3) correlated (as linear or non linear regression) to: fat content 0.64; L –0.58; crude, true, whey protein fractions and casein from 0.38 to 0.57; casein number (CAS-TP); total solids 0.70; fat/ crude protein ratio 0.45; urea concentration (conc.) 0.52;

Table 2. Basic results of the sheep milk quality

MI	Unit	x or xg	SD	vx	min	max
F	g.100g^{-1}	7.58	1.892	25.0	1.28	11.44
L	g.100g^{-1}	4.44	0.380	8.6	3.75	5.16
SNF	g.100g^{-1}	11.40	0.547	4.8	9.33	12.73
DM	g.100g^{-1}	18.98	1.930	10.2	14.01	23.09
SCC	ths.ml^{-1}	948.5	1,404.7	148.1	147	8,877
log SCC		560	0.4012		2.1673	3.9483
U	mg.100ml^{-1}	63.55	10.17	16.0	43.67	83.78
A	mg.l^{-1}	11.10	7.638	68.8	0.82	34.41
log A		8.06	0.3955		-0.0862	1.5367
AS	ml	0.49	0.216	44.1	0.09	1.06
TA	ml.0.25 mol.l^{-1}	12.26	3.328	27.1	6.91	20.51
EC	mS.cm^{-1}	4.36	0.348	8.0	3.36	5.31
pH		6.50	0.405	6.2	5.31	7.02
MFP	°C	-0.6048	0.0691	11.4	-0.7843	-0.4645
RCT	s	115.43	79.443	68.8	31	390
CQ	class	1.33	0.572	43.0	1	4
CF	cm	0.90	0.464	51.6	0.20	1.80
WV	ml	27.68	3.657	13.2	18	35
SW	g.cm^{-1}	1.0355	0.0027	0.3	1.0284	1.0449
CP	g.100g^{-1}	6.32	0.546	8.6	4.67	7.47
CAS	g.100g^{-1}	4.96	0.373	7.5	3.93	5.81
TP	g.100g^{-1}	5.91	0.560	9.5	4.44	7.08
WP	g.100g^{-1}	0.95	0.308	32.4	0.51	1.59
NPNM	g.100g^{-1}	0.42	0.120	28.6	0.20	0.64
UNR	%	48.25	15.073	31.2	24.73	85.76
F/CP		1.20	0.271	22.6	0.17	1.63
CAS-CP	%	78.55	2.988	3.8	66.37	84.05
CAS-TP	%	84.16	4.010	4.8	73.16	90.39
CA	mmol.l^{-1}	6.77	1.475	21.8	2.49	10.22
Ca	mg.kg^{-1}	1,914.50	296.998	15.5	980	2,287
P	mg.kg^{-1}	1,596.73	128.234	8.0	938	1,800
Na	mg.kg^{-1}	740.13	157.848	21.3	418	1,047
Mg	mg.kg^{-1}	192.53	17.094	8.9	150	217
K	mg.kg^{-1}	1,296.75	123.843	9.6	1,094	1,603
I	ug.l^{-1}	164.18	52.179	31.8	45	276
Mn	mg.kg^{-1}	0.073	0.028	38.4	0.029	0.175
Fe	mg.kg^{-1}	0.40	0.192	48.0	0.16	1.16
Cu	mg.kg^{-1}	0.12	0.083	69.2	0.04	0.38
Zn	mg.kg^{-1}	5.23	1.070	20.5	2.28	9.71
Ni	mg.kg^{-1}	0.03	0.006	20.0	0.02	0.04

n – 60 bulk milk samples for all milk indicators with the exception of I = 50 and Ni = 10; MI – milk indicator; x – arithmetical mean; xg – geometrical mean (at $\log_{10}$ form) is placed only at log transformed milk indicators (SCC and A) and is introduced in the same unit as arithmetical mean; SD – standard deviation; vx – variation coefficient (in %); min – minimum; max – maximum

Table 3. Regressions and correlations between log SCC and other milk indicators

MI	Regression equation	R^2	r	Significance
F	y = 3.0266x – 0.7349	0.4121	0.64	***
L	y = –0.5506x + 5.957	0.3378	–0.58	***
SNF	y = 0.9918Ln(x) + 10.408	0.0640	0.25	*
DM	y = 3.3659x + 9.7334	0.4896	0.70	***
U	y = 13.256x + 27.126	0.2734	0.52	***
A	y = 2.2312x + 4.9664	0.0137	0.12	Ns
log A	y = 0.1566x + 0.476	0.0252	0.16	Ns
AS	y = 0.2446Ln(x) + 0.2495	0.0249	0.16	Ns
TA	y = 6.0959Ln(x) + 6.1571	0.0654	0.26	*
EC	y = 0.3339x + 3.4388	0.1485	0.39	**
pH	y = –0.5155x + 7.9135	0.2609	–0.51	***
MFP	y = –0.1059x – 0.3138	0.3778	-0.61	***
RCT	y = –3.9935x + 126.41	0.0004	–0.02	Ns
CQ	y = 0.1808x + 0.8366	0.0160	0.13	Ns
CF	y = –0.2278x + 1.5292	0.0388	–0.20	Ns
WV	y = –1.2825x + 31.207	0.0198	–0.14	Ns
SW	y = –0.0011x + 1.0386	0.0286	–0.17	Ns
CP	y = 0.78x + 4.1792	0.3279	0.57	***
CAS	y = 0.5022x + 3.5781	0.2917	0.54	***
TP	y = 0.7939x + 3.7266	0.3236	0.57	***
WP	y = 0.2908x + 0.1501	0.1437	0.38	**
NPNM	y = –0.0144x + 0.4546	0.0023	–0.05	ns
UNR	y = 10.329x + 19.862	0.0756	0.28	*
F/CP	y = 0.3016x + 0.3721	0.1996	0.45	**
CAS-CP	y = –1.8364x + 83.598	0.0608	–0.25	ns
CAS-TP	y = –2.9349x + 92.223	0.0862	–0.29	*
CA (%)	y = –0.021x + 0.185	0.0986	–0.31	*
Ca	y = –341.07x + 2,851.7	0.2123	–0.46	**
P	y = 84.863x + 1,363.5	0.0705	0.27	*
Na	y = 492.92x0.3842	0.0650	0.26	*
Mg	y = 16.906x + 146.08	0.1574	0.40	**
K	y = –145.18x + 1,695.7	0.2212	-0.47	**
I	y = –25.408x + 234.18	0.0421	–0.21	ns
Mn	y = –0.0232x + 0.1366	0.1081	–0.33	*
Fe	y = 0,9022e-0,33x	0.0863	0.29	*
Cu	y = 0.0021x + 0.1099	0.0001	0.01	ns
Zn	y = –0.865x + 7.6059	0.1052	–0.32	*
Ni	y = 0.0052x + 0.0192	0.0591	0.24	ns

R^2 – determination coefficient; r – correlation coefficient; *, ** and *** – statistical significance $P \leq 0.05$, ≤ 0.01 and ≤ 0.001; ns – $P > 0.05$

Table 4. Regressions and correlations between electrical conductivity and other milk indicators

MI	Regression equation	R^2	r	Significance
L	y = –0.5391x + 6.7925	0.2431	–0.49	***
SNF	y = 0.8676x + 7.6211	0.3037	0.55	***
U	y = 14.238x + 1.5265	0.2369	0.49	***
A	y = –9.4898x + 52.438	0.1865	–0.43	**
AS	y = 0.2228x – 0.4763	0.1281	0.36	**
TA	y = 4.4103x – 6.9539	0.2123	0.46	**
pH	y = –0.3611x + 8.0699	0.0961	–0.31	*
MFP	y = –0.0891x – 0.2168	0.2006	–0.45	**
SW	y = 0.004x + 1.0181	0.2597	0.51	***
CP	y = 1.1x + 1.5306	0.4896	0.70	***
CAS	y = 0.6143x + 2.282	0.3277	0.57	***
TP	y = 1.1821x + 0.7588	0.5386	0.73	***
WP	y = 0.5698x – 1.5331	0.4142	0.64	***
UNR	y = 20.71x – 41.974	0.2282	0.48	**
F/CP	y = –0.2926x + 2,4754	0.1411	-0.38	**
CAS-CP	y = –3.9531x + 95.773	0.2115	-0.46	**
CAS-TP	y = –6.2061x + 111.19	0.2895	–0.54	***
P	y = 401.42Ln(x) + 1,007.3	0.0651	0.26	*
Na	y = 119.94e0.4127x	0.4653	0.68	***
Mg	y = 25.483x + 81.519	0.2686	0.52	***
K	y = –100.21x + 1,733.3	0.0791	–0.28	*
I	y = --43.451x + 352.23	0.0903	–0.30	*
Cu	y = 0.2558Ln(x) – 0.2597	0.0635	0.25	*
Zn	y = –1.1645x + 10.302	0.1432	–0.38	**

citric acid conc. –0.31; EC 0.39; MFP –0.62; pH acidity –0.51; titration acidity 0.26; Ca conc. –0.46; P conc. 0.27; Na conc. 0.26; Mg conc. 0.40; K conc. –0.47; Mn conc. –0.33; Fe conc. 0.29; and Zn conc. –0.32.

In the case of milk fat, the correlation (0.64; $P < 0.001$) could be caused probably by reduction of milk yield due to subclinical mastitis as somatic cell count was higher along higher fat. Leitner *et al.* (17) investigated reduced milk yield from 0.76 to 0.36 kg per milking as the sheep mammary glands were infected in comparison to uninfected. The lactose content in sheep milk was reduced by subclinical or clinical mastitis and in this respect the lactose reduction was linked with higher log SCC value (–0.58; $P < 0.001$; Tab. 3; Fig. 1) very clearly, similarly in comparison to the case of cow's milk (–0.43; $P < 0.001$) but more tightly. The decrease of lactose content by 0.50 % (from 5.00 to 4.50 %) could be connected with the increase of log SCC from 1.7381 to 2.6462 (SCC by 388 from 55 to 443 ths.ml^{-1}). It is equal to lactose reduction by 0.55 % during increase of log SCC by log ones. It means that 33.8 % of the variability in lactose

Table 5. Regressions and correlations between lactose content and other milk indicators

MI	Regression equation	R^2	r	Signifi-cance
F	y = –2.3317x + 17.944	0.2195	–0.47	**
DM	y = –2.3765x + 29.544	0.2190	–0.47	**
U	y = –8.4794x + 101.24	0.1004	–0.32	*
TA	y = –3.6341x + 28.41	0.1722	–0.42	**
pH	y = 0.4388x + 4.5466	0.1697	0.41	**
MFP	y = 0.0719x – 0.9242	0.1561	0.40	**
WV	y = 3.6198x + 11.596	0.1415	0.38	**
SW	y = 0.002x + 1.0265	0.0793	0.28	*
CP	y = –0.7859x + 9.8154	0.2988	–0.55	***
CAS	y = –0.3417x + 6.4766	0.1212	–0.35	**
TP	y = –0.9381x + 10.077	0.4054	–0.64	***
WP	y = –0.5984x + 3.6088	0.5459	–0.74	***
NPNM	y = 0.1529x – 0.2644	0.2347	0.48	***
UNR	y = –26.511x + 166.07	0.4469	–0.67	***
F/CP	y = –0.2277x + 2.2129	0.1021	–0.32	*
CAS-CP	y = 4.1896x + 59.932	0.2840	0.53	***
CAS-TP	y = 7.3607x + 51.446	0.4867	0.70	***
CA	y = 1.4679x + 0.2458	0.1430	0.38	**
Na	y = 8,463.6x-1.6523	0.4600	0.68	***
Mg	y = –30.026x + 325.98	0.4457	–0.67	***
K	y = 269.61x + 98.55	0.6846	0.83	***
Zn	y = 1.6455x – 2.0839	0.3417	0.58	***

content was explainable by variability in values of log SCC. The identical correlation between SCC and L was relatively less close in goat milk (–0.28; $P < 0.01$; 10) than in cow (15; –0.43; $P < 0.001$) and especially sheep milk (–0.58; $P < 0.001$, Tab. 3). The regular measurement and combination of interpretation of values of various milk indicators according to hereto confirmed rules in milk recording system, could improve the control and prevention of occurrence of milk secretion disorders in sheep flocks as it is mostly used already in cow herds. Also the combination of somatic cell count and lactose obtained results with electrical conductivity (EC) values could be efficient because the relationship of log SCC and EC was relatively close in sheep (0.39; $P < 0.01$; Tab. 3; Fig. 1) as well. Some of obtained regression (prediction) equations (Tab. 3, 4 and 5) could be built into specific softwares for advisory service purposes in sheep dairying in the future.

Positive correlations of log SCC to milk protein fractions were probably caused by reduction of milk yield due to subclinical mastitis (higher SCC) similarly as at the fat finding. A little closer relationships of log SCC to crude protein and true protein contents (0.57 and 0.57; $P < 0.001$; Tab. 3) in comparison to casein content (0.54; $P < 0.001$; Tab. 3) could be given by whey protein increasing during milk secretion disorders and SCC increase simultaneously (0.38; $P < 0.01$; Tab. 3). In consequence of the significantly positive correlations of log SCC to fat and crude protein, the total solids increased along log SCC scale as well (0.70; $P < 0.001$; Tab. 3). However, the solids non fat increased insignificantly (0.25; $P > 0.05$; Tab. 3).

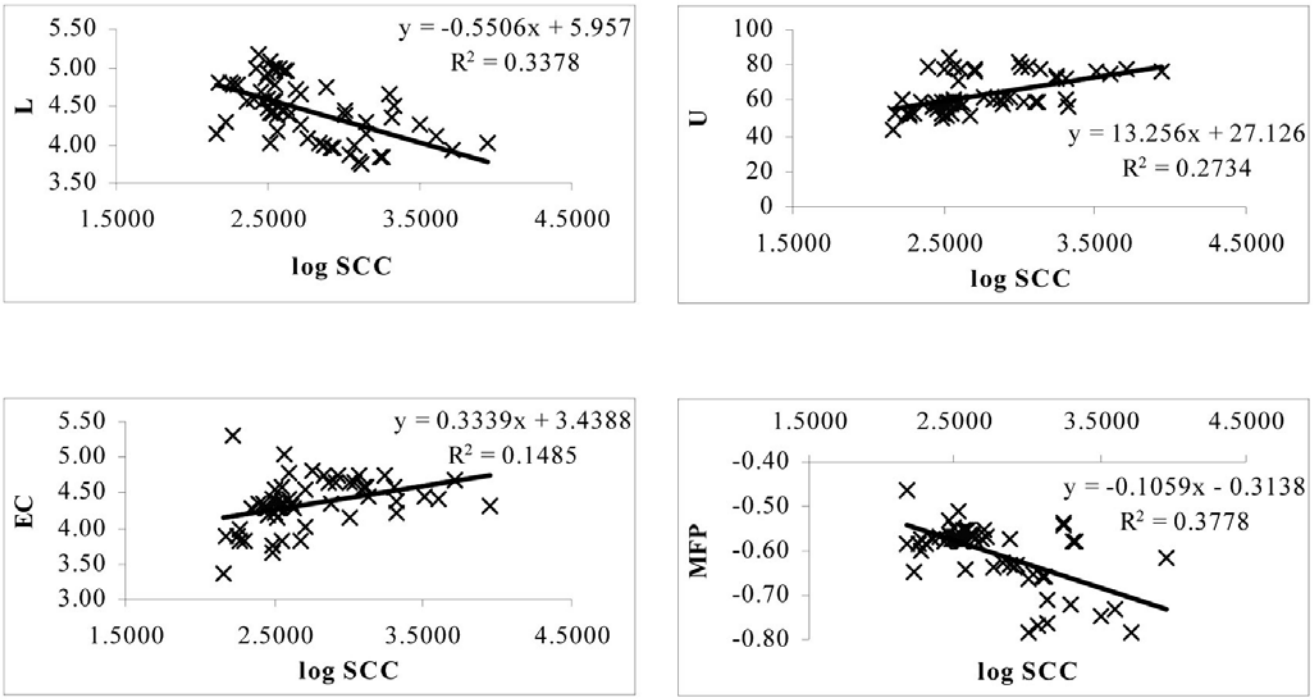

Fig. 1. Relationship between log SCC (SCC in ths.ml^{-1}) and lactose (L; %) content, electrical conductivity (EC; mS.cm^{-1}), urea (U; mg.100ml^{-1}) concentration and milk freezing point (MFP; °C) in sheep bulk milk samples

It is interesting that urea concentration increased along log SCC scale very significantly (0.52; $P < 0.001$; Tab. 3; Fig. 1). It means that 27.3 % of the variations in urea values could be determined by variations in log SCC values. The increase of milk urea by 10 mg.100ml^{-1} (from 60 to 70 mg.100ml^{-1}) could be caused by the increase of log SCC from 2.4799 to 3.2343 (it is equal to SCC increase by 1,413 from 302 to 1,715 ths.ml^{-1}). Perhaps these facts could be caused by the physiological point of view due to higher permeability of mammary gland secretion epitel for blood components (for instance urea) during mastitis disorders. The significant relationship of log SCC to citric acid concentration (–0.31; $P < 0.05$; Tab. 3) showed the opposite orientation in comparison to urea concentration. The significant relationship between log SCC and fat/crude protein ratio (F/CP; 0.45; $P < 0.01$; Tab. 3) means that 20.0 % of the variations in F/CP ratio values could be explainable due to variations in log SCC values. Higher urea concentration, higher F/CP ratio value and also lower milk CA concentration could be expressed on a lower level of energy feeding in animals and also on a higher risk for ketosis occurrence. Therefore, the obtained results indicated that higher secretion disorder occurrence (higher log SCC value) could be linked with lower sheep energy maintenance. It could be a way of logically explaining the obtained results.

The significant correlation between log SCC and titration acidity (0.26; $P < 0.05$; Tab. 3) was not too close. The higher degree of sheep mastitis state (higher log SCC value) was relatively illogically connected with higher value of titration acidity (TA). Cow milk titration acidity is usually reduced with SCC increase or there is no significant relationship in the case of data set with lower SCC mean (12). This fact could be caused and also explained by simultaneously increased crude protein in milk (Tab. 3) which could be a source of acidity.

The relationship between log SCC value and milk freezing point (–0.62; $P < 0.001$; Tab. 3) is very interesting. It is a relatively very close relationship in regard to the physiological point of view. It means that 37.8 % of the variations in milk freezing point (MFP) values could be explainable by variations in log SCC values (Fig. 1). An improvement of milk freezing point by 0.1 °C (from 0.6 to 0.7 °C) could be caused due to SCC increase by 3,930 ths.ml^{-1}, as back transformed from log SCC. So a deteriorating sheep mastitis state (higher log SCC value) is linked with better (lower) milk freezing point as an indicator of raw milk quality. A similar finding is possible also in cow milk. However, the relationship is much closer in sheep than in dairy cows (–0.36; $P < 0.05$; 16), probably because of higher values and higher variation range of SCC in general in sheep. Only at first sight this fact could be a paradoxical phenomenon. It is explainable by the higher permeability of mammary gland secretion epitel for blood ions such as Na^+ (Tab. 3) and Cl^-, which pass into milk in the case of lactose content reduction (Tab. 3; Fig. 1) due to higher SCC and deteriorating udder mastitis state. Also more significant relationships between log SCC and macro- and microelements in sheep milk (Tab. 3) were observed.

Insignificant ($P > 0.05$) relationships of log SCC values were found to: casein number (CAS-CP); specific mass (weight, SW); acetone concentration; alcohol stability; cheeseability (rennet coagulation time (RCT), curds cake quality (CQ), cheese curds firmness (CF), whey volume (WV)); non protein nitrogen matters (NPNM) conc.; I conc.; Cu conc.; and Ni conc. It is is quite surprising that investigated sheep milk technological properties were influenced by log SCC mostly insignificantly (Tab. 3). Also Novotná *et al.* (22) found no significant effect of SCC on quality of curd and cheeseability. However, they mentioned the fact that the shortest rennet coagulation time was associated in the group of samples with almost the highest SCC. Also the RCT increased in sheep milk with increased SCC level (15) as the SCC differences between sample groups were very expressive (< 100, 100–1,000, > 1,000 ths.ml^{-1}). In general, according to our results the more deteriorating udder health by the SCC, does not adversely affect the milk protein recovery and protein and fat indicators in such essential way in sheep milk as compared to cow milk.

The significant relationship between electrical conductivity (EC) and other milk indicators are shown in Tab. 4. EC values were first of all interestedly correlated (as linear or non linear regression) to protein fractions ($P < 0.001$), from 0.64 (whey protein) to 0.73 (true protein) and of course higher EC was logically linked with better (lower) milk freezing point. In accordance with the mutual relationships between SCC and electrical conductivity and lactose the EC in sheep milk was relatively closely correlated with lactose (–0.49; $P < 0.001$). Also the relationships of electrical conductivity to some macroelements, especially Na (correlation 0.68), K (–0.28) and Mg (0.52) were interesting. In the previous first two cases (Na and K) these could be caused by very well known subclinical or clinical mastitis impacts on possible milk macroelement changes (Na increase and K reduction).

The significant relationship between lactose and other milk indicators are shown in Tab. 5. First of all, the lactose values were correlated (as linear or non linear regression) to protein fractions similarly as electrical conductivity values were, but in the opposite orientation. The correlation coefficients varied between –0.35 ($P < 0.01$) and –0.74 ($P < 0.001$). The higher lactose could be probably linked with higher daily milk yield and thereby with lower crude protein including casein. Similarly, it may be explained simultaneously by lower electrical conductivity due to a lower level of mineral salts at higher milk yields. Oravcová *et al.* (23) observed negative correlations between milk yield and fat (from –0.21 to –0.30) and crude protein (from –0.17 to –0.37) in three sheep breeds. Naturally, there could be an interference in terms of lower lactose because of higher SCC (worse mastitis state) simultaneously along higher whey protein (–0.74; $P < 0.001$). It could be caused

by higher permeability of secretion epitel of mammary gland for blood protein substances, which create consequently whey protein in milk in the case of higher SCC. The higher Na values along lower lactose are logical in terms of the known physiology of milk secretion (0.68; $P < 0.001$). Similarly, the logical relationship was observed to K conc. in sheep milk (0.83; $P < 0.001$). The negative correlation between lactose and urea (–0.32; $P < 0.05$) could be probably linked with energy metabolism of the animals. Higher urea and lower lactose could be seen along poor maintenance of energy level. Surprisingly, the higher lactose were connected with worse (higher) milk freezing points (0.40; $P < 0.01$), probably because of lower salts concentration as it has been explained previously in relationships between electrical conductivity, lactose and protein fractions and lower content of solid milk matters during higher sheep milk yield as observed also by Oravcová *et al.* (23).

CONCLUSIONS

On the basis of the results obtained, it may be possible to improve the practical intepretation rules for better monitoring and prevention of milk secretion disorders and increase protein recovery in sheep milk production. Better possibilities for relevant interpretation of relationships between occurrence of milk secretion disorders and changes of sheep milk composition and properties are presented. For instance, by the combination of somatic cell count, lactose and electrical conductivity values, and their use of their predictive equations, improvements in the framework of milk recording and milk quality advisory system in sheep dairying may lead to significant benefits.

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PROBIOTIC SURVIVAL IN YOGHURT MADE FROM COW'S MILK DURING REFRIGERATION STORAGE

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ABSTRACT

This study evaluated the stability of *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis* in yoghurts during their storage. Yoghurts were incubated at 43 °C for 3.5 h and stored at 4 °C. Survival of *Lactobacillus* and *Bifidobacterium*, changes of acidity (pH and SH°), and the microbiological properties of probiotic yoghurts were evaluated on day 1, 3, 7, 14 and 21 of storage. The experiments were performed from the 5th of April, 2008 to the 23rd of January, 2009. The results showed substantial differences between the various types of probiotic bacteria. The monitoring of the samples allowed for determination of the reproductive capacity of the probiotic microorganisms. Following the analysis there was an initial increase in their counts. *Bifidobacterium animalis* ssp. *lactis* counts increased from 0.3×10^9 CFU.g^{-1} to 1.7×10^9 CFU.g^{-1} during the first 7 days independent of the season. The counts of the bacteria *Lactobacillus acidophilus* increased from 0.8×10^9 CFU.g^{-1} to 5.5×10^9 CFU.g^{-1} during the first 7 days of storage during all four seasons. Significant differences were found between decreasing pH and increasing counts of individual bacterial species in the samples monitored.

Key words: *Bifidobacterium animalis* ssp. *lactis; Lactobacillus acidophilus;* probiotics; yoghurt

INTRODUCTION

Because of a renewed interest in probiotics, different types of products have been proposed as carrier foods for probiotic microorganisms by which consumers can take in large amounts of probiotic cells for their therapeutic effect. Yoghurt has long been recognized as a product with many desirable effects for consumers, and it is important that most consumers consider yoghurt to be "healthy" (9).

Yoghurt is a fermented milk product that has been prepared traditionally by allowing milk to sour at 40–45 °C. Modern yoghurt production is a well-controlled process that utilizes the ingredients of milk, milk powder, sugar, fruit, flavours, colouring, emulsifiers, stabilizers and specific pure cultures of lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*) to conduct the fermentation process. The conventional yoghurt starter bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*, lack the ability to survive passage through the intestinal tract and consequently do not play a role in the human gut (6).

Lactobacillus acidophilus and *Bifidobacterium bifidum* are given into yoghurt to add extra nutritional-physiological value. *Lactobacillus acidophilus* and *Bifidobacterium bifidum* are normal inhabitants of the intestine of many animals including humans. It is estimated that more than 70 products containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, including sour cream, buttermilk, yoghurt, milk powder and frozen dessert, are produced worldwide (14).

It has been suggested that to have any therapeutic effect, the minimum number of viable *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in a product should be $\geq 10^5$ g^{-1} (18). It is important to maintain the viability of these organisms until the products are consumed in order to ensure delivery of live organisms. It seems reasonable to assume that the beneficial effects of *Lactobacillus acidophilus* and *Bifidobacterium bifidum*

can be expected only when the ingesting viable cells can reach and survive in the human gut.

The most commonly used species in commercial products are *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus GG*, *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Bifidobacterium breve* and *Bifidobacterium infantis* (19).

Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* is affected by the low pH of the environment. *Bifidobacterium bifidum* is not as acid-tolerant as *Lactobacillus acidophilus* and the growth of *Bifidobacterium bifidum* is significantly retarded below pH 5.0 (4). The growth of *Lactobacillus acidophilus* ceases below pH 4.0 (17).

The aim of this study was to investigate stability and viability of *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis* in laboratory manufactured yoghurts during prolonged storage (three weeks) when refrigerated close to 4 °C. The yoghurts were made from raw cow's milk in four different seasons. The milk used for the experiment was obtained from the same farm.

MATERIALS AND METHODS

Detection of raw milk quality

In raw cow's milk we determined fat, solids non-fat (SNF), density, proteins, water content percentages, temperature (°C), pH, conductivity and total solids using infrared analyzers – Ultrasonic Milk-analyzers (Bulgaria). The somatic cell count (SCC) was determined by the Fossomatic 90 (Denmark). Residues of inhibitory substances were investigated by the reaction of the DELVO test method, which was performed on the basis of the growth of test microbial strain *Bacillus stearothermophilus* var. *calidolactis.*

The standard plate count (SPC) in raw cow milk was determined using standard agar PCA – Plate Count Agar (Hi Media) (24) and the coliform counts on Endo agar Base (HI Media) (25).

Preparation of yoghurts

Raw cow's milk (3.5 % fat content, 8.5 % solids content) from the university farm was pasteurized at 85 °C for 15 seconds under standard laboratory conditions. Pasteurized milk was cooled to 45 °C and skim milk powder was added with high speed stirring, to make 18 g.100 g^{-1} total solids in the yoghurt mix. Heating was continued to 85 °C and the yoghurt mix was held at this temperature for 20 min. It was then cooled quickly to 43 ± 2 °C and 2 g.100g^{-1} (w/w) freeze-dried yoghurt starter culture (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) was added.

The probiotic culture (10^7 CFU.g^{-1}) 1 g.100 g^{-1} (w/w) of freeze-dried *Lactobacillus acidophilus* (LA-5 Chr. Hansen) (sample A) and *Bifidobacterium animalis* ssp. l*actis* (BB-12 Chr. Hansen) (sample B) was added. A portion of the mix was distributed into 150 ml plastic yoghurt cups to make plain natural yoghurts and sealed. Incubation was carried out at 43 °C until a pH of 4.6 was reached at which time the yoghurts were cooled in an ice water bath. The yoghurts were stored in a refrigerator (4 °C) throughout the shelf life (21 days).

Time interval specifications for samples

The "1" day (day 1) analyses were carried out after incubation of yoghurt samples. Subsequently analyses were carried out after 1, 3, 7, 14, and 21 days of refrigerated storage (4 °C).

Enumeration of probiotic bacteria

One container of yoghurts (sample A and sample B) was taken weekly to enumerate the viable probiotic bacteria. One gram of yoghurt sample was weighed directly into a sterile 10 ml tube and mixed with 9 ml of 2 % sodium citrate. The sample was thoroughly mixed using a vortex (BagMixer 400 W, Merci Czech). Serial dilutions were performed in sterile test tubes using 2 % sodium citrate as the diluents for selective enumeration of *Lactobacillus acidophilus* LA-5; then one hundred microlitres were spread plated on MRS-salicin with duplicate plates (anaerogen, Oxoid) and incubated anaerobically at 37 °C for 72 hours.

For the selective enumeration of *Bifidobacterium animalis* ssp. *lactis*, MRS agar supplemented with 0.5 % L-cysteine HCl at 10 %, 0.5 % at 0.1 % modified (5) was used. A spread plate technique was used for this medium, with duplicate plates made for the appropriate dilutions. Plates were incubated anaerobically (anaerobic glove box, Anaerogen, Oxoid) at 37 °C for 48 hours. In order to confirm the identity of the enumerated bacteria, the cell morphology was microscopically observed. The average count of the duplicate plates was used for statistical analysis.

Determination of pH

The pH of both yoghurt samples (A, B) was determined using a digital pH meter (pH 340i/ SET). The pH meter was calibrated using reference pH 4.0 and 7.0 buffered solutions. The yoghurt samples were blended before pH measurements.

Determination of titratable acidity

The titratable acidity of yoghurts (sample A, sample B) was determined after mixing the yoghurt samples with 10 ml of hot distilled water (~ 90 °C) and titration by 0.1 *N* NaOH solutions.

All physico-chemical analyses were carried out in triplicate.

Statistical analysis

Data analysis was conducted using SPSS 12 Statistical Software and the data were statistically tested using analysis of variance (ANOVA) to determine if statistical difference existed at P = 0.05 and the Least Significance Difference (LSD) was used to identify statistical differences among the averages.

RESULTS AND DISCUSSION

Somatic cell count and microbial population of raw milk for yoghurt manufacturing before heat treatment are given in Table 1.

The highest SCC in the raw milk was observed in the summer period (1 633 000) which is above the set maximum limit according to EU legislation (Regul. 853/2004).

Table 1. Somatic cell count and microbial count of raw milk for yoghurt manufacturing before heat treatment

Season	Somatic cell count CFU.ml^{-1}	Total bacterial count CFU.ml^{-1}	Coliform bacteria CFU.ml^{-1}
Spring	286 000	45 000	19 600
Summer	1 633 000	65 900	10 000
Autumn	360 000	32 600	8 300
Winter	156 000	51 200	6 280

Table 2. Effect of milk pasteurization

Season	Total bacteria (%)	Coliform bacteria (%)
Spring	99.21	100
Summer	99.45	100
Autumn	99.99	100
Winter	99.36	100

Table 3. Probiotic cultures in yoghurts after 1, 3, 7, 14 and 21 days storage in various seasons

Days	Yoghurts							
	with *Lactobacillus acidophilus*				with *Bifidobacterium animalis* ssp. *lactis*			
	spring	summer	autumn	winter	spring	summer	autumn	winter
1	2.3 x 10^9	1.6 x 10^9	0.8 x 10^9	0.1 x 10^9	0.4 x 10^9	0.3 x 10^9	0.7 x 10^9	0.3 x 10^9
3	5.2 x 10^9	1.8 x 10^9	1.1 x 10^9	0.4 x 10^9	0.8 x 10^9	0.4 x 10^9	1.1 x 10^9	0.6 x 10^9
7	5.5 x 10^9	2.5 x 10^9	3.4 x 10^9	1.1 x 10^9	1.7 x 10^9	0.6 x 10^9	1.2 x 10^9	1.1 x 10^9
14	4.1 x 109	0.5 x 10^9	1.6 x 10^9	0.9 x 10^9	0.8 x 10^9	0.5 x 10^9	0.5 x 10^9	0.8 x 10^9
21	2.2 x 10^9	0.4 x 10^9	0.8 x 10^9	0.6 x 10^9	0.1 x 10^9	0.1 x 10^9	0.2 x 110^9	0.4 x 10^9

Table 4. Statistical analysis of pH and titratable acidity in yoghurts with probiotic bacteria

Ratings	Yoghurt			
	with *Lactobacillus acidophilus*	With *Bifidobacterium animalis* ssp. *lactis*	with *Lactobacillus acidophilus*	with *Bifidobacterium animalis* ssp. *lactis*
	pH		Titratable acidity	
A vs B	0.27**	n.s.	-16.14***	n.s.
A vs C	0.37***	0.50***	n.s.	-38.86***
A vs D	0.64***	0.44***	-23.22**	-29.90***
B vs C	0.09*	0.52***	n.s.	-36.18***
B vs D	0.37***	0.45***	-7.08*	-27.22***
C vs D	0.27**	-0.06**	-10.36*	8.96***

Ratings: A – spring average scores, B – summer average scores, C – autumn average scores, D – winter average scores;
* – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$ – average of differences

The appearance of mesophilic colonies varied in size and colour throughout the medium and TBC did not exceed the maximum value set for cow's milk (≤ 100 000 ml^{-1}). Coliform bacteria were observed in the minimum value of 6 280 ml^{-1} of milk during the winter period.

The effect of milk pasteurization for total bacterial count ranged from 99.21 % (spring season) to 99.99 % in autumn. The effect of pasteurization for coliform bacteria (Table 2) was 100 %.

Residues of inhibitory agents were not detected anywhere in raw cow's milk used for the yoghurt processing in the experiment.

The results of the determinations for fat, solids non-fat, density, proteins, water content percentages,

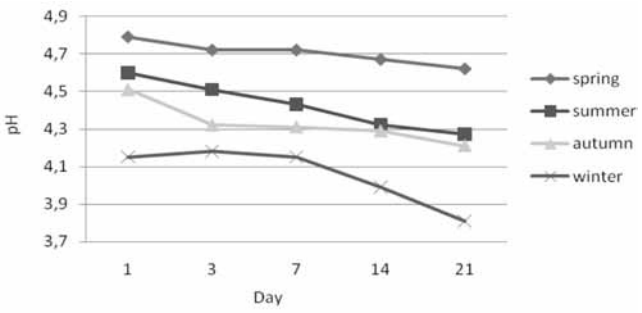

Fig. 1. Change in pH in yoghurt samples A over four seasons

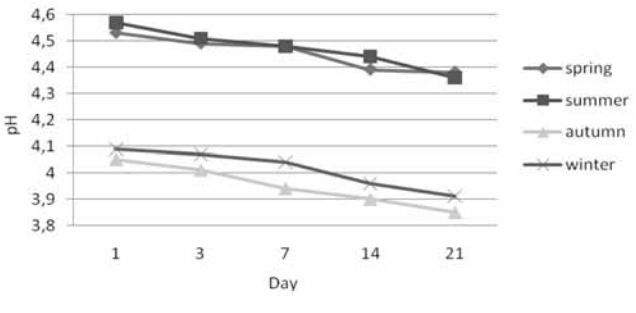

Fig. 2. Change in pH in yoghurt samples B over four seasons

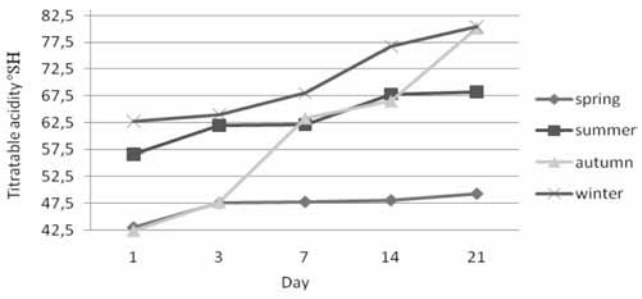

Fig. 3. Change in titratable acidity in yoghurt samples A over four seasons

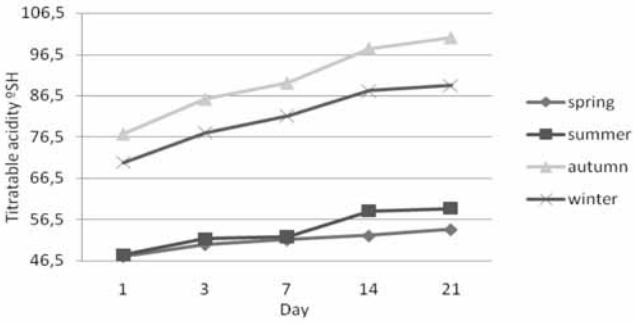

Fig. 4. Change in titratable acidity in yoghurt samples B over four seasons

temperature, pH, conductivity and total solids are not presented, but all of them complied with the national Slovak technical standards (23) used for the payment for raw cow's milk.

In spring, during the storage, the average viable cell counts of *Lactobacillus acidophilus* decreased from 2.3×10^9 CFU.g^{-1} on day 0 to 2.2×10^9 CFU.g^{-1} on day 21; whereas those of *Bifidobacterium animalis* ssp. *lactis* decreased from 0.4×10^9 CFU.g^{-1} on day 0 to $0.1 \times 10^9 \times$ CFU.g^{-1} on day 21 (Table 3).

Overall, all yoghurts stored contained greater than 10^8 CFU.g^{-1} of viable *Lactobacillus acidophilus* at the end of 21-day experiment. The maximum average viable cell counts of *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis* (5.5×10^9 CFU.g^{-1}and 1.7×10^9 CFU.g^{-1}, respectively) was observed on day 7 (Table 3).

Thus in this study, all yoghurt samples contained considerably more probiotic bacteria than the recommended level (10^6–10^7 CFU.g^{-1}) of both species of incorporated probiotic bacteria (11, 20).

During storage, the count of *Bifidobacterium animalis* ssp. *lactis* decreased considerably more than *Lactobacillus acidophilus* and continued to decline until day 21.

This is not in agreement with the published literature data. Antunes *et al.* (1) observed that the *Bifidobacterium* genus had lower viability during storage, especially in acidic foods, such as yoghurt and cultured milk. Also, Antunes *et al.* (2) obtained counts lower than 5 $\log_{10}$ CFU.ml^{-1} in developed probiotic yoghurt.

Barreto *et al.* (3) obtained the same results when evaluating the viability of Bifidobacteria in yoghurts and cultured milk product sold on the Brazilian market. Similarly, Gueimonde *et al.* (7) observed Bifidobacteria counts lower than 10^5 in some probiotic products sold in Spain. Shin *et al.* (21) and Kailasapathy *et al.* (13) reported Bifidobacteria counts higher than 10^6 at the expiry date of yoghurts.

The pH and titratable acidity changes during yoghurt storage are shown in Figures 1–4. An overall decline in the pH of all the stored yoghurts occurred during the study. The initial pH (day 0) ranged between 4.1 and 4.75 with an average of 4.45, and the final pH ranged from 3.8 to 4.6, with an average of 4.25. There was a significant difference ($P < 0.05$) in pH between yoghurts containing *Lactobacillus acidophilus* (sample A) and *Bifidobacterium animalis* ssp. *lactis* (sample B).

The relationship between pH and the number of viable *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis* during storage of all yoghurts over a 21-day period at 4 °C was studied. The analyses of the results showed a relationship between the pH and the viable probiotic counts for both probiotic strains used in these experiments. Viability of both organisms was lower when yoghurt pH declined. Viability of *Lactobacillus acidophilus* was higher when yoghurt pH was between 4.1 and 4.8, during storage at 4 °C.

The viability of *Bifidobacterium animalis* ssp. *lactis* was higher when the pH was 4.05–4.55. This is in contrast

with the result published by Kailasapathy *et al.* (13) who reported that their results showed that *Bifidobacterium animalis* ssp. *lactis* B-94 needed higher pH for better cell viability and cellular stability during storage of yoghurt compared to *Lactobacillus acidophilus* L10. The decline in the pH was presumably due to continued fermentation by the lactic acid bacteria in yoghurts during storage.

The final pH of yoghurt can affect the growth and viability of *Lactobacillus* and *Bifidobacterium*, but particularly that of *Bifidobacterium* (15, 10). It has been found that acid production ability by lactic acid bacteria, especially post-incubation (post-acidification), affected the cell viability of both *Lactobacillus* and *Bifidobacterium* (12).

Titratable acidity increased significantly ($P < 0.05$) on day 21 of storage (Table 4.). Higher titratable acidity was observed in yoghurt B with *Bifidobacterium animalis* ssp. *lactis.* These results are in agreement with Tarakci and Erdogan (22) who reported increased acidity of yoghurt over the storage period. Guler and Mutlu (8) also observed an increase in titratable acidity during the storage period. Similar results were obtained by Maľa *et al.* (16) in the samples of commercial network yoghurts in which the titratable acidity increased within two weeks of storage to 5° SH on average.

When the samples of yoghurt with *Lactobacillus acidophilus* were compared among seasons, significant differences were observed between spring and summer ($P < 0.05$), spring and autumn ($P < 0.01$) and spring and winter ($P < 0.01$). In the yoghurts with *Bifidobacterium animalis* ssp. *lactis* significant differences were observed only between summer and winter ($P < 0.05$). It seems that these changes may be associated with the quality of the raw milk, mainly with the SCC.

The viability of both *Lactobacillus* and *Bifidobacterium* species diminishes markedly during refrigerated storage at low pH levels.

CONCLUSIONS

This study evaluated the stability and viability of *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis* in yoghurt manufactured under standard laboratory conditions.

In raw cow's milk we determined fat, solids non-fat, density, proteins, water content percentages, temperature, pH, conductivity and total solids. Results of all these parameters complied with the Slovak technical standards (23).

The counts of probiotic *Lactobacillus acidophilus,* observed during the storage period (21 days), were significantly increased during the first seven days in spring. Also the counts of probiotic *Bifidobacterium animalis* ssp. *lactis* showed an increase during the first seven days of storage in spring, autumn and winter.

The results obtained allowed us to state that the content of all observed bacteria (BMK, *Lactobacillus acidophilus, Bifidobacterium animalis* ssp. *lactis*) in white yoghurts, produced under laboratory conditions in four seasons of the year, reached the minimum count of 10^6 CFU.ml^{-1} at the end of the storage period and thus complied with the therapeutic minimum level of probiotic bacteria in products intended for human nutrition.

The presence of the investigated micro-organisms was proved in all samples during the entire expiry period in total counts greater than 10^6 CFU.g^{-1} in yoghurts produced by the conventional method.

However, more carefully controlled studies in which energy intake and expenditure are measured needs to be conducted before any conclusions can be drawn regarding the positive effect of cultured dairy foods in humans and on weight gain and feed efficiency in animals.

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