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PROF. DR. JOŽE FERČEJ (1917–2009)

Štirinajstega oktobra smo se na ljubljanskih Žalah poslovili od staroste slovenskih govedorejcev, profesorja dr. Jožeta Ferčeja. Profesor Ferčej je bil zadnji iz skupine strokovnjakov, ki so v Sloveniji po drugi svetovni vojni izvedli pasemsko rajonizacijo in razširili umetno osemenjevanje krav.

Rodil se je 28. februarja 1917 na Blejski Dobravi. Gimnazijo je končal v Kranju, študij kmetijstva pa 1941, dva dni pred napadom na Jugoslavijo, na Kmetijsko – gozdarski fakulteti v Beogradu. Tam je leta 1941 diplomiral in 1963 tudi doktoriral s področja selekcije rjave pasme v Sloveniji. Po vojni je bil referent za živinorejo v Celju in Mariboru ter na Ministrstvu za kmetijstvo. Bil je tudi tajnik Zveze živinorejskih zadrug za sivo-rjavo govedo in tajnik zveze živinorejskih selekcijskih zadrug. Od leta 1953 do leta 1974 je bil znanstveni sodelavec, znanstveni svetnik in predstojnik Zavoda za živinorejo na Kmetijskem inštitutu Slovenije. V letih 1955 in 1956 je bil pol leta na izpopolnjevanju v Kopenhagnu na Danskem. Od leta 1974 in do upokojitve v letu 1987 je bil redni profesor za govedorejo na Oddelku za živinorejo, Biotehniške fakultete v Ljubljani. V letih 1971 in 1972 je bil profesor Ferčej predsednik Jugoslovanske skupnosti znanstvenoraziskovalnih organizacij za področje živinorejskih ved. Večkrat je bil tudi delegat Jugoslavije na sestankih in konferencah Evropske zootehnične federacije. Intenzivno je deloval v Evropskem združenju rejcev rjavega goveda, v dveh obdobjih je bil tudi predsednik tega združenja in je organiziral konferenci združenja v Sloveniji.

Za realizacijo razvojnih programov na področju govedoreje se je znal povezati z govedorejskimi strokovnjaki po Sloveniji, poskrbel pa je tudi za razvoj mladih sodelavcev, Janeza Pogačarja in Slavka Čepina, ki sta kasneje doktorirala in postala univerzitetna profesorja. S skrbjo za mlajše sodelavce je nadaljeval tudi na Biotehniški fakulteti. V Sloveniji bi težko našli govedorejca, ki se s profesorjem ni kdaj srečal. Dobre, prijateljske stike je znal navezovati tudi z naprednimi kmeti. Aktiven je ostal tudi po upokojitvi leta 1987.

Izjemno plodno delo profesorja ni ostalo neopaže-

no v široki javnosti. Prejel je številna domača in mednarodna odlikovanja, priznanja in nagrade, med njimi Red dela z zlatim vencem (1968), Red zaslug za narod z zlato zvezdo (1987), Priznanje švicarske zveze rejcev rjavega goveda (1969), Jesenkovo priznanje Biotehniške fakultete (1977), Nagrado Sklada Borisa Kidriča za tehnično izboljšavo – s sodelavci (1981), Kidričevo nagrado za življenjsko delo v govedoreji (1987).

Profesor je bil ploden pisec. Seznam bibliografskih navedb od leta 1947 presega 450 enot. Slovenski govedorejci, kmetijski strokovnjaki in prijatelji smo prof. dr Jožetu Ferčeju hvaležni za vse kar je naredil in napisal za slovenskega kmeta, kmetijstvo in zlasti za govedorejo. S svojim delom si je postavil trajen spomenik.

In Memoriam Prof. Dr. Jože FERČEJ (1917–2009)

Professor Jože Frečej, the doyen of Slovenian cattle breeders was buried on Ljubljana cemetery Žale on October 14. Professor Ferčej belonged to the group of experts who after the Second World War introduced artificial insemination and regionalisation of cattle breeds to Slovenia.

He was born on February 28 1917 at Blejska Dobrava, finished high school in Kranj and university study at the Faculty for Agriculture and Forestry in Belgrade in 1941, two days before German air force attacked Yugoslavia. From the same Faculty he received his PhD in 1963.

After the Second World War, he was employed as an expert for cattle breeding in Celje, Maribor and at the Slovenian Ministry for agriculture. He was also secretary of the Society for brown cattle breeding and secretary of the Society for cattle selection. Between 1955 and 1974 he was researcher and head of the unit for animal breeding at the Agricultural institute of Slovenia. In the years 1955 and 1956, he spent six months as a guest researcher in Copenhagen, Denmark. From 1974 to his retirement in 1987 he vas full professor for cattle breeding at the Department of Animal Science at Biotechnical Faculty, University of Ljubljana. In the years 1971 and 1972 was professor Ferčej president of the Yugoslav zootecnical research community. He represented Yugoslavia several times at the meetings of the European brown cattle Association, was two times its president and organized two annual meetings of the Association in Slovenia.

He developed vivid contacts to researchers and experts in the field of cattle breeding in Slovenia as well as abroad. He was mentor of several graduate students, among tem also Janez Pogačar and Slavko Čepin, who both later became university professors. Professor Ferčej remained active also after his retirement in 1987. For his professional and research work he received numerous awards, among them "*Red dela z zlatim vencem*" (1968), "*Red zaslug za narod z zlato zvezdo*" (1987), Award of the Swiss Brown Cattle Breeder Association (1969), Jesenko Award of the Biotechnical Faculty (1977), Award of the Boris Kidrič Foundation for technical innovation – with co-workers (1981) and Kidrič award for his lifework in the field of cattle breeding (1987).

Professor Ferčej was a fruitful writer. His entire bibliography contains more than 450 units and Slovenian cattle breeders, experts, farmers and friends appreciate his contribution to the development of Slovenian agriculture. With his work he will remain with us.

Prof. dr. Jože Osterc

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ODPOR DO ŽIVIL MED SLOVENSKIMI OSNOVNOŠOLCI

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Odpor do živil med slovenskimi osnovnošolci

Članek predstavlja analizo vzrokov za odpor do živil med slovenskimi osnovnošolci, starimi od 10 to 15 let, glede na spol, razred in kraj bivanja. V anketni raziskavi je sodelovalo 628 osnovnošolcev iz 16 naključno izbranih šol v Sloveniji. Ugotovili smo, da osnovnošolci največ odklanjajo živila zaradi senzoričnih lastnosti, kot sta okus in vonj. Med dejavniki okolja imajo na odklanjanje živil največji vpliv prehranske navade v družini. Anketirana dekleta so bolj izbirčna kot fantje. Manj odklonilen odnos imajo le do zelenjave. Starejši učenci bolj odklanjajo živila živalskega izvora, predvsem notranjih organov (npr. možgani, jetrca, vampi), kot njihovi mlajši kolegi. Razlike med osnovnošolci iz zahodne in vzhodne Slovenije se kažejo v odporu do mesa mehkužcev in dvoživk. Osnovnošolci v zahodni Sloveniji imajo manjši odpor do tovrstne hrane.

Ključne besede: prehrana ljudi / osnovnošolci / odpor do hrane / prehranske navade / Slovenija

Food dislikes among Slovenian schoolchildren

The article presents analysis of reasons for food dislikes among Slovenian primary schoolchildren aged 10 to 15, by gender, age and permanent residence. Altogether 628 primary school children from 16 randomly chosen schools in Slovenia filled the questionnaire. We found out that the most influential origins of food dislikes among schoolchildren were sensory characteristics, especially taste and smell. Among environmental factors were the most influential eating habits in families. Girls were more particular about their food then boys, except when it comes to vegetables. The study revealed that older schoolchildren disliked more organ meat than their younger colleagues. Differences among schoolchildren from western and eastern Slovenia were significant in dislikes toward meat from molluscs and amphibians. Schoolchildren from western Slovenia were less rejectable toward this kind of food.

Key words: human nutrition / primary school children / food dislikes / eating habits / Slovenia

1 UVOD

Pravilna prehrana in redna telesna dejavnost sta ključna dejavnika za optimalno rast in razvoj mladostnika (Reinhardt in Brevard, 2002; Story in Neumark-Sztainer, 1999). Naklonjenost oziroma odpor do hrane pomembno vplivata na prehranske navade, še posebej v otroštvu (Drewnowski in Hann, 1999; Wardle in Cooke, 2008). Prehranske navade in vedenje so odraz številnih dejavnikov, ki jih lahko v grobem delimo na genetske in okoljske (Wardle in Cooke, 2008).

Med genetskimi dejavniki, ki so predvsem odvisni od naše dedne zasnove, gre izpostaviti tri. Na prvem mestu je brez dvoma okus. Raziskovalci ugotavljajo, da imajo otroci in odrasli raje sladke in slane okuse (Beauchamp in Moran, 1982; Destor in sod., 1977) kot pa kisle ali grenke (Destor in sod., 1975; Steiner, 1979). Naklonjenost do nekaterih okusov najverjetneje kaže človekovo evolucijsko prilagajanje na s sladkorjem in energijo bogata živila ter odklanjanje kisle in grenke hrane, ki bi lahko bila okužena z nevarnimi bakterijami ali toksini (Wardle in Cooke, 2008). Kot vse kaže je dedno pogojena tudi želja po uživanju gostih, visoko energijskih živil (Birch, 1992; Gibson in Wardle, 2003). Genetski dejavnik

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naj bi bil, vsaj deloma, tudi izbirčnost in neofobija (strah oziroma odklanjanje nove, nepoznane hrane). Neofobija naj bi negativno vplivala na sam vnos zelenjave, sadja in mesnin ter skupen vnos energije z živili (Cooke, 2004; Cooke in sod., 2006), nima pa vpliva na količino zaužitih mlečnih izdelkov in škrobnih živil (Cooke in sod., 2003). Rozin in Fallon (1980), Rozin (1991) ter Letarte in sod. (1997) ugotavljajo, da so meso in mesni izdelki najpogosteje omenjena skupina živil, ki je ljudje ne marajo. Najbolj pogosto odklanjajo meso organov, medtem ko sta perutnina in govedina najbolj priljubljeni vrsti mesa (Letarte in sod., 1997). Odpor do mesa in mesnih izdelkov je pogostejši pri ženskah (Kubberod in sod., 2002; Letarte in sod., 1997).

Otroci v procesu interakcije z okoljem razvijajo svojo naklonjenost do živil; izpostavljeni so novim živilom, teksturam, okusom in vonjem (Birch, 1999). Med senzoričnimi lastnostmi ponujenih živil je za sprejemanje pomemben videz in barva živila oziroma jedi (Cardelo, 1996). Učijo se doma, v šoli in pri prijateljih (Skinner in sod., 1998). Zelo pomemben okoljski dejavnik je družinsko okolje, še posebej prehranske navade mater (Scaglioni *et al.*, 2008). Starši oblikujejo otrokove prehranske navade in vedenja s svojim lastnim zgledom in izbiro živil, ki jim jih ponujajo (Cutting *et al.*, 1999), kot tudi z vedenjem in načinom hranjenja otroka (Birch *et al.*, 2001; Johnson in Birch, 1994). Nezanemarljivi dejavniki okolja so tudi vpliv medijev (Byrd-Bredbener in Grasso, 2000), prehransko izobraževanje v šoli in šolska prehrana (Neumark-Sztainer *et al.*, 1999, Skinner *et al.*, 2002).

V raziskavi smo obravnavali nenaklonjenost do živil pri slovenskih osnovnošolcih, starih med 10 in 15 let. Zanimalo nas je, kateri so najpomembnejši dejavniki, ki vplivajo na nenaklonjenost ter kakšne so razlike v nenaklonjenosti do živil glede na spol in razred, ki ga obiskujejo. Želeli smo tudi ugotoviti, ali so opazne razlike v odporu do hrane med učenci iz vzhodnega in zahodnega dela Slovenije.

2 MATERRIAL IN METODE

2.1 VZOREC

Anketni vprašalnik je izpolnilo 628 učencev iz 16-ih naključno izbranih osnovnih šol iz različnih krajev Slovenije (Murska Sobota, Maribor, Novo mesto, Ljubljana, Kranj, Nova Gorica, Celje, Veržej, Slovenska Bistrica, Dolenjske Toplice, Dol pri Ljubljani, Bled, Ajdovščina, Štore, Marezige, Mirna). Podatki o spolu in starosti anketiranih učencev so v preglednicah 1 in 2.

Preglednica 1: *Spol*
Table 1: *Gender*

Spol	Frekvenca	Delež
Fantje	298	47,5
Dekleta	330	52,5

Preglednica 2: *Starost*
Table 2: *Age*

Starost	Frekvenca	Delež	Kumulativni delež
10	32	5,1	5,1
11	277	44,1	49,2
12	18	2,9	52,1
13	109	17,4	69,4
14	185	29,5	98,9
15	7	1,1	100,0

2.2 OPIS INSTRUMENTA

Anketni vprašalnik smo sestavili na podlagi rezultatov preizkusnega vprašalnika. V preizkusnem vprašalniku so bila navedena živila, za katere smo na podlagi poklicnih izkušenj predvidevali, da jih učenci odklanjajo. V anketni vprašalnik smo vključili 20 živil, za katere so učenci v preizkusnem vprašalniku najpogosteje označili, da jih ne marajo. Za oceno odpora do živil smo uporabili Likertovo petstopenjsko lestvico (Likert, 1932). V nadaljevanju so nas zanimali vzroki, zakaj ne jedo naštetih živil. Izbirali so lahko med 15-imi navedenimi vzroki, ki smo jih prav tako oblikovali na osnovi rezultatov preizkusnega vprašalnika. Anketirani so imeli na voljo tudi odgovor drugo, kjer so lahko dodali vzrok, ki ni bil naveden. Od anketiranih smo pridobili podatek o starosti, spolu, razredu in kraju šolanja. Anketirani so podali tudi svoje podatke o višini in teži.

2.3 POSTOPEK IZVAJANJA ANKETE

Anketni vprašalnik smo preizkusili na skupnem vzorcu 80-tih učencev. Na osnovi rezultatov smo oblikovali končni vprašalnik in ga poslali po pošti na 16 naključno izbranih slovenskih osnovnih šol. V navodilih za izvajalce anket na osnovnih šolah je bilo navedeno, da naj anketo izvedejo v enem šestem razredu in v enem devetem razredu. Do razlik v številu anketirancev med kraji šolanja je prišlo zaradi različnega števila učencev na posamezni šoli in posledično različnega števila učencev v razredih na šolah. Izpolnjene anketne vprašalnike smo po pošti prejeli z vseh 16-ih osnovnih šol.

2.4 OBDELAVA PODATKOV

Podatki vprašalnika so bili obdelani na nivoju deskriptivne in inferenčne statistike. Pri tem smo uporabili frekvenčno distribucijo spremenljivk, osnovno deskriptivno statistiko spremenljivk (mere srednje vrednosti, mere razpršenosti), Levene preskus homogenosti varianc (F-preskus), t-preizkus za neodvisne vzorce, faktorsko analizo (z varimax rotacijo). Podatki in rezultati so predstavljeni v preglednicah in z grafi.

3 REZULTATI IN DISKUSIJA

3.1 NENAKLONJENOST / ODKLONILEN ODNOS DO ŽIVIL IN VZROKI

Za 20 vrst živil, ki so jih osnovnošolci v preizkusnem vprašalniku najpogosteje označili, da jih ne marajo, so anketirani podali svojo oceno na petstopenjski lestvici (ocena 1 pomeni maksimalno nenaklonjenost; ocena 5 pomeni maksimalno naklonjenost do živila). Iz preglednice 3 je razvidno, da anketirani osnovnošolci ocenjujejo z največjim odporom polže, možgane, žabje krake in vampe. Anketirani so si najbolj enotni glede odpora do polžev (SD = 0,821) in možganov (SD = 0,858), ki, glede na njihove ocene, veljata za najbolj nepriljubljeni živili. Med navedenimi živili anketirani najmanj odklonilno ocenjujejo rozine, bučke, ocvirke, krvavice, školjke, vampe in kozje mleko. Z ozirom na povprečne vrednosti za posamezne spremenljivke (živila) in interval zaupanja lahko ugotovimo, da anketirani, razen v primeru buck in rozin, večinoma odklanjajo navedena živila.

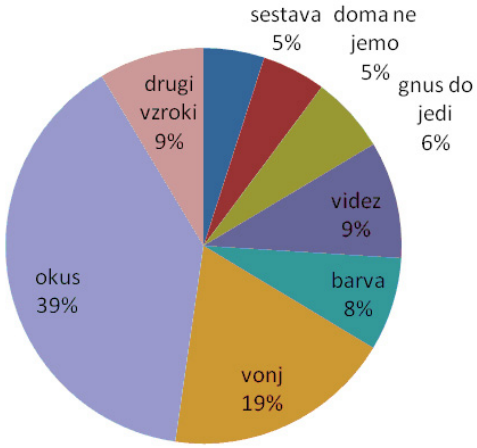
S faktorsko analizo (z varimax rotacijo) smo izračunali manjše število linearnih kombinacij merjenih spremenljivk (pregl. 4) z lastno vrednostjo (ang. eigenvalue) večjo od 1,5. Tri živila (olive, rozine, kozje mleko), katerih vrednosti so bile manjše od 0,35, so bila izločena iz nadaljnje obravnave (Anastasi, 1996). Izračunani faktorji so "o*dpor do zelenjave*" (α = 0,79) s šestimi spremenljivkami, "o*dpor do mesa, predvsem notranjih organov*" (α = 0,68) s šestimi spremenljivkami in "o*dpor do mesa mehkužcev ali dvoživk*" (α = 0,68) s štirimi spremenljivkami. Ena spremenljivka (račje meso) je bila izločena iz nadaljnje obravnave, ker so bile njene vrednosti večje od 0,35 v več kot enem od treh izračunanih faktorjev (Palaigeor-

Preglednica 3: *Odpor do hrane pri slovenskih osnovnošolcih (N = 628)*
Table 3: *Food dislike among Slovenian schoolchildren (N=628)*

	Povpr.	SD	Standardna napaka
Brokoli	2,289	1,421	0,056
Cvetača	2,571	1,510	0,061
Ohrovt	2,065	1,325	0,052
Olive	2,412	1,578	0,062
Por	2,420	1,463	0,058
Bučke	2,998	1,588	0,063
Jajčevci	2,421	1,535	0,061
Rozine	3,496	1,511	0,063
Krvavice	2,761	1,535	0,063
Ocvirki	2,847	1,589	0,063
Kunčje meso	2,377	1,576	0,062
Račje meso	2,590	1,605	0,064
Polž	1,221	0,820	0,033
Kraki	1,399	1,055	0,042
Vampi	1,757	1,350	0,053
Možgani	1,226	0,858	0,034
Jetra	2,382	1,601	0,063
Školjke	2,761	1,771	0,071
Hobotnica	2,507	1,681	0,067
Kozje mleko	2,746	1,531	0,061

Preglednica 4: *Faktorska analiza (z varimax rotacijo)*
Table 4: *Factorial analysis (using varimax rotation)*

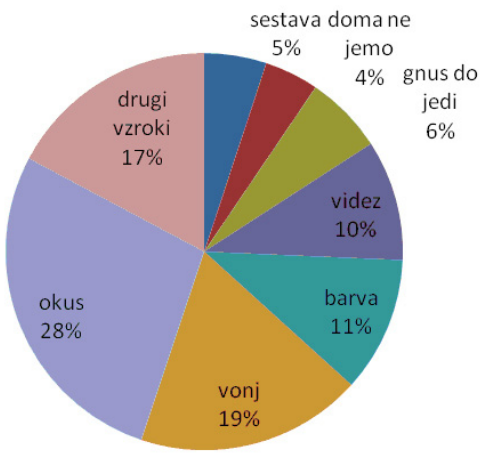
	Odpor do zelenjave	Odpor do mesa, predvsem notranjih organov	Odpor do mesa mehkužcev in dvoživk
Bbrokoli	0,819	0,037	0,013
Cvetača	0,783	0,045	−0,031
Ohrovt	0,735	0,052	−0,032
Por	0,603	0,100	0,128
Bučke	0,659	0,051	0,141
Jajčevci	0,538	0,056	0,262
Krvavice	0,011	0,719	−0,113
Ocvirki	0,031	0,651	0,013
Kunčje meso	0,122	0,552	0,274
Račje meso	0,088	0,488	0,388
Vampi	0,075	0,570	0,211
Možgani	−0,116	0,379	0,158
Jetrca	0,085	0,616	0,132
Polži	0,012	0,108	0,718
Žabji kraki	−0,040	0,152	0,737
Školjke	0,284	0,091	0,654
Hobotnica	0,220	0,146	0,653
Lastna vrednost	4,314	2,413	1,524



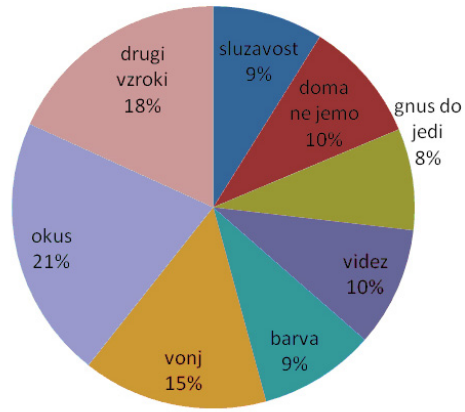
Slika 1: *Glavni vzroki za odpor do zelenjave.*
Figure 1: *Main reasons for resufing vegetables.*

giou in sod. 2005). Skupni Crombach alfa koeficient za instrument (brez štirih spremenljivk) je 0,87.

V nadaljevanju smo anketirane osnovnošolce spraševali po vzrokih za odklonilen odnos do živil. Najpomembnejši vzroki za odklanjanje navedenih živil so njihove senzorične lastnosti. Iz primerjave posameznih dejavnikov (slika 1, slika 2, slika 3) je razvidno, da sta glavna vzroka za odpor do živil okus in vonj, s čimer se naše ugotovitve ujemajo z rezultati tujih raziskav (Beauchamp in Moran, 1982, Destor in sod., 1977). Najpomembnejši okoljski dejavnik, ki vpliva na odklonilen odnos osnovnošolcev do možganov, žabjih krakov, polžev ter še nekaterih drugih živil živalskega izvora, so prehranske navade v družini. Nezanemarljiv vzrok za odklanjanje kunčjega in račjega mesa je ljubezen do živali, ki jo čutijo anketirani osnovnošolci. Jedi, kot so npr. vampi, možgani, žabji kraki, polži in krvavice, anketirani odklanjajo tudi zaradi gnusa. Odklanjanje živil zaradi nepoznavanja le teh, kar v strokovni literaturi imenujejo neofobija (Cooke, 2004; Cooke in sod., 2006), lahko opazimo v odporu anketiranih osnovnošolcev do živil, kot so npr. možgani, žabji kraki in polži.



Slika 2: *Glavni vzroki za odpor do mesa, predvsem notranjih organov.*
Figure 2: *Main reasons for refusing meat, mainly inner organs.*



Slika 3: *Glavni vzroki za odpor do mesa mehkužcev in dvoživk.*
Figure 3: *Main reasons for refusing meat from molluscs and amphibians.*

Iz slike 1 je razvidno, da sta med slovenskimi osnovnošolci okus in vonj daleč najpomembnejša vzroka za odklonilen odnos do zelenjave, kar je najverjetneje posledica dovzetnosti/naklonjenosti otrok in odraslih za sladke in slane okuse ter odpor do kislih in grenkih okusov. Pri odporu do mesa, predvsem notranjih organov, so vzroki bolj enakomerno razporejeni (slika 2). Poleg vonja in okusa pomembno vplivata na odpor tudi videz in barva. Med drugimi vzroki (17 %) anketirani omenjajo vegetarijanstvo, ime jedi, nepoznavanje jedi, verske razloge, ljubezen do živali itd. Tudi v odporu do mesa mehkužcev in dvoživk so vzroki raznoliki (slika 3). Poleg okusa, vonja, videza in barve živila anketirani odklanjajo živila tudi zato, ker jih ne pripravljajo doma, se jim žival (jed) gnusi oziroma so sluzaste. Med drugimi vzroki (18 %) pogosteje omenjajo nepoznavanje jedi, njeno sestavo, ime živila in ljubezen do živali.

3.2 RAZLIKE GLEDE NA SPOL IN RAZRED OSNOVNE ŠOLE

S pomočjo t-testa smo ugotavljali, kakšne so razlike v odklanjanju živil glede na spol. Iz preglednice 5 je razvidno, da so razlike med spoloma statistično značilne. Fantje imajo bolj odklonilen odnos do navedenih vrst zelenjave ($p = 0,020$). Dekleta pa imajo bolj odklonilen

Preglednica 5: *Odklanjanje živil glede na spol*
Table 5: *Food dislike regarding sex*

	Spol	N	Povpr.	SD	t	df	p-vrednost
Odpor do zelenjave	M	298	2,360	0,9812	−2,329	626	0,020
	Ž	330	2,552	1,0888			
Odpor do mesa, predvsem notranjih organov	M	298	2,540	0,9421	8,763	566	0,000
	Ž	330	1,940	0,7491			
Odpor do mesa mehkužcev in dvoživk	M	298	2,091	1,0405	2,825	605	0,005
	Ž	330	1,865	0,9581			

odnos do živil živalskega izvora – odpor do mesa, predvsem notranjih organov (p = 0,000), ter odpor do mesa mehkužcev in dvoživk (p = 0,005). Po rezultatih sodeč so dekleta veliko bolj izbirčna od fantov in odklanjajo večje število živil obravnavanih v raziskavi. Rezultati potrjujejo ugotovitve raziskav Kubberod in sod. (2002) in Letarte in sod. (1997), da ženske pogosteje odklanjajo meso in mesne izdelke kot moški. Manj odklonilen odnos imajo le do zelenjave, kar je verjetno posledica sodobnih modnih trendov, ki dekletom zapovedujejo suhe postave. Uživanje nizko kalorične hrane, kot je zelenjava, pomaga ohranjati želena telesna razmerja.

Ugotavljali smo tudi razlike v odporu do hrane med šestošolci (Mo = 11 let) in devetošolci (Mo = 14 let). Ugotovili smo, da imajo devetošolci večji odpor do mesa, predvsem notranjih organov, (t = 2,126; df = 624; p = 0,034) kot pa šestošolci (pregl. 6). Druge razlike niso statistično značilne.

3.3 RAZLIKE MED VZHODNO IN ZAHODNO SLOVENJO

Pri ugotavljanju razlik v odporu do hrane med osnovnošolci iz vzhodnega in zahodnega dela Slovenije smo v analizo izmed 16 šol vključili samo tri najbolj vzhodne (Murska Sobota, Maribor, Veržej) in tri najbolj zahodne (Marezige, Ajdovščina, Nova Gorica) šole. S pomočjo t-testa smo primerjali regionalne razlike za tri izračunane faktorje odpora do hrane.

Preglednica 6: *Odpor do hrane glede na razred osnovne šole*
Table 6: *Food dislike regarding age (school grade)*

	Razred	N	Povpr.	SD	t	df	p-vrednost
Odpor do zelenjave	6.	317	2,459	1,039	0,058	624	0,954
	9.	309	2,454	1,040			
Odpor do mesa, predvsem notranjih organov	6.	317	2,297	0,921	2,126	624	0,034
	9.	309	2,145	0,865			
Odpor do mesa mehkužcev in dvoživk	6.	317	1,950	0,976	−0,420	624	0,675
	9.	309	1,983	1,019			

Preglednica 7: *Razlike med osnovnošolci iz vzhodne (vzh) in zahodne (zah) Slovenije v odporu do hrane*
Table 7: *Differences in food dislike between schoolchildren from eastern and western part of Slovenia*

	Slovenija	N	Povpr.	SD	t	df	p-vrednost
Odpor do zelenjave	Vzh.	143	2,374	0,975	−1,188	250	0,236
	Zah.	109	2,527	1,066			
Odpor do mesa, predvsem notranjih organov	Vzh.	143	2,301	0,775	0,248	250	0,804
	Zah.	109	2,275	0,848			
Odpor do mesa mehkužcev in dvoživk	Vzh.	143	1,902	0,951	−2,292	250	0,023
	Zah.	109	2,172	0,891			

Vzhodna (Vzh) Slovenija: Murska Sobota, Maribor, Veržej; Zahodna (Zah) Slovenija: Marezige, Ajdovščina, Nova Gorica

Iz preglednice 7 je razvidno, da so razlike med vzhodno in zahodno Slovenijo statistično značilne pri odporu do mesa mehkužcev in dvoživk ($t = -2{,}292$; $df = 250$; $p = 0{,}023$). Osnovnošolci iz zahoda Slovenije imajo manjši odklonilen odnos do tovrstne hrane kot osnovnošolci iz vzhoda Slovenije. Razlike so bile pričakovane, saj so školjke, polži in hobotnice mediteranska hrana. Ta je veliko bolj dostopna v zahodni Sloveniji, ki se razteza proti Jadranskemu morju. Tudi sama kulinarična raznovrstnost slovenskega prostora vpliva na razlike v odnosu do mediteranske hrane v prid učencev iz zahodnega dela Slovenije.

4 SKLEPI

Na osnovi rezultatov raziskave lahko naše ugotovitve strnemo v naslednje sklepe:

- osnovnošolci odklanjajo živila največkrat zaradi senzoričnih lastnosti, kot sta okus in vonj;
- med dejavniki okolja imajo največji vpliv na sprejemanje ali odklanjanje živil prehranske navade v družini;
- anketirana dekleta so bolj izbirčna kot fantje; manj odklonilen odnos imajo le do zelenjave;
- devetošolci bolj odklanjajo živila živalskega izvora, predvsem notranjih organov (npr. možgani, jetrca, vampi), kot šestošolci;
- razlike med osnovnošolci iz zahodne in vzhodne Slovenije se kažejo v odporu do mesa mehkužcev in dvoživk. Osnovnošolci v zahodni Sloveniji imajo manjši odpor do tovrstne hrane, kar je lahko odraz poznavanja tradicionalne prehrane okolja in boljše dostopnosti mediteranske hrane na zahodu Slovenije.

Ker preko 95 % učencev v Sloveniji uživa dopoldansko malico, ki jo pripravljajo v šolski kuhinji, okoli 60 % učencev pa tudi kosila (Zavod Republike Slovenije za šolstvo, 2009), rezultati raziskave lahko služijo za usmerjanje načrtovanja šolskih jedilnikov, predvsem pri ponudbi živil.

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USE OF HERBS AND SPICES AND THEIR EXTRACTS IN ANIMAL NUTRITION

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Use of herbs and spices and their extracts in animal nutrition

The ban on nutritive antibiotic use in Europe and the increased awareness of the consumers triggered a need for natural and safe feed additives to achieve better production results of farm animals. Plant extracts are used in animal nutrition as appetite and digestion stimulants, stimulants of physiological functions, for prevention and treatment of certain pathological conditions, as colorants and antioxidants. This article is a review of present literature data on the usage of plant extracts in poultry, pig and ruminant nutrition.

Key words: animal husbandry / pigs / ruminants / poultry / animal nutrition / herbs / spices / plant extracts

Uporaba zelišč in začimb ter njihovih ekstraktov v prehrani živali

Prepoved uporabe nutritivnih antibiotikov v prehrani živali v Evropi in naraščajoča zavest potrošnikov je sprožila potrebo po uporabi naravnih in zdravih prehranskih dodatkov za doseganje boljših proizvodnih rezultatov. Rastlinske izvlečke v prehrani živali uporabljamo kot stimulatorje apetita in prebave, za preprečevanje in zdravljenje nekaterih bolezenskih stanj, za stimuliranje fizioloških funkcij, kot barvila in kot antioksidante. Predstavljen članek je pregled dosedanjih znanstvenih dognanj o uporabi rastlinskih ekstraktov v prehrani perutnine, prašičev in prežvekovalcev.

Ključne besede: živinoreja / prašiči / prežvekovalci / perutnina / prehrana živali / zelišča / začimbe / rastlinski izvlečki

1 INTRODUCTION

Only quality feed together with proper hygiene, potable water and management can ensure the production of nutritious animal products with desired organoleptic properties (Saxena, 2008). Keeping farm animals healthy is necessary to obtain healthy animal products. For the last decade the use of additives of natural origin in animal and human nutrition has been encouraged. Numerous researches focused on the clarification of the biochemical structures and physiological functions of various feed additives like probiotics, prebiotics, organic acids and plant extracts.

Herbs, spices and their extracts were already used thousands of years ago in Mesopotamia, Egypt, India, China and old Greece, where they were appreciated for their specific aroma and various medicinal properties (Greathead, 2003). When discussing the use of herbs and spices as feed additives, we can hardly rely only on old believes about health impact of certain herbs and spices or their active components. We need a scientific proof of their beneficial effect on health and performance of the animals to justify their use. The technological progress enables us to more easily determine the structure and function of yet unidentified active molecules of plant origin.

To gain advantageous effects of herbs and spices, they can be added to feed as dried plants or parts of plants and as extracts. The composition of extracts from the same plant depends on the method of extraction and

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the properties of the extraction solvent used. Depending on the chemical characteristics of extraction solvents we can extract only certain molecules. There is also a difference between purified and unpurified extracts. Unpurified extracts contain a number of different molecules extracted with certain solvent, which can affect the action of each other, while purified extracts contain only one active component. The purified active molecules extracted from plants can be sometimes substituted by synthetic naturally identical molecules. Plants mainly contain one or some predominant active molecules (secondary metabolites), which are responsible for certain biological effects. The amount of these molecules varies depending on the variety of plant, growing conditions, harvest time etc. When we need the effect of a specific active component, it is more efficient to use a purified molecule alone than a dried plant or unpurified extract. But we have to be aware, that the potency of an unpurified extract often exceeds the potency of a purified one because of synergistic effect among the molecules in it. When talking about plant extracts, we must mention also essential oils. These are extracts of vaporous oils of strong taste and smell, which are still usually extracted by distillation with steam. Essential oils are very potent molecules and must be used in small quantities. Adversaly they can affect the function of intestinal microflora, can cause allergies, suppress feed intake and can be stored in tissues. With the proper usage, most of essential oils are recognized as GRAS (generally recognized as safe). Today the market offers different extracts of certain aromatic plants, combinations of extracts of different plants, purified active components or combinations of purified active components and synthesized active molecules (naturally identical) (Indresh, 2007).

The effect of active components from herbs and spices depends largely on the dosage used. No effect

Table 1: *Often used plants, its active components and functions (Loo and Richard, 1992; Charalambous, 1994; Kamel, 2000)*
Preglednica 1: *Pogosto uporabljene rastline, njihove aktivne komponente in funkcije (Loo in Richard, 1992; Charalambous, 1994; Kamel, 2000)*

Plant	Used parts	Mayor active component	Function
Aromatic spices			
Nutmeg	Seed	Sabinene	Digestion stimulant, antidiarrhoeic
Cinnamon	Bark	Cimetaldehyde	Appetite and digestion stimulant, antiseptic
Cloves	Cloves	Eugenol	Appetite and digestion stimulant, antiseptic
Cardamom	Seed	Cineol	Appetite and digestion stimulant
Coriander	Leaves, Seed	Linalol	Digestion stimulant
Cumin	Seed	Cuminaldehyde	Digestive, carminative, galactagogue
Anise	Fruit	Anethol	Digestion stimulant, galactagogue
Celery	Fruit, Leaves	Phtalides	Appetite and digestion stimulant
Parsley	Leaves	Apiol	Appetite and digestion stimulant, antiseptic
Fenugreek	Seed	Trigonelline	Appetite stimulant
Pungent spices			
Capsicum	Fruit	Capsaicin	Digestion stimulant
Pepperr	Fruit	Piperine	Digestion stimulant
Horsradish	Root	Allyl izotiocianat	Appetite stimulant
Mustard	Seed	Allyl izotiocianat	Digestion stimulant
Ginger	Rizom	Zingerone	Gastric stimulant
Garlic	Bulb	Allicin	Digestion stimulant, antiseptic
Herbs			
Rosemary	Leaves	Cineol	Digestion stimulant, antiseptic, antioxidant
Thyme	Whole plant	Thymol	Digestion stimulant, antiseptic, antioxidant
Sage	Leaves	Cineol	Digestion stimulant, antiseptic, carminatif
Laurel	Leaves	Cineol	Appetite and digestion stimulant, antiseptic
Mint	Leaves	Menthol	Appetite and digestion stimulant, antiseptic

whatever can be observed at small doses; on the other hand, large amounts can be even toxic.

The search for nutritive antibiotic alternatives in EU and increased awareness and concern of the consumers, further encouraged the precise researches on the possibilities of plant extract use in animal nutrition. The main scope in animal husbandry – to ensure good performance of farm animals and get quality animal products, can be achieved only with the effort to keep the animals healthy. In this aspect, herbs and spices are not just appetite and digestion stimulants, but can, with impact on other physiological functions, help to ensure good health and welfare of the animals, what can positively affect their performance.

2 POSSIBLE USE OF HERBS AND SPICES

2.1 HERBS AND SPICES AS APPETITE AND DIGESTION STIMULANTS

When considering supplementing the feed with herbs and spices or their extracts to stimulate the appetite, we have to know the taste preferences of different animal species. Janz *et al.* (2007) found that pigs preferred the feed supplemented with garlic or rosemary over the feed supplemented with oregano or ginger. Furthermore, Jugl-Chizzola *et al.* (2006) noticed that weaned pigs consumed significantly less feed if it was supplemented with thyme or oregano. If pigs in this experiment had the possibility to choose among feed with or without above mentioned spices, they had chosen the unsupplemented feed. The spices known for their appetite stimulant effect are cinnamon, cloves, cardamom, laurel and mint (Loo and Richard, 1992).

Due to the wide variety of active components, different herbs and spices affect digestion processes differently. Most of them stimulate the secretion of saliva. Curcuma, cayenne pepper, ginger, anis, mint, onions, fenugreek, and cumin enhance the synthesis of bile acids in the liver and their excretion in bile, what beneficially effects the digestion and absorption of lipids. Most of the prelisted spices stimulate the function of pancreatic enzymes (lipases, amylases an proteases), some also increase the activity of digestive enzymes of gastric mucosa (Srinivasan, 2005). Besides the effect on bile synthesis and enzyme activity, extracts from herbs and spices accelerate the digestion and shorten the time of feed/food passage through the digestive tract (Platel and Srinivasan, 2001; Suresh and Srinivasan, 2007).

2.2 ANTIMICROBIAL ACTION OF HERBS AND SPICES

Feed supplements with growth promoting activity increase stability of feed and beneficially influence the gastrointestinal ecosystem mostly through growth inhibition of pathogenic microorganism's growth. Due to improved health status of digestive system, animals are less exposed to the toxins of microbiological origin. Consequently herbs and spices help to increase the resistance of the animals exposed to different stress situations and increase the absorption of essential nutrients, thus improving the growth of the animals (Windisch *et al.*, 2008).

Numerous secondary metabolites formed by plants serve as defence agents against physiological and environmental stressors, predators and pathogenic microorganisms. Several *in vitro* studies showed strong antimicrobial activity of certain plant extracts against Gram– and Gram+ bacteria. Pasqa *et al.* (2006) found a change in long chain fatty acid profile in the membranes of *E. coli* grown in the presence of limonene or cinnamaldehyde. Similar observations were made with *Salomonella enterice* grown in the presence of carvacrol or eugenol and with *Bronchotrix thermosphacta* grown in the presence of either limonene, cinnamaldehyde, carvacrol or eugenol. In the case of *Pseudomonas fluorescens* in *Staphylococcus* aureus none of the tested phytochemicals changed the fatty acid profile. The changes in fatty acid composition can affect surviving ability of microorganisms.

The studies measuring hydrophobicity of *E. coli* (test for measuring the ability of microbial attachment) showed a large increase of hydrophobicity of *E. coli* grown in the presence of St. John's wort or Chinese cinnamon and a moderate increase when medium was supplemented with thyme or Ceylon cinnamon. The differences in hydrophobicity were in good correlation with MIC_{50} values (minimal inhibitory concentration). This confirms the fact that herbs and spices act as antimicrobial agents by changing the characteristics of cell membranes, and causing ion leakage, thus making microbes less virulent (Windisch *et al.*, 2008). The exact antimicrobial action of herbs and spices in *in vivo* situations is hard to evaluate, because of the very complex and balanced microbial populations in gastrointestinal tract and the interaction of active components from herbs and spices with other nutrients. Castillo *et al.* (2006) reported that the mixture of cinnamaldehyde, capsicum oleoresin and carvacrol enhances the growth of lactobacilli, and so increases the ratio of lactobacilli to enterobacteria. So herbs and spices do not posses only the antimicrobial activity, but also modulate the composition of microbial population by prebiotic activity.

2.3 ANTI-INFLAMMATORY ACTION

Extracts of curcuma, red pepper, black pepper, cumin, cloves, nutmeg, cinnamon, mint and ginger showed anti-inflammatory effect in the studies on rats (Srinivasan, 2005; Manjunatha in Srinivasan, 2006). The major active molecules with anti-inflammatory action are terpenoids and flavonoids. These molecules suppress the metabolism of inflammatory prostaglandins. The most known herbs and spices with anti-inflammatory potential in our area are chamomile, marigold, liquorice and anis (Craig, 2001).

2.4 ANTIOXIDATIVE ACTION

Many active components of herbs and spices can prevent lipid peroxidation through quenching free radicals or through activation of antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. Main molecules responsible for the antioxidative properties of herbs and spices are phenolic substances (flavonoids, hydrolysable tannins, proanthocianidins, phenolic acids, phenolic terpenes) and some vitamins (E, C and A). Often used herbs rich in phenolics are: rosemary, thyme, oregano, sage, green tea, chamomile, ginko, dandelion and marigold (Halliwell *et al.*, 1995; Craig, 2001; Ćetković *et al.*, 2004; Škerget *et al.*, 2005; BakIrel *et al.*, 2008; Fasseas *et al.*, 2008).

Herbs and spices can protect the feed against oxidative deterioration during storage. This is a widely used practice in pet food and human food industry. The herb commonly used for feed/food preservation is rosemary (*Rosmarinus officinalis*). It can be used alone or in combination with tocopherols or synthetic antioxidants (Jacobsen *et al.*, 2008).

2.5 IMMUNOSTIMULANT FUNCTION

The immune system generally benefits from the herbs and spices rich in flavonoids, vitamin C and carotenoids. The plants containing molecules which possess immunostimulatory properties are echinacea, liquorice, garlic and cat's claw. These plants can improve the activity of lymphocytes, macrophages and NK cells, they increase phagocytosis or stimulate the interpheron synthesis (Craig, 1999).

3 THE USE OF HERBS AND SPICES IN NUTRITION OF DIFFERENT ANIMAL SPECIES

3.1 POULTRY

How to replace antibiotic growth promoters is also a question for the poultry industry. Some studies on plant extracts are showing promising results. Çabuk *et al.* (2006) measured production parameters of broilers which were supplemented by a mixture of oregano, laurel, sage, anis and citrus essential oils. The mixture of essential oils significantly improved feed conversion, what can be attributed to more effective availability of nutrients due to the changes in intestinal ecosystem.

Lippens *et al.* (2005) tested the efficacy of a mixture of cinnamon, oregano, thyme, cayenne pepper and citrus extracts and a mixture of plant extracts and organic acids in comparison to nutritive antibiotic avilamicin in broiler chickens. Chickens supplemented with plant extracts reached significantly higher body weight than the ones in the control or avilamicin group. Higher body weight was a consequence of increased feed consumption. Feed conversion in group fed plant extracts was 0.4% better than in the group with avilamicin and 2.9% better than in the control group. The authors noticed no synergistic effect between plant extracts and organic acids.

Resistance of coccidia to currently used coccidiostatics to treat coccidiosis represents a serious problem in poultry industry. The use of plant extracts to treat coccidiosis is not a new approach. When searching for the best natural extract to treat coccidiosis, we have to take into account that the extract needs to be at least partially soluble in lipids to penetrate the cellular membrane, because coccidia are located inside the cells. Two Chinese plants, *Dichroa febrifuga* and *Sophora flavescens* are rich in alkaloids which are effective in treating coccidiosis (Youn in Noh, 2001). As infections with *Emeria tenela* include also lipid peroxidation in the intestine, herbs and spices with strong antioxidant potency may represent a good supportive treatment. In one of the latest studies Naidoo *et al.* (2008) studied the capacity of four African plants which would be appropriate to treat coccidiosis: leaves of *Combretum woodii*, leaves and stem of Artemisia afra, a whole plant and seeds of *Vitis vinifera*. Extracts of all chosen plants improved the feed conversion to the same extent as coccidiostatic toltrazuril. The best effect was seen with *Tulbaghia violacea*, which also partially lowered the shedding of oocysts.

The use of herbs and spices as antioxidants is not important only for the health of the animals, but also for the oxidative stability of their products. The effect of oregano essential oil on oxidative stability of chicken and

turkey meat was well studied in the past. Supplementation of turkeys with 200 mg/kg of oregano essential oil significantly decreased lipid peroxidation of cooked and fresh meat during refrigerated storage (Botsoglou *et al.*, 2003b). Essential oil of oregano also efficiently preserved the quality of chicken meat during frozen storage (Botsoglou *et al.*, 2003a). Extracts from herbs and spices in combination with vitamins C and E even more effectively prevent lipid peroxidation in tissues, what was shown in the studies on chickens and turkeys (Papageorgiou *et al.*, 2003; Young *et al.*, 2003). At this time the use of plant extracts instead synthetic or semi-synthetic antioxidants represents higher economical costs, however, this could be avoided with systematic intensified growing of needed plants and new technological processes of extraction.

The colorants for increasing yolk colour in laying hens or skin colour in broilers in intensive production can be of natural (carotenoids) or synthetic origin. Often used forage plants rich in carotenoids are maize and alfalfa. Besides these there are several other plants used for isolation of natural pigments like tagetes and red pepper. The main yellow pigments in tagetes are zeaxantin and lutein, while red pepper contains two important red pigments – capsantin and capsorubin. The extract from tagetes colours the yolk three times less effectively in comparison with the synthetic apo-ester of carotenic acid. Pigments from natural origin also degrade during the feed storage up to 30% (Sirri *et al.*, 2007). Nevertheless, pigments obtained from tagetes or calendula species and red pepper are very suitable as yolk colorants in organic farming.

3.2 PIGS

In the pig production, most problems can be expected in the time of weaning. Weaning can be accompanied by infections, especially with enterotoxic *Escherichia coli*. The use of herbs and spices in piglet nutrition can reduce the incidence of infections. Results from Roselli *et al.* (2007) showed that alicin from garlic protects intestinal cells from increased permeability of membrane in pigs infected with *E. coli*. Garlic also contains active substances which suppress the action of fungi and viruses (Zigger, 2001) and improve the feed intake and daily weight gain of piglets (Janz *et al.*, 2007). Cinnamaldehyde, an active component of cinnamon, possesses antibacterial properties. Zigger (2001) observed larger feed intake and live weight gain of weaned pigs fed feed supplemented with garlic and cinnamon extracts. The mortality due to intestinal disorders dropped from 3.9 to 1.2%. Namkung *et al.* (2004) found that a mixture of cinnamon, thyme and oregano extracts inhibited the growth of coliform bacteria. A brown algae *Ascophyllum nodosum* could be a good feed supplement with growth promoting activity of pigs infected with *E. coli* (Turner *et al.*, 2002).

Table 2: *Lymphocyte DNA damage and urinary 8-OHdG excretion of pigs fed a high PUFA diet with or without Calendula off. extracts*
Preglednica 2: *Poškodbe DNA limfocitov in količina s sečem izločenega 8-OHdG, pri prašičih, krmljenih z visoko vsebnostjo PUFA v krmi z oziroma brez dodatka ekstrakta Calendula off.*

Group	% DNA in the tail of the comet	OTM	8-OHdG (µg/24 h)
Control	7.8[a]	1.74[a]	149.9[ab]
Oil	12.0[b]	4.68[b]	269.4[b]
Calendula off. 1	6.8[a]	1.46[a]	138.9[a]
Calendula off. 2	8.2[a]	2.05[a]	150.6[ab]
Vitamin E	6.6[a]	1.54[a]	216.5[b]
SEM	0.65	0.372	31.44
P-value	< 0.01	< 0.01	0.02

[abc] LS-means – without the same superscript differ significantly, P < 0.05; OTM – Olive tail moment; 8-OHdG – 8-hidroxy-deoxyguanosine.

Combination of carvacrol, cinnamaldehyde and capsicum oleoresin beneficially effected gastrointestinal ecosystem and gastric emptying of weaned pigs (Manzanilla *et al.*, 2004). The same mixture was tested for its antioxidative properties in our laboratory. The mixture effectively protected pig's blood lymphocytes against oxidative DNA damage at the concentration of 271.2 mg/kg of feed. Its effect was comparable to that of 90.4 mg/kg of vitamin E. The concentration of spice mixture supplemented to pigs in this study was not sufficient to fully prevent lipid peroxidation induced by high intake of lightly oxidizable PUFA.

Frankič *et al.* (in press) studied antioxidant capacity of propylene glycol extracts of *Calendula officinalis* (*Calendula off.* 1 – extract from petals, 3 ml/day; *Calendula off.* 2 – extract from whole flowers tops, 3 ml/day) and vitamin E (38.4 mg/day) in the case of oxidative stress induced by high PUFA intake in pigs. The extracts effectively prevented oxidative DNA damage in peripheral lymphocytes (measured as % DNA in the tail of the comet and OTM (Olive tail moment), but did not prevent lipid peroxidation, measured by 8-OHdG (8-hidroxy-deoxyguanosine) (Table 2).

Although most studies concerning the effect of herbs and spices in pig production have been conducted on piglets, Allan *et al.* (2005) carried out an experiment on swine. Swine were fed 1000 ppm of dried oregano leaves and flowers enriched with 500 g/kg of oregano essential oil. Observed beneficial effects of oregano supplementation were: lower mortality rate, less culling during

lactation period, shorter service interval, more live born and less stillborn piglets.

3.3 RUMINANTS

Herbs and spices have been introduced also to ruminant nutrition. Microbial ecosystem in the rumen is composed from complex anaerobic microbial population of bacteria, fungi, protozoa, methanogeneous arhea and bacterifagi. Numerous metabolites produced in rumen during microbial fermentation affect the basic digestive and metabolic functions and productivity of the host. Researchers have been searching for new possibilities to modulate the microbial fermentation in the rumen. The main goal of manipulating the rumen fermentation is to increase the effectiveness of digestion and metabolism of nutrients, to increase the productivity of the animals and to suppress the undesirable processes as methanogenesis. In intensive farming systems the feed additives, including antibiotics, were used to increase the production of milk, meat and wool. The ban on antibiotic use in Europe increases the production costs what triggered the need to search for antibiotic alternatives also in ruminant nutrition.

There are numerous studies showing beneficial effects of herbs and spices on feed intake, immune functions and health, rumen fermentation and productivity of calves, dairy cows, heifers and also beef cattle (Kraszewski *et al.*, 2002; Greathead, 2003; Wawrzynczak *et al.* 2000; Cardozo *et al.* 2006). There are some data of the positive effect of plant supplements in nutrition of sheep and goats (Butter *et al.*, 1999). Extracts of yucca plant contain saponnins and glico-components which are responsible for the increase of rumen fermentation and in some cases for reduction of ammonium synthesis (Ryan, P. and Quinn, T.: http://www.irishscientist.ie/P175.htm). Kudke *et al.* (1999) supplemented calves with powder of *Azadirachta indica* tree. Supplemented calves had higher weight gain than unsupplemented ones. The unsupplemented group had much higher incidence of parasite infections.

Gladine *et al.* (2007) tested the antioxidant effect of marigold, grape, rosemary and citrus extracts in sheep. Lipid peroxidation was induced by continuous infusion of linseed oil into the duodenum. The extracts were applied directly into rumen through the rumen cannula. The results showed that all tested plant extracts kept their antioxidant capacity *in vivo* in sheep. The most bioefficient in limiting lipid peroxidation was marigold extract.

Cardozo *et al.* (2006) studied the effect of alfalfa extract, anise, capsicum, and a mixture of cinnamaldehyde end eugenol on ruminal fermentation in beef heifers. The results indicated that tested concentrations of cinnamaldehyde and eugenol mixture, anise oil and capsicum oil may be used as modifiers of rumen fermentation in beef production systems. Same authors tested six natural plant extracts (garlic, cinnamon, anise, yucca, oregano and capsicum extract) and three secondary plant metabolites (cinnamaldehyde, eugenol, anethole) at five doses and two different pH (7.0 and 5.5) to determine their effect on *in vitro* microbial fermentation using ruminal fluid of heifers (Cardozo *et al.*, 2005). Results demonstrated that the effect of herbs and spices on ruminal fermentation in beef cattle may differ depending on ruminal pH. At pH 5.5, garlic, capsicum, yucca and cinnamaldehyde altered ruminal fermentation in favour of propionate, which is more energetically efficient.

Results obtained in the research of Benchaar *et al.* (2007) showed limited effects of 750 mg/day of essential oil mixture (thymol, eugenol, vanillin, guaiacol and limonene) on nutrient utilization, ruminal fermentation, and milk performance of cows fed diets containing alfalfa or corn silage as a sole forage source. Polish researchers showed that 2% of mixture of *Urtica dioica*, *Pradix teraxci*, *Agrimonia eupatoria*, *Fructus carvi* and *Matrica Chamonilla* improves the quality of milk (Kraszewski *et al.*, 2002).

Tannins, the secondary plant metabolites found in stem, wood, leaves, fruits and seeds of many plant species can positively affect the protein digestion in ruminants. Tannins bind to proteins and form complexes which pass trough the rumen undegraded. These proteins which pass the microbial degradation in the rumen are then successfully utilized by the animal and provide the proteins necessary especially in the special physiological states (like early lactation) and in the cases when feed is not of the best quality (Waghorn *et al.*, 1990). Tannins also prevent bloat of the rumen (Butter *et al.*, 1999) and possess antihelmitic properties (Barry and McNabb, 1999).

Extracts from herbs and spices help to prevent and alleviate different kinds of health problems. They are effective in treatment of endometritis (inflammation of the endometrium) in cows. Esparza-Borges and Ortiz-Márquez (1996) evaluated the effect of extrats of garlic (*Allium sativum*, L), eucalypt (*Eucalyptus globulus*, Labill.) and *Gnaphalium conoideum* on acute endometritis of Holstein cows. The most effective of all extracts was the garlic extract, however, also eucalypt worked beneficially.

4 CONCLUSIONS

The main scope of animal production is to ensure the high productivity, healthy animals and quality animal products, which are stable and appropriate for fur-

ther processing. In this aspect, herbs and spices are not just appetite and digestion stimulants, but can, with impact on other physiological functions, help to sustain good health and welfare of the animals and improve their performance. Current studies show promising results regarding the use of phytochemicals as growth and production promoters. There is still a need to clarify the phytochemical composition and the mechanisms of action for many herbs, spices and their extracts and furthermore, to assess the appropriate dose that should be safely used in specific circumstances and animal species.

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TIME DEPENDENT FORMATION OF MARKERS OF OXIDATIVE STRESS INDUCED BY A HIGH FAT DIET SUPPLEMENTED OR UNSUPPLEMENTED WITH VITAMIN E IN PIGS

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Time dependent formation of markers of oxidative stress induced by a high fat diet supplemented or unsupplemented with vitamin E in pigs

The time dependent formation of oxidative damage induced by polyunsaturated fat in the diet was investigated in an experiment with pigs as a model for humans. The role of vitamin E in the prevention of oxidative stress was also studied. Twenty-four growing pigs were penned individually and after an adaptation period divided into three groups. All groups received isocaloric daily rations composed of a basal diet isocalorically supplemented with: starch, linseed oil or linseed oil and vitamin E. Oxidative stress was evaluated by measuring the degree of lymphocyte and granulocyte nuclear DNA damage, concentration of malondialdehyde (MDA) in blood plasma, 24-hour urine MDA excretion rate and concentration of vitamin E isomers in the blood at the beginning, after 24 hours, after 6 days and at the end of the 22 day experimental period. The results confirmed that a high proportion of polyunsaturated fat in the diet increased lymphocyte and granulocyte DNA damage only after 6 days. The lymphocytes appear to be more sensitive to this type of oxidative stress than granulocytes. The MDA concentration in the blood and urinary MDA excretion after 24 hours of oxidative stress seem to be more accurate indicators than the rate of lymphocyte and especially granulocyte DNA damage. Vitamin E supplementation effectively protects the blood cells against increased DNA damage during the whole course of the experiment, but failed to reduce MDA formation significantly 24 hours and 6 days after the beginning of oxidative load. The study further suggests that supplementation of vitamin E is able to completely prevent DNA damage of both types of investigated blood cells at any time, but is only able to reduce the formation of lipid peroxidation products after prolonged treatment.

Key words: pigs / animal nutrition / oxidative stress / DNA damage / polyunsaturated fatty acids / PUFAs / vitamin E / comet assay / malondialdehyde

Časovna odvisnost tvorbe kazalcev oksidacijskega stresa pri prašičih, povzročenega s prehrano, obogateno z maščobami ter z ali brez dodatka vitamina E

V raziskavi smo spremljali časovno odvisnost oksidacijskega stresa, povzročenega z dodatkom večkrat nenasičenih maščobnih kislin (VNMK) ter vlogo vitamina E pri njegovem zmanjšanju. Poskus smo izvedli na prašičih kot modelu za človeka. V individualne bilančne kletke smo uhlevili 24 mladih rastočih prašičev ter jih po obdobju prilagajanja razdelili v tri skupine. Vse tri skupine so dobivale enake osnovne izokalorične dnevne obroke z dodatki škroba, lanenega olja ali lanenega olja in vitamina E. Oksidacijski stres smo ovrednostili kot stopnjo poškodb jedrne DNK limfocitov in gralulocitov, koncentracijo malondialdehida (MDA) v krvni plazmi, 24-urno izločanje MDA s sečem in koncentracijo izomer vitamina E v krvni plazmi. Vrednosti navedenih parametrov so bile določene na začetku poskusa, po 24 urah, po 6 dneh in na koncu 22 dnevnega poskusa. Rezultati so pokazali, da je visoka vsebnost večkrat nenasičenih maščobnih kislin (VNMK) v prehrani povečala poškodbe jedrne DNK limfocitov in granulocitov že po 6 dneh. Limfociti so se, v primerjavi z granulociti, izkazali kot bolj občutljivi. Koncentracija MDA v krvni plazmi in v 24-tih urah izločena količina MDA s sečem se je v našem primeru izkazala kot boljši pokazatelj oksidacijskega stresa v primerjavi s stopnjo poškodb DNK limfocitov, še posebno granulocitov. Dodatek vitamina E je učinkovito zaščitil krvne celice pred povečanimi poškodbami DNK v celotnem poskusnem obdobju, medtem ko smo po 24 urah in tudi po 6 dneh ugotovili statistično značilno večjo koncentracijo MDA v primerjavi z začetnimi vrednostmi. Na osnovi dobljenih rezultatov lahko sklepamo, da dodatek vitamina E oba tipa preiskovanih krvnih celic v celoti zaščiti pred poškodbami DNK, nastajanje produktov lipidne peroksidacije pa se lahko zmanjša le po dolgotrajnejšem dodajanju.

Ključne besede: prašiči / prehrana živali / oksidacijski stres / poškodbe DNK / večkrat nenasičene maščobne kisline / vitamin E / kometni test / malondialdehid

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1 INTRODUCTION

Diet and nutritional-related lifestyle factors have a great influence on the formation of free radicals in humans and animals, and they are also important for protection against the harmful effects of radicals. Previous studies have shown that oxidative stress induced by too high an intake of dietary polyunsaturated fatty acids (PUFAs) enhances damage to DNA, while increased intake of antioxidants may have a protective function (RDA, 1989). However, to our knowledge no studies were performed to investigate the time dependent course of oxidative stress induced by a high dietary intake of polyunsaturated fat. At the same time, the protective effect of antioxidants in this process has also not been elucidated. Differences in the time dependent formation of various parameters of oxidative stress could be of interest not only from the theoretical point of view but also from practical considerations. Questions such as how fast does oxidative stress occur after increased oxidative load caused by high PUFA intake, how efficiently are antioxidants able to play a protective role, what is the response of different indicators of oxidative stress. The aim of the present study was to investigate time dependent changes of some markers of oxidative stress induced by a high intake of dietary polyunsaturated fat in pig, as a model for humans. At the same time, the potency of vitamin E in preventing these changes was studied.

The hypothesis of the study was that time dependent appearance of different markers of oxidative stress (malondialdehyde concentration in blood plasma and 24-hour urine MDA excretion rate, degree of leukocyte and granulocyte nuclear DNA damage, concentration of vitamin E isomers in the blood) is not the same, and that supplementation of feed with vitamin E provides protection against some of the damaging effects of PUFAs.

2 MATHERIAL AND METHODS

2.1 EXPERIMENTAL ANIMALS, DIETS, BLOOD AND URINE SAMPLES

Twenty-four young growing castrated male crossbreed pigs (live weight 11.9 kg ± 1.0) were included in the experiment. The animals were penned individually in balance cages that allowed separate collection of urine. The experiment was divided into adaptation and experi-

Table 1: *Composition and content of energy and nutrients in daily rations of experimental groups of pigs (estimated for a 12 kg pig)*
Preglednica 1: *Sestava ter energijska in hranilna vrednost dnevnega obroka posamezne poskusne skupine (preračunano na 12 kg prašiča)*

	Group		
	LowFat	HighFat	HighFat+Vit-E
Wheat starch, g/day	227.78	111.58	111.58
Linseed oil, g/day	0.0	53.12	53.12
Maize, g/day	59.80	59.80	59.80
Soybean meal, g/day	117.60	117.57	117.57
Skimmed milk powder, g/day	91.11	91.11	91.11
Mineral – vitamin supplement [1], g/day	2.53	2.53	2.53
Vitamin E, mg/day	0.0	0.0	57.64
Daily feed intake, g/day	498.82	435.71	435.71
Nutritive value:			
Metabolisable energy [2], KJ/day	7423.50	7423.50	7423.50
Proportion of energy from fat [3], %	5	30	30
Proportion of energy from PUFA[3], %	2.9	20.9	20.9
Protein, g/day	86.5	88.7	87.9
Fat, g/day	8.7	56.0	57.1
Total dietary fibre, g/day	44.0	45.2	45.9

[1] Calculated to meet nutritional requirements according to NRC (1998). Mineral-vitamin supplement provided daily: 2.0 g Ca, 3.4 g P, 0.15 g Na, 5500 IU vitamin A, 7.6 IU vitamin E.
[2] The energy value of feedstuffs and diets was estimated according to GEH (1988).
[3] Estimated.

mental periods that lasted for 14 and 22 days, respectively. The animals were fed 2.5 times the maintenance requirement (Prosky *et al.*, 1992). At the beginning of the experimental period, the animals were randomly assigned to three groups. All the groups received isocaloric daily rations composed of an equal amount of the basal diet which was supplemented according to the different dietary treatments: LowFat with starch, HighFat with linseed oil, HighFat+VitE with linseed oil and vitamin E (Table 1). The part of energy requirements which were met by fat in the LowFat group and both linseed oil supplemented groups was 5 and 30%, respectively. The amount of vitamin E in the HighFat+Vit-E diet should cover the increased needs for vitamin E because of the higher PUFA intake was calculated according to Muggli (1994).

The composition and analysis of daily rations in different groups is presented in Table 1. The feed was fed in the form of a feed mixture. All ingredients of the mixture, except linseed oil, were mixed together weekly. The linseed oil was added and mixed to the diet of individual animals before every feeding.

During the adaptation period the animals adapted to the rearing system and all of them received the same diet (LowFat). The animals were fed twice a day. Water was provided at libitum. At the beginning and at the end of the experiment, the pigs were weighted.

At the beginning, after 24 hours, after 6 days and at the end of the 22 days experimental period blood samples were taken from the jugular vein and 48-h urine was collected (except 24 hours after the beginning of the experiment when the collection time was 24 hours).

The content of protein, fat and fibre was determined by standard procedures published by Neumann and Bassler (1997). The fatty acid composition of diets was analyzed by a gas chromatographic method after transesterification of lipids as described previously (Fidler *et al.*, 2000).

2.2 LYMPHOCYTE AND GRANULOCYTE DNA DAMAGE – SINGLE-CELL GEL ELECTROPHORESIS – COMET ASSAY

Blood samples for single cell gel electrophoresis (Comet assay) were collected in 4.5 ml evacuated tubes containing $EDTAK_3$ anticoagulant. Blood samples were stored on ice for a maximum of 1 hour before the separation procedure. Lymphocytes and granulocytes were separated from the blood samples on a discontinuous Percoll gradient according to a modified procedure described by Hjorth *et al.* (1988) and Kjeldsen *et al.* (1999). A partially modified procedure of Singh *et al.* (1988) was implemented for the comet assay. Olympus CH 50 epifluorescent microscope at 200 × magnification was used for the examination of leukocyte nuclei in the microgels (100 W Hg lamp, excitation filter of 480–550 nm and barrier filter of 590 nm). The images were captured by Hamamatsu Orca 1 CCD camera, analyzed and the nuclear DNA damage estimated by a dedicated computer program Comet 4 (Single Cell Gel Electrophoresis, Kinetic Imaging Ltd., 2000). For each treatment, two slides were prepared and 50 cells (total 100 cells) were examined.

2.3 PLASMA AND URINE MALONDIALDEHYDE (MDA) CONCENTRATION

The blood samples for MDA concentration analysis and urine were collected and prepared as described previously (Pajk *et al.,* 2006). The methodology of Wong *et al.* (1987) modified by Chirico (1994) and Fukunaga *et al.* (1995) was used to measure the concentrations of malondialdehyde (MDA) in blood plasma and urine by HPLC using a Waters Symmetry C_{18} chromatography column (5 µm, 4.6 × 150 mm) and a Waters Symmetry C_{18} guard column (5 µm, 3.9 × 20 mm). A Waters Alliance 2690 apparatus equipped with a Waters Dual λ Absorbance Detector 2487 was applied. The results of the analysis were evaluated by the Millenium[32] Chromatography Manager program.

2.4 VITAMIN E CONCENTRATION IN PLASMA

Blood samples for vitamin E concentration were collected in 10 ml evacuated tubes containing $EDTAK_3$ anticoagulant. Plasma was prepared by centrifugation (400 × g for 10 min.) at 4 °C and transferred to micro centrifuge tubes. The samples were stored at –70 °C. According to Abidi (2000) and Aust *et al.* (2001) vitamin E (as α– and β+γ- tocopherols) was extracted from plasma by hexane, after precipitation of proteins with ethanol containing 0.3% (w/v) tert.-butyl-p-cresol (BTH) to prevent oxidation. Samples were analyzed by HPLC (Waters Alliance 2690), using a Waters Symmetry C_{18} chromatography column (5 µm, 4.6 × 150 mm) and an ODS C_{18} guard column (4 mm L × 3.0 mm ID). A Waters Dual λ Absorbance Detector 2487 and Waters Scanning Fluorescence Detector 474 were used.

2.5 STATISTICAL ANALYSIS

The data were analyzed by the General Linear Model (GLM) procedures from SAS® software (SAS, 2000).

Comparisons between the different treatments were made by contrasts provided by the GLM procedure. The data were expressed as least square means ± standard error. A least significant difference of 0.05 was used to separate the treatment means.

3 RESULTS

During the experiment, the animals had no health or other problems, consumed feed without residues and normal body weight gain was observed in all groups (337 ± 61 g per day).

While at the beginning of the experimental period no statistical differences among groups in any of the measured parameters could be observed, already 24 hours after the nutritional intervention some very important differences among dietary treatments were found.

3.1 PLASMA AND URINE MALONDIALDEHYDE CONCENTRATION

The concentration of MDA in plasma and the urinary MDA excretion rate in the LowFat group remained at almost the same level during the whole experimental period (Table 2). In contrast, both MDA parameters increased in both linseed oil supplemented groups significantly already 24 hours after dietary intervention. Six days afterwards and at the end of the 22 day experimental period, the MDA concentration in plasma and the MDA excretion rate in urine in HighFat and HighFat+Vit-E groups were also significantly higher than in the LowFat group.

While 24 hours and six days after the beginning of the experiment increased MDA excretion rates in urine were at the same level in the HighFat and HighFat+Vit-E groups, on the 22nd day the value in the HighFat group

Table 2: *Effect of high polyunsaturated fat and vitamin E intake on plasma malondialdehyde concentration and malondialdehyde excretion in urine during the experiment*
Preglednica 2: *Vpliv zauživanja večkrat nenasičenih maščobnih kislin in vitamina E na koncentracijo malondialdehida v krvni plazmi in količino dnevno izločenega malondialdehida v obdobju poskusa*

	Group		
	LowFat	HighFat	HighFat+Vit-E
MDA in plasma, nmol/ml:			
At the beginning	0.25 ± 0.09	0.26 ± 0.09	0.28 ± 0.07
After 24 hours	0.22^{a} ± 0.10	0.64^{b} ± 0.28	0.47^{c} ± 0.18
After 6 days	0.24^{a} ± 0.14	0.67^{b} ± 0.35	0.69^{b} ± 0.25
After 22 days	0.24^{a} ± 0.12	0.66^{b} ± 0.31	0.48^{c} ± 0.12
MDA urine excretion, nmol/24 hour:			
At the beginning	2 225 ± 1 261	2 193 ± 1 089	2 315 ± 877
After 24 hours	$2\,115^{a}$ ± 652	$8\,589^{b}$ ± 1 679	$7\,363^{b}$ ± 1 519
After 6 days	$2\,519^{a}$ ± 1 126	$10\,067^{b}$ ± 5 342	$8\,659^{b}$ ± 5 279
After 22 days	3402^{a} ± 1421	$20\,588^{b}$ ± 11362	$10\,704^{ab}$ ± 5 100

[a, b] Means with different superscripts in the same line differ significantly; P≤0.05

Table 3: *The percentage of DNA in head of comets in lymphocytes during the experiment*
Preglednica 3: *Odstotek DNK v glavi kometov v limfocitih v obdobju poskusa*

	Group		
	LowFat	HighFat	HighFat+Vit-E
Percentage of DNA in head of comets in lymphocytes			
At the beginning	95.1 ± 0.83	94.9 ± 1.30	94.9 ± 0.66
After 24 hours	93.6^{a} ± 1.06	91.9^{b} ± 1.71	93.9^{a} ± 1.14
After 6 days	93.3^{a} ± 0.94	88.3^{b} ± 0.68	92.9^{a} ± 0.77
After 22 days	91.6^{a} ± 1.85	82.7^{b} ± 2.72	91.3^{a} ± 0.93

[a, b] Means with different superscripts in the same line differ significantly; P≤0.05

Table 4: *The percentage of DNA in head of comets in granulocytes during the experiment*
Preglednica 4: *Odstotek DNK v glavi kometov v granulocitih v obdobju poskusa*

	Group		
	LowFat	HighFat	HighFat+Vit-E
Percentage of DNA in head of comets in granulocytes			
At the beginning	92.9 ± 1.2	92.6 ± 1.9	93.0 ± 2.2
After 24 hours	92.0 ±0.45	91.0 ± 2.15	91.7 ± 0.65
After 6 days	92.3^{a} ±1.0	88.6^{b} ± 0.4	92.0^{a} ± 1.1
After 22 days	92.3^{a} ± 1.8	87.6^{b} ± 2.0	91.7^{a} ± 1.2

$^{a, b}$ Means with different superscripts in the same line differ significantly; P≤0.05

was significantly higher. At this time MDA excretion with urine in the vitamin E supplemented HighFat+Vit-E group did not significantly differ from either the LowFat or the HighFat group. Also the value for plasma MDA concentration was in between that of the other two groups. In this case the difference from the LowFat and to HighFat groups was significant.

3.2 NUCLEAR DNA DAMAGE OF LYMPHOCYTES AND GRANULOCYTES

The results of DNA damage of lymphocytes and granulocytes are presented in Table 3 and 4 as a percentage of DNA in the head of the comet.

The experiment confirmed that a high proportion of polyunsaturated fat in the diet (group HighFat) increased lymphocyte and granulocyte DNA damage. An absolutely small, but significant decrease of degree of lymphocytes DNA damage was observed even after 24 hours. In both types of cells the effect was more strongly expressed after six days. While the decrease in the percentage of DNA in the head of lymphocyte and granulocyte on the 6th day was similar, on the 22nd day of the experimental period the percentage of lymphocyte DNA in the head was lower. In both types of cells degree of lymphocytes DNA damage in the HighFat+Vit-E group remained on the same level during the experiment as in the LowFat group.

3.3 VITAMIN E CONCENTRATION IN PLASMA

Plasma concentrations of α- and β+γ-tocopherol throughout the experiment are reported in Table 5. During the experimental period there was a significant effect of the type of diet consumed. The effect was observed even after 24 hours. While the plasma α–tocopherol concentration in the LowFat and HighFat groups was unchanged during the whole experimental period, the con-

Table 5: *Concentration of α- and β+γ-tocopherol in plasma during the experiment*
Preglednica 5: *Koncentracija α- in β+γ-tokoferola v plazmi v obdobju poskusa*

	Group		
	LowFat	HighFat	HighFat+Vit-E
α–tocopherol (ppm)			
At the beginning	2.46 ± 1.01	2.45 ± 1.03	2.17 ± 0.41
After 24 hours	2.21^{a} ± 1.12	2.02^{a} ±0.79	3.23^{b} ± 1.14
After 6 days	2.08^{a} ± 0.80	2.02^{a} ± 0.93	5.33^{b} ± 1.93
After 22 days	1.83^{a} ± 0.87	1.86^{a} ± 0.99	4.53^{b} ± 1.37
β+γ-tocopherol (ppm)			
At the beginning	0.034 ± 0.011	0.041 ± 0.019	0.051 ± 0.029
After 24 hours	0.032^{a} ± 0.013	0.249^{b} ± 0.0114	0.252^{b} ± 0.112
After 6 days	0.034^{a} ± 0.013	0.180^{b} ± 0.108	0.123^{c} ± 0.097
After 22 days	0.029^{a} ± 0.015	0.237^{b} ± 0.198	0.135^{c} ± 0.068

$^{a, b, c}$ Means with different superscripts in the same line differ significantly; P≤0.05

centration significantly increased in the HighFat+Vit-E group even 24 hours after the beginning of the experiment and remained so afterwards.

The concentration of β+γ-tocopherol in plasma in the LowFat group remained at the same level during the whole experimental period. β+γ-tocopherol concentration significantly increased in the HighFat and HighFat+Vit-E groups even 24 hours after dietary intervention and remained so also on the 6th and 22nd days of the experiment. At this time the concentration in the HighFat+Vit-E group was significantly lower than in the HighFat group.

4 DISCUSSION

The time dependent formation of oxidative stress induced by a high dietary intake of polyunsaturated fat is currently not well known. At the same time, the protective effect of vitamin E in this process has not yet been elucidated.

In the present study oxidative stress was induced by the selection of linseed oil which contains 73 wt. % of PUFA (Rezar *et al.*, 2003). The energy supply from PUFA was approximately 19% (Table 1). It is known that a high intake of PUFA increases the nutritive requirements for antioxidative vitamins (Muggli, 1994). The oxidative stress in both groups fed linseed oil was additionally increased by the fact that the supply of supplemented antioxidative vitamins was not increased.

As expected, feeding linseed oil in the HighFat group increased the oxidative stress by increasing not only the formation of products of lipid peroxidation but also the rate of blood cell DNA damage. The increased presence of MDA in plasma and urine reflects the products of lipid oxidation originating from diet and formed in the tissues (Guichardant *et al.*, 1994). Our previous studies (Rezar *et al.*, 2003) showed plasma MDA concentration and especially MDA excreted in the urine to be sensitive biochemical markers of the extent of lipid peroxidation.

A study by Marnett (2002) found that lipid peroxidation is one of the major sources of endogenous DNA damage in humans that may contribute to cancer and other chronic diseases linked to lifestyle and dietary factors. The results of the present experiment show that the high intake of PUFA in the HighFat group significantly increased not only the concentration of MDA in blood plasma and the urinary MDA excretion rate but also the degree of both lymphocyte and granulocyte DNA damage. The results obtained clearly demonstrate the harmful effects of polyunsaturated fat in the diet on the oxidative status of pigs, which in view of their metabolism and digestion may serve as a good model for humans (Darcy-Vrillon *et al.*, 1993).

While the effect of oxidative stress induced by a high fat intake on lipid peroxide formation has already been shown (Yang *et al.*, 1997; Rezar *et al.*, 2003), the present study is, to our knowledge, the first to demonstrate *in vivo* time dependent effects and the different effects on MDA formation and lymphocyte and granulocyte DNA integrity.

As assumed, the degree of lymphocyte and granulocyte DNA damage was not the same. According to their different physiological roles and life span, these two cell types can exhibit rather different sensitivities to chemical, physical or biological insults in DNA damage (Giovannelli *et al.*, 2003) and probably different DNA repair capacities (Šrám *et al.*, 1998). A difference between the DNA damage of lymphocyte and granulocyte was observed even after 24 hours and was even greater on the 22nd day of the experiment (Table 2, 3). In the present study, lymphocytes, which have a longer life-span, were found to respond faster and to accumulate DNA damage with prolonged PUFA exposure. The rate of DNA damage in lymphocytes increased with prolonged oxidative stress. Tice (1995) found that short-lived granulocytes may provide information only on current exposure whereas lymphocytes might also give information on past exposure. The results thus provide some evidence that lymphocytes are more sensitive to oxidative stress caused by PUFA than granulocytes, as a result of their DNA repair system and/or longer life span.

While the level of lymphocyte and granulocyte DNA damage did not change 24 hours after the beginning of the experiment, the plasma MDA concentration and urine MDA excretion rate already at that time showed a significantly higher rate of lipid oxidation (Table 4). This indicates that at least in the early stage of such a type of increased oxidative stress MDA measurements are more sensitive parameters of increased oxidative stress. During the experiment the MDA concentration in the blood and the rate of MDA excretion with urine increased. It is obvious that the increase in the latter was much more pronounced and correlates better with the rate of DNA damage of lymphocytes than granulocytes.

Determination of α- and β+γ-tocopherols in plasma may contribute information on the antioxidant status of an individual and may be useful for evaluation of nutritional status and risk of degenerative diseases (Aust *et al.*, 2001). It is known from other investigations (Mileva *et al.*, 2002) that as a consequence of increased oxidative load occurs a decrease in the concentration of antioxidative substances in the blood. On that account it was expected that the concentration of α-tocopherol in plasma would decrease in the HighFat group. But that was not

the case. The reason for this might be a low α-tocopherol concentration of the basal diet and might indicate that in the experiment the applied NRC (1998) recommendations are too low. Since linseed oil is a poor source of α-tocopherol (8.59 mg/100 g) and a good source of β+γ-tocopherol (106, 93 mg/100 g), an increase in plasma β+γ-tocopherol concentration in the HighFat group was expected and actually observed.

As expected, the consumption of vitamin E in the form of α-tocopherol in the HighFat+Vit-E group also significantly increased the concentration of α-tocopherol in plasma during the whole course of the experiment (Table 5). The increase in plasma α-tocopherol was also positively associated with the observed parameters of oxidative stress (Tables 2, 3, 4). The degree of lymphocyte and granulocyte DNA damage in the HighFat+Vit-E group was significantly lower than in the HighFat group. Moreover, the degree of lymphocyte and granulocyte DNA damage in the HighFat+Vit-E group was at the same level as in the LowFat group and was not influenced by the high unsaturated fat intake during the whole experimental period. The positive effect of vitamin E on oxidative stress was also demonstrated as a reduction in MDA concentration in plasma and urine (HighFat+Vit-E group) (Table 4). But in contrast to the protective effect in blood cells, a significant protective effect was not observed earlier than at the 22nd day of the experiment. At this point the vitamin E supplementation was able to reduce MDA formation by approximately 50%. Some other investigators also found that supplementing the diet with vitamin E reduces the plasma or urinary MDA level as well as liver MDA concentration (Cadenas *et al.*, 1996; Naidoo *et al.*, 1998; Kirimlioglu *et al.*, 2006). Since the amount of MDA excreted in the urine correlates positively with its synthesis in the body (Siu and Draper, 1982), and the measurement of urinary-excreted MDA is a more precise indicator of the plasma MDA concentration (Guichardant *et al.*, 1994; Kosugui *et al.*, 1994), the reduced oxidative load in the vitamin E supplemented group could be regarded as even more important.

5 CONCLUSIONS

The results confirmed that a high proportion of polyunsaturated fat (PUFA) in the diet increased the measured parameters of oxidative stress. The lymphocytes proved to be a more sensitive indicator of this type of oxidative stress but the difference was observed only after longer exposure to this type of oxidative stress (22 days). The concentration of MDA in plasma and the rate of urine MDA excretion proved to be very sensitive indicators of oxidative stress, since they responded to an increased unsaturated fatty acid load even after 24 hours. The study further suggests that supplementation of vitamin E is able to completely prevent the formation of DNA damage of both types of investigated blood cells at any time, but is only able to reduce the formation of products of lipid peroxidation after prolonged treatment.

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EFFECTS OF DIETARY PECTIN ON PROTEIN DIGESTION AND METABOLISM IN GROWING RATS

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Effects of dietary pectin on protein digestion and metabolism in growing rats

In an attempt to clarify the relationships between the digestive and metabolic effects of additional feeding of citrus pectin, the intestinal contents, tissues weights, nitrogen excretion and retention were studied in laboratory rats. Twenty-four growing male Wistar rats (98.8 g ± 5.3 g of body weight) were fed *ad libitum* for 20 days with balanced diets containing casein as the source of protein. In the experimental diet 96 g of wheat starch was replaced by 80 g of citrus pectin and 16 g of vegetable oil. Apparent digestibility and apparent protein biological value were calculated, the weights of digestive tissues and digestive organ content weights were also determined. All tissues of different parts of the digestive tract were heavier in the pectin fed group, and small intestine and caecum were statistically significant different as compared to control group. The contents of the small intestine and caecum were significant heavier in the pectin group. Pectin significantly lowered dry matter intake and growth rate and significantly increased faecal excretion of nitrogen and significantly decreased urinary nitrogen excretion. The consequence of alteration in the nitrogen excretion route was significantly lower apparent protein digestibility and apparent net protein utilisation, but apparent protein biological value was unchanged.

Key words: proteins / digestibility / metabolism / pectin / small intestine / large intestine / laboratory rats

Vpliv pektina na prebavo beljakovin in metabolizem pri rastočih podganah

Da bi poskusili razložiti povezavo in vpliv krmljenja pektina iz limonine lupine na prebavo in presnovo, smo izmerili maso vsebine prebavil, maso tkiv prebavil ter izločen in absorbiran dušik pri laboratorijskih podganah. Štiriindvajset rastočih laboratorijskih podgan moškega spola seva Wistar (s povprečno telesno maso 98,8 g ± 5,3 g) smo 20 dni krmili *ad libitum* z uravnoteženo krmo, ki je vsebovala kazein kot vir beljakovin. V poskusni krmi smo 96 g pšeničnega škroba zamenjali z 80 g pektina iz limonine lupine in 16 g mešanice rastlinskih olj. Izračunali smo navidezno prebavljivost beljakovin in navidezno biološko vrednost beljakovin ter določili maso posameznih tkiv in vsebine prebavil. V poskusni skupini, ki je imela v krmi pektin, smo izmerili večjo maso tkiv posameznih delov prebavil v primerjavi s kontrolno skupino, s statistično značilnimi razlikami pri tankem in slepem črevesju. Masa vsebine tankega in debelega črevesja je bila statistično značilno večja pri skupini s pektinom. V skupini s pektinom v krmi so živali zaužile značilno manj suhe snovi krme in imele manjše priraste, značilno več izločenega dušika v blatu, pa tudi značilno manj izločenega dušika preko seča. Posledica razlik v izločanju dušika preko blata ali seča je značilno manjša navidezna prebavljivost in navidezna neto izkoristljivost beljakovin, medtem, ko je navidezna biološka vrednost beljakovin ostala nespremenjena.

Ključne beseda: beljakovine / prebavljivost / presnova / pektin / tanko črevo / debelo črevo / laboratorijske podgane

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1 INTRODUCTION

Pectin is a component of many feedstuffs. Especially citrus and sugar beet pulp are rich in pectin and to a limited degree it is present in grains and legumes. Pectin improves the stability and tight connection of plant cells, their osmolality and water content, and together with hemicellulose, cellulose and lignin it reinforces the cell matrix.

The name pectin is derived from the Greek word "pectos", which means gelatinizing or swelling and is related to the main physical and chemical character of this molecule in establishing gels. Most authors point out the resistance of pectin to the digestive enzymes of mammals and include it among the non-starch polymers (Eastwood, 1992). A logical approach is the classification of pectin as part of dietary fibre (Trowell *et al*., 1976; Eastwood, 1978; Schneemann, 1986; McDougall *et al*., 1996), but only a small part of pectin belongs to the cell wall. The rest is part of the cell cytoplasm and most fractions of pectin are soluble. After isolation, pectin can be used as a special dietetic feed composition.

A large number of investigations have been carried out to study the effect of dietary fibre on the digestibility of nutrients. Most authors report that the fibre content of diet can impair the apparent digestibility of nutrients. In particular, the effect of dietary fibre differs with the source and nature of the fibre and relates to its chemical composition as well as to its physical and chemical properties. The effect of the viscous nature of dietary fibre, like pectin, on digestibility is contradictory. Already Murray *et al*. (1977) reported a decrease in the apparent digestibility of nitrogen in pigs after feeding gel-forming polysaccharides (methyl-cellulose or pectin), whereas the replacement of starch by cellulose caused no decrease. Other authors observed no effect of gel-forming polysaccharides on nitrogen digestion in rats (Larsen *et al*., 1994) and in pigs (Li *et al*., 1994). Soluble polysaccharides have been found generally to be without significant effect on the apparent digestibility of nutrients in pigs (Huisman *et al*., 1985). For other authors, not only the amount of nitrogen excreted in faeces, but also the nitrogen excreted in urine was found to be affected by the type and fermentability of carbohydrate. Pastuszewska *et al*. (2000a) found that faecal nitrogen excretion in rats was increased by all carbohydrates (potato starch, pectin or cellulose) when substituted for cereal starch, but only pectin decreased urinary nitrogen excretion. Carbohydrates significantly altered the routes of nitrogen excretion in protein-free diets too (Pastuszewska *et al*., 2000b).

The present study was designed to examine the effect of pectin on protein utilisation and on the development of the gastrointestinal tract to obtain a better picture of the nutritive functions of pectin in growing rats.

2 MATERIAL AND METHODS

2.1 DIETS

Two diets were prepared, a control diet and a pectin diet in which a fraction of the wheat starch was replaced by pectin from citrus peel (Fluka ref. No. 76280, degree of esterification 63–66%, MW 30 000–100 000). The diets contained different amounts of an oil mixture to adjust the diets' energy concentrations. The protein source in the diets was casein purchased from Union des Caséineries de Charente Maritime (La Rochelle, France) and the diets were calculated to contain 110 g of crude protein. Diets (Table 1) were designed to meet the nutritional requirements of growing rats (NRC, 1995). Weende analysis, mineral content and dietary fibre analysis (Lee *et al.*, 1992) of the diets were performed.

2.2 ANIMALS AND EXPERIMENTAL PROCEDURE

All procedures were performed according to current legislation on animal experimentation in Slovenia. Permission for the experiment was granted by the Veterinary Administration of the Republic of Slovenia (VURS) under the number 323-02-215/2004/2. Twenty four male Wistar rats (98.8 g ± 5.3 body weight) reared in the Lek laboratory animal unit (Ljubljana, Slovenia) were housed in cages placed in a room kept at about 21 °C and 60% humidity (checked and recorded each day), with light automatically regulated on a 12-hour light/dark cycle starting at 7.00 a.m. After a 4 days adaptation period, in which the rats received a control diet, rats were separated into two equal groups (n = 12), with average body weight 120.1 ± 5.5 g and 120.1 ± 5.2 g in the control and pectin groups, respectively. Animals were individually housed in metabolic cages which permitted the collection of urine and faeces separately during the experiment, and had free access to drinking water. They received *ad libitum* control or pectin semi-synthetic diet during an 18 or 20-day period.

On the 6th day of the experimental period the 5-day balance study began (Orešnik and Cvirn, 1984; Orešnik *et al.*, 1982; Stekar *et al.*, 1984). Each day animals received a new weighed daily meal and the residue from the day before was weighed. Body weights were recorded on the first day of the balance experiment, on the third day and on the last day. Urine was collected in a bottle after filtra-

Table 1: *Diets (g/kg)*
Preglednica 1: *Krma (g/kg)*

	Control Kontrola	Pectin Pektin
Casein / Kazein	120	120
Wheat starch / Pšenični škrob	616	520
Premix / Premiks [1]	40	40
Sugar (sucrose) / Sladkor (saharoza)	50	50
Mixture of vegetable oils Mešanica rastlinskih olj [2]	54	70
Agar-agar / Agar-agar	40	40
Pectin / Pektin	0	80
Mineral mixture Mešanica rudninskih snovi [3]	70	70
Vitamin mixture Mešanica vitaminov[4]	10	10
Sum / Vsota	1000	1000

[1] Mixture of wheat starch and L-cystine 55 mg/g (2.2 g of cystine added in each diet) / Mešanica pšeničnega škroba in L-cistina 55 mg/g (2,2 g cistina dodanega vsaki krmi)
[2] Rape oil, groundnut oil and sunflower oil (50:45:5) / Repično olje, arašidovo olje in sončnično olje (50:45:5)
[3] Minerals UAR 205 b (UAR, Villemoisson-sur-Orge, France) / Rudninske snovi UAR 205 b
[4] Vitamins UAR 2.00 / Vitamini UAR 2,00

tion. This bottle contained 10 ml of 6 M HCl for each cage to stop all reactions in the urine and to prevent losses of nitrogen. The faeces samples were collected in a different vessel. On the last day (5^{th}) of the balance study, the urine was transferred to a prepared plastic bottle, weighed and stored at −20 °C until analyses were performed. Faeces samples were also stored in prepared plastic bottle, weighed and frozen.

Before taking an aliquot of the sample for analysis, faeces were homogenised in a ceramic holder. Urine was homogenised by shaking to prevent stratification. In diets, faeces and urine nitrogen was determined by the Kjeldahl method. Dietary crude protein (CP) was evaluated as N × 6.25. Dry matter and crude ash were determined in diets and faeces. Based on the amount of N intake, N in faeces and N in urine, the following indices of N utilisation were calculated: nitrogen apparent digestibility = (N intake − N in faeces) / N intake; apparent protein biological value = (N intake − N in faeces − N in urine) / (N intake − N in faeces) and apparent net protein utilisation (NPU) = (N intake − N in faeces − N in urine) / N intake; protein efficiency ratio (PER) = growth rate / CP intake. Digestibility of organic matter (OM) = OM intake − OM in faeces / OM intake and dry matter efficiency: growth rate / dry matter intake × 100 were also calculated.

2.3 SAMPLING OF TISSUES

Half of the animals in each group were sacrificed on the 18^{th} day of the experiment and the other half on the 20^{th} day in the morning between 9 and 11 o'clock. Rats were anaesthetized with an abdominal injection of Pentothal (0.1 ml/100 g of body weight, Sigma, Saint Louis, Mo., USA). The digestive tract was quickly removed. Each part of the digestive tract (stomach, small intestine, colon and caecum) was weighed with its content. The digestive content of the small intestine was collected. The small intestine wall was rinsed with a cold 2% TCA solution, wiped and weighed. Stomach, colon and caecum treatment was similar to treatment of the small intestine.

2.4 DATA ANALYSIS

Data were analysed by the General Linear Models (GLM) procedure (SAS/STAT, 2000), taking into consideration the diet as the main effect, and in the case of the relative weight of intestinal tissues and gastrointestinal content also the day of slaughter. Data are expressed as least square means (LSM) ± standard deviation (SD). Significance was considered established at $P < 0.05$.

Table 2: *Relative weight of digestive tissues (g per 100 g of the body weight) (average ± SD)*
Preglednica 2: *Relativna masa tkiv prebavnega trakta (g / 100 g telesne mase) (povprečje ± SD)*

	Control / Kontrola (12)	Pectin / Pektin (12)	P-value / p-vrednost *
Stomach / Želodec	0.63 ± 0.14	0.66 ± 0.06	0.5410
Small intestine / Tanko črevo	2.94 ± 0.29	3.63 ± 0.84	**0.0151**
Caecum / Slepo črevo	0.41 ± 0.07	0.65 ± 0.10	< **0.0001**
Colon / Kolon	0.63 ± 0.07	0.70 ± 0.11	0.0656
Whole intestine / Celotno črevesje	3.97 ± 0.33	4.97 ± 0.88	**0.0016**
Digestive tract / Prebavni trakt	4.60 ± 0.42	5.63 ± 0.88	**0.0016**

* Diet and day of slaughtering as two main effects / Krma in dan žrtvovanja kot dva glavna vpliva

Table 3: *Weights (g) of the gastrointestinal content (average ± SD)*
Preglednica 3: *Mase (g) vsebine prebavil (povprečje ± SD)*

	Control Kontrola (12)	Pectin Pektin (12)	P-value p-vrednost *
Content of stomach / Vsebina želodca	4.40 ± 5.71	3.06 ± 3.08	0.1634
Content of small intestine / Vsebina tankega črevesja	1.42 ± 0.78	3.34 ± 2.57	**0.0069**
Content of caecum / Vsebina slepega črevesja	2.36 ± 0.72	3.91 ± 0.72	**< 0.0001**
Content of colon / Vsebina kolona	1.44 ± 0.58	1.60 ± 0.50	0.4928
Content of whole intestine / Vsebina celotnega črevesja	5.23 ± 1.40	8.85 ± 3.07	**0.0002**
Content of digestive tract / Vsebina prebavnega trakta	9.70 ± 6.21	11.91 ± 5.69	0.1028

* Diet and day of slaughtering as two main effects / Krma in dan žrtvovanja kot dva glavna vpliva

3 RESULTS

In Table 2 the weights of different organs of the digestive tract are expressed as relative weights (g/100 g of body weight) to minimise the body size effect. The average relative weights of the small intestine and caecum were significantly ($P < 0.05$) higher in the pectin group as compared to the control. The relative weight of the stomach was similar in both groups. The average colon relative weight was higher in the pectin group than in the control, but the difference was not significant. The average relative weight of the whole intestine or whole digestive tract was also significantly ($P < 0.05$) higher in the pectin group.

Similar results were found for the weights of the digestive contents. There was significantly ($P < 0.05$) more digestive contents in the small intestine, caecum and whole intestine in the pectin fed rats compared to the control group (Table 3).

After the 5-day pre-experimental period, the average body weight of animals in the pectin group was significantly ($P < 0.05$) lower as compared to the control group. Since the dry matter intake in pectin group was lower all the time of experimental period, the difference become significant already at the beginning of the balance experiment (15.9 g ± 11.04 g) and increased (30.7 g ± 13.98 g) until the end of the balance experiment (Table 4). The result was significantly lower growth rate in pectin group as compared to control group. The problem of different body weight could be minimise, if animals of the control group have some lower body weight at the beginning of experimental period, but in this case the age of animals will be different and the difference in the growth rate even bigger. The differences in the dry matter efficiency and PER value were also significantly lower in the pectin group than in the control group.

Since the dry matter intake was lower in the pectin group, the nitrogen intake was also significantly ($P < 0.05$) lower than in the control group (Table 5). The amount of faeces excreted per day, the amount of N excreted in faeces per day and the amount of urine excreted per day were significantly ($P < 0.05$) higher in the pectin group as compared to the control group. On the other hand, the nitrogen excreted in urine was significantly lower in

Table 4: *Body weight, dry matter intake and growth rate (average ± SD) in 5 days balance measurements*
Preglednica 4: *Telesna masa, zaužita suha snov in prirast (povprečje ± SD) v 5 dneh bilančnih meritev*

	Control Kontrola (6)	Pectin Pektin (6)	P-value p-vrednost
Initial body weight (g) / Telesna masa ob začetku poskusa (g)	149.3 ± 7.11	133.4 ± 6.27	0.0021
Final body weight (g) / Telesna masa ob koncu poskusa (g)	179.2 ± 8.10	148.5 ± 7.22	**< 0.0001**
Dry matter intake (g DM/day) / Zaužita suha snov (gSS/dan)	17.0 ± 0.70	13.9 ± 0.46	**< 0.0001**
Growth rate (g/day) / Prirast (g/dan)	6.0 ± 0.51	3.0 ± 0.40	< 0.0001
DMI/average body weight (g/g) / ZSS/povprečno telesno maso (g/g)	0.103 ± 0.004	0.099 ± 0.005	0.0734
Dry matter efficiency (%) / Izkoristek suhe snovi krme (%)	35.22 ± 2.34	21.75 ± 2.32	**< 0.0001**
PER (g growth/g CP) / PER (g prirasta/g SB)	3.0 ± 0.20	1.6 ± 0.20	**< 0.0001**

DMI – dry matter intake / ZSS – zaužita suha snov; PER – protein efficiency ratio / učinkovitost beljakovin za prirast; CP – crude protein / SB – surove beljakovine

Table 5: *Balance experiment, digestibility, protein biological value and apparent net protein utilisation (average ± SD)*
Preglednica 5: *Bilančni poskus, prebavljivost, biološka vrednost beljakovin in navidezna neto izkoristljivost beljakovin (povprečje ± SD)*

	Control Kontrola (6)	Pectin Pektin (6)	P-value p-vrednost
N intake (mg/day) Zaužiti N (mg/dan)	323 ± 13	259 ± 9	< **0.0001**
N intake/average body weight (mg/g) Zaužiti N/povprečno telesno maso (mg/g)	2.0 ± 0.1	1.8 ± 0.1	**0.0188**
Excreted faeces (g fresh mass/day) Izločeno blato (g svežega blata/dan)	2.4 ± 0.2	3.1 ± 0.6	**0.0016**
N in faeces (mg/day) N v blatu (mg/dan)	28.0 ± 3.2	44.1 ± 5.9	**0.0002**
N in faeces/average body weight (mg/g) N v blatu/povprečno telesno maso (mg/g)	0.171 ± 0,023	0.314 ± 0.050	< **0.0001**
Excreted urine (g/day) Izločen seč (g/dan)	19.5 ± 2.9	24.4 ± 2.7	**0.0120**
N in urine (mg/day) N v seču (mg/dan)	86.8 ± 9.3	66.2 ± 7.1	**0.0015**
N in urine/average body weight (mg/g) N v seču/povprečno telesno maso (mg/g)	0.529 ±0.053	0.471 ± 0.057	0.0980
N balance (mg/day) Bilanca N (mg/dan)	208 ± 10	149 ± 13	< **0.0001**
N balance/average body weight (mg/g) Bilanca N/povprečno telesno maso (mg/g)	1.27 ±0.05	1.06 ± 0.08	**0.0003**
Apparent digestibility of protein (%) Navidezna prebavljivost beljakovin (%)	91 ± 1	83 ± 2	< **0.0001**
Digestibility of organic matter (%) Prebavljivost organske snovi (%)	95 ± 1	92 ± 1	< **0.0001**
Apparent protein biological value (%) Navidezna biološka vrednost beljakovin (%)	71 ± 2	69 ± 4	0.4519
Apparent net protein utilisation (%) Navidezna neto izkoristljivost beljakovin (%)	64 ± 2	57 ± 4	**0.0047**

N – nitrogen / dušik

the pectin group. Consequently, the nitrogen balance was significantly decreased in the pectin group (on average only 71.5% of the value in the control group). Results expressed per 1 g of average body weight show significant increase excretion of N in faeces (for 84%) and decrease consumed N (for 10%) and N balance (for 16%) in pectin group, but no significant differences in excretion of N through urine.

The apparent protein digestibility, digestibility of dry matter and organic matter decreased significantly ($P < 0.0001$) as a result of pectin addition to the diet (Table 5). On the contrary, the apparent biological value of protein was not affected by pectin, the average values were not different in the two groups ($P = 0.4519$), but apparent net protein utilisation was also significantly decreased in the pectin group as compared to the control.

4 DISCUSSION

It is well recognized that dietary non-starch polysaccharides, especially soluble ones, such as pectin, can decrease the apparent digestibility of whole protein or of amino acids in pigs (de Lange *et al.*, 1989; Mosenthin *et al.*, 1994; Zhu *et al.*, 2005; Libao-Mercado *et al.*, 2006). Such effects are likely to be related to endogenous nitrogen losses, which can be seen in increased secretion and impaired reabsorption in the lower part of the gastrointestinal tract in pigs (Grela *et al.*, 1998) and in rats (Larsen *et al.*, 1993), and in stimulation of the rate of microbial fermentation in the gut of monogastric animals (Eggum, 1995; Schulze *et al.*, 1995; McCullough *et al.*, 1998). The present study demonstrates that the course of

digestion and also the enlargement of the intestinal tissues are influenced by pectin.

It has been found that the addition of pectin to the diet significantly increases endogenous nitrogen flow in rats (Pastuszewska *et al.*, 2000b) and in pigs (de Lange *et al.*, 1989; Libao-Mercado *et al.*, 2006) which includes digestive gland secretions, desquamated cells from active replacement of the gastrointestinal mucosal lining, and secretion of plasma components (urea and a small amount of plasma protein) (Shah *et al.*, 1982). Indeed pectin has been shown to stimulate enterocyte turnover (Fukunaga *et al.*, 2003; Chun *et al.*, 1989), and may increase mucin secretions because of stimulation by the increase in caecal short chain fatty acid production (Barcelo *et al.*, 2000) in laboratory rats.

The effect of pectin on protein flow (of dietary and endogenous origin) could also be the result of an interference with luminal protein digestion either of dietary or of endogenous origin, as shown for other dietary fibers that enhance endogenous nitrogen secretion (Schulze *et al.*, 1995). Endogenous amino acids may not be available for absorption because of the physical and chemical adsorptive properties of pectin (Souffrant, 2001). El Kossori *et al.* (2000) suggested that protein hydrolysis could be prevented by interactions between protein or enzyme and fiber without modification of the viscosity, and would depend on the kind of fiber. Animals producing more mucus could have a slower absorption rate, because it has been postulated that mucus contributes to the apparent thickness of the unstirred layer and affords protection to the mucosal surface (Nimmerfall and Rosenthaler, 1980). However, increased luminal mucin did not disturb glucose or ovalbumin absorption (Morita *et al.*, 2006).

Undigested endogenous or dietary nitrogenous compounds can be transformed by microorganisms in the large intestine. In the intestinal tract of pigs, microbes degrade up to 90% of pectin by fermentation (Drochner *et al.*, 2004). Such microbes use pectin as an energy source and also use most of the luminal nitrogen, which is consequently excreted in faeces. However, this phenomena cannot be responsible for the enlargement of excreted nitrogen in the faeces unless the increased microbial fermentation, evidenced by the increase in the production of short chain fatty acids (SCFA) in the large intestine (Pirman *et al.*, 2007), induces a stimulation of endogenous nitrogen secretions. Beside that, SCFAs have some other important roles in the intestinal lumen. Research on rats showed that an increased concentration of SCFA in the intestinal lumen, because of the ingestion of non-starch polysaccharides or non-digestible oligosaccharides decreased the pH value in the large intestine, leading to the conversion of NH_3 to NH_4^+. This form of ammonia cannot diffuse through the intestinal wall (Younes *et al.*, 1995), leading to the change of the nitrogen metabolism, since the more nitrogen is excreted by faeces, the less nitrogen is transferred to urea in the liver and consequently less nitrogen is excreted by urine (Mosenthin *et al.*, 1992). On the other hand, where microbial fermentation is very intensive (because of the presence of soluble dietary fiber, like pectin), the utilization of urea by microflora in the intestinal lumen is increased and again nitrogen is excreted through faeces (microbial mass) and less nitrogen is excreted through urine.

In the present study, the enlarged faecal nitrogen loss seems to be of endogenous origin. Indeed, faecal nitrogen loss was increased by 57% by pectin feeding, which is very similar to the increase described for endogenous losses (53%) in rats fed a pectin nitrogen–free diet when compared with a control nitrogen-free diet (Pastuszewska *et al.*, 2000b). This conclusion suggests that the increase in nitrogen faecal loss induced by pectin feeding is unlikely to result from an increase in faecal loss of dietary nitrogen. Consequently most dietary amino acids in our experiment would have been absorbed as with the control diet.

All the carbohydrates (potato starch, pectin, cellulose and tannic acid) used in the study on laboratory rats of Pastuszewska *et al.* (2000a) tended to decrease the blood urea nitrogen concentration as compared to the control (wheat starch as carbohydrate), and nearly 20% nitrogen excreted by urine in a control group (319 mg vs. 254 mg in urine of pectin group) was obviously metabolized in the large intestine (72 mg vs. 110 mg in faeces of pectin group). If such a proportion was applied in the present study, on average 16.54 mg of endogenous urea nitrogen (blood urea nitrogen excreted by faeces) would be redirected from urinary excretion to fecal excretion. This value is close to the difference between nitrogen faecal excretion in the pectin (28.00 mg/d) and control rats (44.05 mg/d). Nitrogen originating from endogenous urea may be a major component of the increase in nitrogen faecal losses by pectin feeding. Another consequence of this exchange is that the assessments of protein nutritional value need to be corrected to take into account this exchange between urine and faecal nitrogen excretion. In this case, the corrected nitrogen digestibility becomes 91.31% and 89.37% for the control and the pectin diets, respectively, while the corrected biological value becomes 70.58% and 64.21% in the control and pectin diets, respectively. Consequently the main difference in protein and diet nutritional value between the control and the pectin diets seems to be not at absorption level but rather in differences in urea excretion pathways and possibly in amino acid metabolism.

This could be a consequence of an increase in the requirements for a specific nutrient (energy, amino ac-

ids like threonine) due to the high protein turnover in intestinal tissues observed in pectin fed rats, which may impair amino acid utilization in other organs (Pirman *et al.*, 2007). Indeed the weights of intestinal tissues in rats were increased as a result of addition of pectin to the diet, as it was found in a previous study (Pirman *et al.*, 2007). When different fibers were compared, the greatest enlargement of caecal digests and tissue was observed after addition of pectin to the diet (Pastuszewska *et al.*, 2000a). This intestinal hypertrophy corresponded to hyperplasia in the small intestine (Brown *et al.*, 1979), to an increase in villous cell exfoliation and crypt cell proliferation (Jacobs 1983), and to alteration of gut morphology by increasing the number of goblet cells and mucus production (Cassidy *et al.*, 1981) in laboratory rats. In our previous study on addition of pectin to the diet of the laboratory rats (Pirman *et al.*, 2007) the villous height in the small intestine and crypt depth in small intestine, caecum and colon were significantly increased as compared to the control diet. In a previous study, the strong stimulation of intestinal protein turnover corresponded to a slight decrease in muscle protein turnover (Pirman *et al.*, 2008). In the study of Zhu *et al.* (2005) on pigs the utilization of threonine in whole body protein deposition was linearly decreased with the dietary pectin level, but not of lysine. This was connected to the high amount of threonine in mucoproteins, so the influence of pectin on digestive physiology operates through amino acid and nitrogen utilization at the whole body level.

5 CONCLUSIONS

Pectin is an important factor affecting the proportions of faecal and urinary nitrogen excretion and ultimately both apparent protein digestibility and corrected apparent protein biological value. This effect is related to the fermentability of pectin, especially in the large intestine. Furthermore, the study confirmed the effects of pectin on digestive physiology, namely increased urea excretion from blood to intestine and reduced urea excretion by urine. Both consequences (in digestive tract and in kidney function) of the presence of pectin in the diet are of benefit for health status in animals and men.

6 POVZETEK

Pektin spada v skupino topne vlaknine in ga najdemo v mnogih in različnih vrstah hrane in krme. V literaturi najdemo različne in nasprotujoče učinke na prebavljivost hranljivih snovi pri dodajanju topne vlaknine v obrok. V večini primerov povečajo izločanje dušika preko blata, a le redko zmanjšajo izločanje dušika preko seča. Z našo raziskavo smo želeli ugotoviti vpliv pektina na izkoristljivost beljakovin in na razvoj posameznih delov prebavil, s tem pa bi dobili boljšo predstavo o prehranski vlogi pektina. Pripravili smo dve krmi, v katerih je bil kazein kot vir beljakovin. V poskusni krmi smo del pšeničnega škroba zamenjali s pektinom iz limonine lupine in mešanico rastlinskih olj, da sta bili krmi izoenergijski. 24 rastočih laboratorijskih podgan moškega spola smo po 4 dnevih predposkusa razdelili v dve homogeni skupini (telesna masa 120,1 g ± 5,5 g in 120,1 g ± 5,2 g za kontrolno in pektinsko skupino) in jih 20 dni *ad libitum* krmili z eno od pripravljenih krmnih mešanic in merili zauživanje krme in priraste. V času poskusa smo 5 dni zaporedoma ločeno zbirali blato in seč (bilančni poskus). Na koncu poskusa smo živali žrtvovali, odvzeli tkiva prebavil in jih stehtali polne in prazne. Izračunali smo navidezno prebavljivost beljakovin in organske snovi, navidezno biološko vrednost beljakovin in navidezno neto izkoristljivost beljakovin. Pektin vpliva na povečanje mase tkiv prebavil in vsebine prebavil, kar v največji meri velja za slepo črevo. Pektin je tudi pomembno vplival na porazdelitev izločenega dušika preko blata oz. preko seča, kar ima vpliv na navidezno prebavljivost beljakovin in korigirano biološko vrednost beljakovin. To je povezano s fermentabilnostjo pektina. Naši rezultati potrjujejo vpliv pektina na fiziologijo prebave, saj povečuje izločanje sečnine (dušika) preko krvnega obtoka nazaj v prebavila in zmanjšuje izločanje le-te preko seča. Oboje ima ugoden vpliv na zdravstveno stanje živali in ljudi.

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UPORABA DNA OZNAČEVALCEV ZA PREVERJANJE POREKLA PRI OVCAH IN DOLOČANJA OČETOVSTVA V ČREDAH Z VEČ PLEMENSKIMI OVNI

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Uporaba DNA označevalcev za preverjanje porekla pri ovcah in določanja očetovstva v čredah z več plemenskimi ovni

Rodovniški podatki kontroliranih tropov so bistvenega pomena za rejce ovc in izvajanje selekcijskih programov. V tej študiji smo preverili, če je možno z osmimi molekularno genetskimi označevalci ter uporabo računalniškega programa ATLAS preveriti skladnost podatkov v več-generacijskih rodovnikih jezersko-solčavske in oplemenjene jezersko-solčavske pasme. Ugotovili smo 90,9 % točnost beleženja rodovniških podatkov, medtem ko smo neskladja ugotovili pri štirih družinah od 44-ih. V dveh primerih je bil potomcu nepravilno pripisan oče ali mati, pri ostalih dveh primerih pa sta bila jagnjetu napačno pripisana oba starša. V drugem delu raziskave smo poskušali z istim nizom mikrosatelitnih označevalcev določiti očetovstva štirim jagnjetom istrske pramenke, kjer v času paritev v trop pripuščajo dvajset ovnov in tako jagnje nima poznanega očeta. Trem jagnjetom smo lahko nedvoumno določili očeta z izločitvijo ostalih devetnajstih. V enem primeru smo ugotovili, da niz osmih označevalcev ni dovolj informativen za določitev očeta potomcu, zato smo analizo razširili in z vključitvijo dodatnih štirih mikrosatelitnih označevalcev uspešno določili očetovstvo tudi v tem primeru. Z izbranim nizom molekularnih označevalcev in obdelave podatkov je možno učinkovito preverjati obstoječe rodovniške podatke in napovedati očetovstvo jagnjetom v tropih, kjer pripuščajo večje število ovnov. Rezultati take analize so lahko v pomoč rejcem in selekcijskim službam za izboljšanje točnosti rodovniških podatkov in učinkovitejše načrtovanje reje in selekcije pri ovcah.

Ključne besede: ovce / poreklo / očetovstvo / molekularna genetika / genetski označevalci

Molecular genetics markers used for parentage verification and paternity determination in multiple-sire sheep pedigrees

Pedigree data from recorded flocks are of importance for the proper flock management as well as for the selection programmes and accurate performance prediction. In this study we examined if the use of eight molecular markers and computer analysis package ATLAS can be applied to verify known pedigrees of Jezersko-Solčava and Improved Jezersko-Solčava sheep breed from Slovenia. 90.9 % of pedigree data were in concordance with molecular genetic tests whereas in four pedigrees discordant parentage tests were obtained. In two cases, a different father or mother was assigned, whereas in the other two pedigrees both parents were discordant with molecular test results. In the second part of this study we aimed to determine the paternity for four lambs of Istrian Pramenka breed, in which a random mating scheme with 20 rams was used and hence the lambs had no father assigned. Using the same set of eight microsatellite markers, we were able to unequivocally determine paternity for 3 out of 4 lambs. In one case the analysis was not informative enough but with inclusion of 4 more microsatellite markers its sire could be determined. With the chosen set of microsatellite markers and data analysis programme ATLAS it is therefore possible to efficiently perform pedigree data validation as well as paternity prediction for lambs from flocks, where a large number of rams are used in a random mating system. Applying such molecular tests could help sheep breeders in flock management and improve efficiency of selection programmes.

Key words: sheep / parentage / paternity / molecular genetics / genetic markers

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1 UVOD

Preverjanje porekla, še zlasti očetovstva, z uporabo molekularnih označevalcev je relativno uveljavljena metoda pri nekaterih vrstah domačih živalih, kot so konji, govedo, psi in eksotične živali. Pri teh vrstah živali se je ugotavljanje porekla z uporabo molekularnih označevalcev razvilo predvsem zaradi cenovne vrednosti plemenskih živali ter zaradi omejitve pri trgovanju in prometu z le tistimi linijami živali, ki imajo predložen rodovniški certifikat. Velik pomen ima tudi pri laboratorijskih oziroma poskusnih živalih predvsem zaradi večje zanesljivosti in predstavljivosti rezultatov poskusa. Pri ovcah je imelo v preteklosti preverjanje porekla manjši pomen. Molekularno genetske študije so bile bolj v rabi za oceno genetske sorodnosti oziroma diverzitete populacij ovc. V sedanjem času pa, ko je vse več populacij v selekcijskih programih ter v programih obveznega genotipiziranja za prionski gen (PrP), bi bilo zaželeno imeti metode za dodatno preverjanje porekla. Na primer, v velikih čredah ovc, kjer v čredi plodi več ovnov hkrati, je celo nemogoče določiti očeta, poleg tega pa se tudi v tropih s kontroliranimi pripusti zgodi, da je zapisan napačen oče. Če je jagnje, ki ima dvomljiv rodovnik, nato izbrano za test, je zaželeno, da z dodatno molekularno genetsko metodo ugotovimo poreklo vsaj po očetu. To je pomembno tako z vidika ocenjevanja plemenske vrednosti očeta kot nadzora inbridinga, torej zaradi izogibanja nadaljnjega parjenja v sorodstvu in zanesljivejše genotipizacije za PrP lokus.

Preverjanje porekla, gensko kartiranje, forenzične preiskave in genska diagnostika so le nekatere izmed možnosti za uporabo tipiziranja DNA (Dovč, 1994). Ker je identiteta očeta velikokrat bolj vprašljiva kot identiteta matere, je večina postopkov bolj usmerjena v preverjanje očetovstva. V ta namen uporabljamo molekularno genetske metode na osnovi genetskih označevalcev (Dovč, 1994). Genetski označevalec je lahko katerikoli odsek v genomu, kjer obstajajo razlike v zaporedju nukleotidov v DNA. Na ravni populacije se ta variabilnost kaže v razlikah med osebki znotraj populacije. Vendar razlike nimajo bistvenega vpliva na fenotip živali, kljub temu pa nam razkrivajo genetsko sorodnost med osebki. Pomembne značilnosti, s katerimi ocenjujemo informativne genetske označevalce, so velika stopnja variabilnosti, enakomerna in številčna razporeditev po genomu ter enostavnost in cenenost identifikacije ter uporabe. Mikrosatelitni označevalci izpolnjujejo vse te zahteve, in sicer so visoko variabilni, številni, razporejeni so naključno po genomu ter relativno tehnično nezahtevni (Kavar in Dovč, 1999). Z uporabo sodobne tehnologije za analizo mikrosatelitov lahko postopke standardiziramo in avtomatiziramo ter tako hitro in enostavno pridemo do rezultatov. Pri odčitavanju genotipa (števila baznih parov) posameznega označevalca upoštevamo, da potomec podeduje dva alela, enega po očetovi, drugega pa po materini strani. Če posameznemu osebku združeno obravnavamo genotipe na več lokusih, dobimo sestavljen genotip, ki predstavlja molekularni opis osebka na izbranih lokusih. S primerjanjem sestavljenih genotipov osebkov, ki so domnevno sorodstveno povezani, lahko sorodstvene zveze potrdimo ali ovržemo. Z uporabo večjega števila mikrosatelitov (8 in več) se zelo zmanjša verjetnost, da bi imela dva osebka po naključju enak sestavljen genotip. S kombinacijo več visoko polimorfnih lokusov lahko dobimo specifičen genotip za vsako žival, razen za enojajčne dvojčke ali visoko inbridirane linije (Kavar, 2001). Informativnost oz. polimorfnost označevalca v populaciji lahko predstavimo z vrednostjo PIC (*Polymorphism Information Content*), ki je odvisna od števila alelov na lokusu in porazdelitve alelov v populaciji.

Pri ovcah je doslej znanih že skoraj tisoč mikrosatelitov (http://rubens.its.unimelb.edu.au/~jillm/jill.htm) in so jih že uporabili v študijah preverjanja porekla (Achmann in sod., 1998). V čredah drobnice, kjer je pri parjenju prisoten en plemenski oven, običajno ne prihaja do rodovniških neskladij vsaj glede določanja očetovstva.. Drugače je v rejah, kjer se v času pripustov velikih čred uporablja več plemenskih ovnov hkrati. V teh primerih lahko predstavlja rodovništvo in kontrola porekla problem. Take črede lahko z leti dosežejo visoko stopnjo inbridiranosti, čigar posledice se lahko kažejo v slabši plodnosti, slabši proizvodnosti, pogostejšem pojavljanju raznih infektivnih in dednih bolezni, povezanih z večjo pogostostjo izražanja recesivnih mutacij (Laughlin in sod., 2003). Laughlin in sod. (2003) so v ta namen z uporabo mikrosatelitnih označevalcev ugotavljali očetovstvo na potomcih iz čred, kjer je bilo pri pripustih prisotnih več samcev hkrati. Ugotovitve raziskave kažejo, da so lahko ob primerjavi genotipov na treh mikrosatelitnih označevalcih lahko določili očeta 73 jagnjetom od 79 jagnjet (92 %), če so poznali tudi genotip matere. Kadar so bili genotipi označevalcev ocenjeni brez informacije genotipa matere, so lahko določili očeta 58 jagnjetom od 79 jagnjet (73 %). V tem primeru torej očetovstva niso mogli določiti pri 27 % potomcev.

Najnovejše raziskave pri genetskem kontroliranju porekla drobnice so usmerjene v pomnoževanje večjega števila mikrosatelitnih lokusov hkrati v eni PCR reakciji. Do sedaj so bile pri ovcah izvedene kontrole porekla z mikrosateliti v laboratoriju Gruppi Sanguigni (LGS, Cremona, Italija), kjer so razvili dvodelni sistem, pri katerem so v eno PCR reakcijo v prvem setu združili 7, v drugem pa 8 mikrosatelitov (Glowatzki-Mulis in sod., 2007). Študija je uspešno dodelila posamezne živali pravi pasmi. Ocenjena verjetnost, da sta genotipa dveh naključno iz-

branih posameznikov iz populacije identična, je nihala med $5{,}23 \times 10^{(-7)}$ in $2{,}24 \times 10^{(-18)}$.

Leta 2004 je v Sloveniji stekel postopek kontrole porekla z molekularnimi metodami za trop istrskih pramenk iz Centra za sonaravno rekultiviranje Vremščica. Analizo izvajajo na Veterinarski fakulteti v Ljubljani, kjer uporabljajo standardni set mikrosatelitnih lokusov (enake označevalce, kot smo jih uporabljali v naši raziskavi), ki omogoča izločitev napačnih prednikov z več kot 99 % verjetnostjo (Cotman, 2007). V genetskem laboratoriju Oddelka za zootehniko so bili prvi poskusi genotipizacije z mikrosateliti usmerjeni na določanje genetske variabilnosti med avtohtonimi populacijami ovc ter identifikacijo osebkov in preverjanje porekla (Kavar in Dovč, 1999; Kavar in sod. 2002). Z namenom, da bi ocenili genetsko sorodnost med tremi slovenskimi avtohtonimi pasmami ovc, so Kavar in sod. (2002) z uporabo mikrosatelitov genotipizirali ovce istrskih pramenk, bovških ovc in jezersko solčavskih ovc (Kavar in sod., 2002). Rezultati kažejo, da razlike med pasmami niso zelo velike in da sta si bovška in jezersko solčavska ovca genetsko bližji kot istrska pramenka. Rezultati analize AMOVA so pokazali, da večina variance izhaja iz razlik znotraj pasem, le približno 6 % se je da razložiti z razlikami med pasmami (Kavar in sod., 2002). Zato predpostavljajo skupen nastanek vseh treh pasem oz. še posebej bovške in jezersko-solčavske ovce.

Našo raziskavo smo izvedli z namenom, da bi z uporabo molekularnih genetskih označevalcev preverili poreklo pri jezersko-solčavski in oplemenjeni jezersko-solčavski pasmi ter primerjali rezultate z že znanimi rodovniškimi podatki. Ocenili smo tudi informativno vrednost osmih mikrosatelitnih označevalcev za istrsko pramenko in s programom ATLAS določili očetovstvo v tropu istrskih pramenk, kjer je v času parjenja prisotnih več plemenskih ovnov. Z analizo frekvenc alelov in stopnje heterozigotnosti pri istrski pramenki, jezersko-solčavski in oplemenjeni jezersko solčavski pasmi smo ocenili tudi informativnost uporabljenega niza mikrosatelitnih označevalcev.

2 MATERIAL IN METODE

2.1 VZORČENJE

V študijo je bilo vključenih 24 družin (oče, mati in njuni potomci) oplemenjene jezersko-solčavske pasme, 19 družin jezersko-solčavske pasme in 4 družine istrske pramenke. Skupno smo genotipizirali 124 živali, vzrejenih pri osmih rejcih (pregl. 1).

Vsi potomci so bili vključeni v kontrolo porekla in proizvodnje za drobnico. Potomcem (odbrana moška jagnjeta za testiranje), starim od 90 do 120 dni, je veterinar odvzel kri na karantenski postaji Horjul in testni postaji v Logatcu iz zunanje vratne vene (*v. jugularis externa)*. Staršem, to je plemenskim ovcam in ovnom pri rejcih in Centru za sonaravno rekultiviranje Vremščica, smo s posebnim ščipalcem odvzeli majhen košček rovaša (približno 4 mm^2).

Preglednica 1: *Živali v študiji po pasmah in rejcih*
Table 1: *Animals used in the study listed by breed and breeder*

Rejec	Pasma	Št. potomcev	Št. mater	Št. očetov
Rejec 1	JSR	13	12	3
Rejec 2	JSR	9	8	1
Rejec 3	JSR	2	2	1
Rejec 4	JS	4	4	1
Rejec 5	JS	3	3	1
Rejec 6	JS	10	9	2
Rejec 7	JS	3	3	1
Rejec 8	IP	4	4	20

JS – jezersko-solčavska; JSR – oplemenjena JS; IP – istrska pramenka

2.2 IZOLACIJA DNA, IZBIRA MIKROSATELITOV IN VERIŽNA REAKCIJA S POLIMERAZO PCR

Odmrznjeni košček ušesnega tkiva smo po odstranitvi dlak lizirali z dodatkom 300 µl pufra za lizo s 5 µl proteinaze K (Kavar s sod. 2002). Vzorec smo inkubirali na 55 °C za najmanj 4 ure pri občasnem mešanju ter izolirali DNA s standardno fenolno ekstrakcijo ter alkoholno precipitacijo. Pri vzorcih krvi smo v reagenčne posodice odpipetirali 200 µl krvi in dodali 800 µl TE pufra. Raztopino smo centrifugirali 30 sekund na 12000g in supernatant odlili. Sediment smo ponovno resuspendirali v 800 µl TE pufra in prejšnji korak trikrat ponovili. Očiščeni celični sediment smo resuspendirali z 200 µl liznega pufra in 4 µl proteinaze K. Ostali del protokola je enak kot pri ekstrakciji DNA iz ušesnega tkiva.

Mikrosatelitne lokuse smo določili glede na predhodne študije (Kavar in sod. (2002) in izbrali osem polimorfnih mikrosatelitnih označevalcev (pregl. 2). PCR reakcijsko mešanico smo pripravili v ločenem prostoru, kjer ni nevarnosti kontaminacije s produkti predhodnih PCR reakcij in ostalih DNA molekul. Reakcijski pogoji so prikazani v preglednici 3.

Reakcijo PCR smo izvajali v mikrotiterskih ploščah v mikroprocesorsko vodenem cikličnem termostatu PTC – 100 (MJ Research, Watertown MA, USA) in uporabili program, ki se je začel z denaturacijo na 95 °C 5 min, sledilo je 13 ciklov denaturacije pri 95 °C 15 s, prileganja

Preglednica 2: *Seznam uporabljenih mikrosatelitnih označevalcev, predviden razpon dolžin PCR produktov, ter zaporedja začetnih oligonukleotidov*
Table 2: *List of microsatellite markers, range of PCR product lenghts and nucleotide sequences of both primers*

Ime označevalca	Dolžine PCR produktov		Zaporedje začetnih oligonukleotidov (*Primers*)
	Min.	Max.	
MAF214	181	250	5′- GGG TGA TCT TAG GGA GGT TTT GGA GG -3′
	181	250	5′- AAT GCA GGA GAT CTG AGG CAG GGA GG -3′
MAF65	124	142	5′- AAA GGC CAG AGT ATG CAA TTA GGA G -3′
	124	142	5′- CCA CTC CTC CTG AGA ATA TAA CAT G -3′
McM42	79	101	5′- CAT CTT TCA AAA GAA CTC CGA AAG TG -3′
	79	101	5′- CTT GGA ATC CTT CCT AAC TTT GGG -3′
McM527	168	178	5′- GTC CAT TGC CTC AAA TCA ATT C -3′
	168	178	5′- AAA CCA CTT GAC TAC TCC CCA A -3′
TGLA53	116	136	5′- CAG CAG ACA GCT GCA AGA GTT AGC -3′
Govedo	116	136	5′- CTT TCA GAA ATA GTT TGC ATT CAT GCA -3′
OarAE119	144	181	5′- CTC AGC AAA TGG TTC CTG GGC ACC -3′
	144	181	5′- TTT TAT AGT GAG GTG ACC ACT TGA TG -3′
OarCP49	77	103	5′- CAG ACA CGG CTT AGC AAC TAA ACG C -3′
	77	103	5′- GTG GGG ATG AAT ATT CCT TCA TAA GG -3′
OarFCB11	119	142	5′- GGC CTG AAC TCA CAA GTT GAT ATA TCT ATC AC-3′
	119	142	5′- GCA AGC AGG TTC TTT ACC ACT AGC ACC -3′

začetnih oligonukleotidov pri 60 °C 30 s, in podaljševanja pri 72 °C 1 min ter 21 ciklov denaturacije pri 95 °C 15 s, prileganja pri 52 °C 30 s, in podaljševanja pri 72 °C 1 min, ter zaključnim podaljševanjem pri 72 °C, 10 min. Genotipizacijo smo izvedli z avtomatskim sekvenatorjem ABI Prism 310 (Perkin – Elmer Applied Biosystems, Foster City, ZDA). Ker aparat ABI PRISM™ 310 omogoča istočasno zaznavanje štirih različnih fluorescentnih barvil v eni kapilari, smo pri analizi združevali po štiri PCR produkte v dve mešanici, ki sta vsebovali različno označene začetne oligonukleotide (pregl. 4). Mešanico, ki smo jo nanašali na sekvenator, smo pripravili z združitvijo 3µl PCR produkta štirih mikrosatelitnih označevalcev. Pred nanosom v sekvenator smo odpipetirali 5 µl združene PCR mešanice v reagenčno posodico, dodali 12 µl formamida in 0.4 µl standarda Rox 350. Po kratkem centrifugiranju smo vzorce za 3 min. denaturirali na 95 °C in jih takoj po tem postavili na led. Tako pripravljene vzorce smo razvrstili v sekvenator za genotipizacijo.

Preglednica 3: *PCR reakcijska mešanica*
Table 3: *Reaction mixture for the PCR reaction*

Koncentracije PCR reagentov	Količina reagenta / vzorec, µL
Deionizirana H_2O (Sigma)	1,42
1 × Taq pufer (Feramentas)	1,00
25 mM $MgCl_2$ (Feramentas)	1,00
2 mM dNTP	1,00
0,25 uM 5’ začetni oligonukleotid	0,25
0,25 uM 3’ začetni oligonukleotid	0,25
0,4 enote polimeraze Ampli Taq (Fermentas)	0,08
PCR mešanica skupaj	5,00
DNA (5 ng/µl)	5,00
Celotna količina reakcijske mešanice	10,00

2.3 ANALIZA GENOTIPOV S PROGRAMOM ATLAS IN STATISTIČNE OBDEALVE

Zbiranje podatkov in določanje genotipov smo izvedli z uporabo programske opreme GENESCAN Analysis Software, verzija 3.7. Zbrane podatke genotipov (alel smo označili kot velikost produkta PCR) v Excelu smo vnesli v program ATLAS (Perez in sod., 2004), s katerim smo preverili ujemanje genotipov med starši in potomci. Pri določanju očetovstva v tropu istrske pramenke, kjer smo imeli na razpolago 20 potencialnih plemenskih ovnov, smo z orodjem predlaganje prednikov (ang.: *Propose parents*) potomcu določili ovna znotraj skupine

Preglednica 4: *Shema združevanja mikrosatelitnih označevalcev za analizo na sekvenatorju*
Table 4: *Pooling of microsatellite PCR products for genotyping*

Mešanica 1 / Mix1		Mešanica 2 / Mix2	
FAM	MCM 42 (79–101) bp	OarCP 49 (77–103) bp	FAM
	TGLA 53 (116–136) bp	OarAE 119 (144–181) bp	
TAMRA	MAF 65 (124–142) bp	OarFCB11 (119–142) bp	TAMRA
	McM 527 (168–178) bp	MAF 214 (181–250) bp	JOE

potencialnih staršev. Na koncu, ko so bili očetje določeni ustreznim družinam, smo z orodjem izris rodovnika (ang.: *draw pedigree*) grafično predstavili izris genotipov osebka in njegovih najbližjih sorodnikov (staršev, bratov, sester ipd.)

Da bi določili stopnjo polimorfizma označevalcev pri pasmah, smo izračunali frekvence alelov in delež heterozigotnosti z uporabo programa GENETIX (Belkhir in sod., 1998). Preučili smo odnose med osebki znotraj populacij oz. informativnost (polimorfnost) označevalcev za posamezne pasme. Pomembna značilnost genetskega označevalca je njegova heterozigotnost, torej verjetnost, da je nek osebek heterozigoten za ta označevalec. Mera za informativnost označevalca nam pove pričakovan delež heterozigotnih osebkov. Večja, kot je heterozigotnost, bolj je označevalec informativen. Visoko polimorfni označevalec naj bi imel odstotek heterozigotnosti večji od 70 % (Ott, 1992). Pričakovani delež heterozigotnosti smo izračunali iz števila posameznih alelov v populaciji glede na Hardy-Weinbergovo ravnotežje po naslednjem obrazcu:

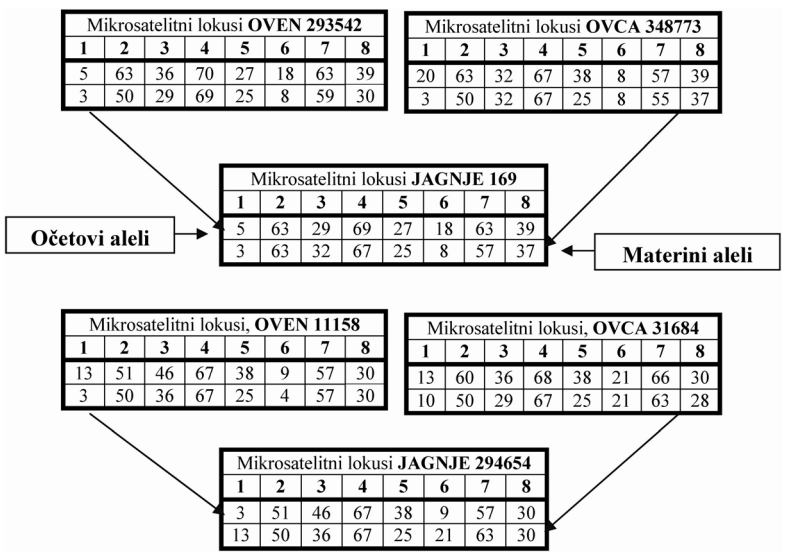
$$H = 1 - \sum_{i=1}^{k} \times p_i^2$$

k = število alel
p_i = pogostost i-te alele

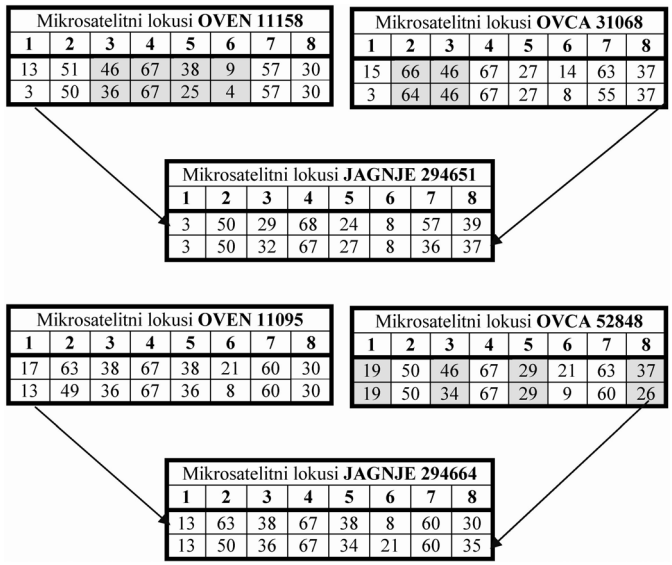
3 REZULTATI

3.1 ANALIZA POREKLA ZA JEZERSKO-SOLČAVSKO IN OPLEMENJENO JEZERSKO-SOLČAVSKO PASMO

Z namenom, da bi preverili rodovniške podatke, smo s pomočjo programa ATLAS najprej testirali tiste živali, za katere smo imeli znano poreklo. Preverili smo rodovniške podatke, ki jih zapisujejo rejci sami ter podatke selekcijske službe. Oboje smo primerjali z rezultati, ki smo jih dobili pri testiranju genotipov. V 40-tih od 44-tih družin smo potrdili pravilno zapisovanje rodovniških podatkov. Na sliki 1(levo) je prikazana jezersko-solčavska družina rejca 6, desno pa oplemenjena jezersko-solčavska družina rejca 1. Vsaka žival je predstavljena z



Slika 1: *Slikovni prikaz (program ATLAS) dveh rodovnikov, kjer je genotipska analiza potrdila pravilno zapisovanje rodovniških podatkov.*
Figure 1: *Scheme drawn by programme ATLAS for two pedigrees with concordant results between listed pedigree and genotype test.*



Slika 2: *Primer neskladja genotipov staršev z genotipom potomca pri rejcu 1 – jagnje je bilo pripisano napačni družini (zgoraj). V spodaj primeru je bila jagnjetu pripisana napačna mati. Osenčeni lokusi pri določenem osebku predstavljajo neskladja genotipov med predvidenimi starši in potomcem.*

Figure 2: *A case of two pedigrees where genotype analysis demonstrated discordant results with enlisted pedigress. Progeny of the left pedigree was assigned to a wrong family (shadowed boxes), whereas progeny of the right pedigree was not assigned to correct dam.*

identifikacijsko številko, npr. OČE rejca 6 ima ID 293542. Pri potomcu so za vsakega od osmih lokusov (alel prikazan kot število CA ponovitev) prikazani aleli, ki jih potomec podeduje po strani očeta (zgornja vrsta) in po strani matere (spodnja vrsta).

V 4 primerih od 44 –tih (9,1 %) smo odkrili razhajanja med rezultati genetske analize rodovnikov in zapisanih rodovniških podatkov. V dveh primerih sta bila potomcu nepravilno pripisana oče ali mati, pri ostalih dveh primerih pa sta bila jagnjetu napačno pripisana oba starša (slika 2). Pri dveh družinah smo opazili neskladja na šestih mikrosatelitnih lokusih, pri ostalih dveh družinah pa na petih oziroma štirih lokusih.

3.2 DOLOČANJE OČETOVSTVA ZA ISTRSKO PRAMENKO

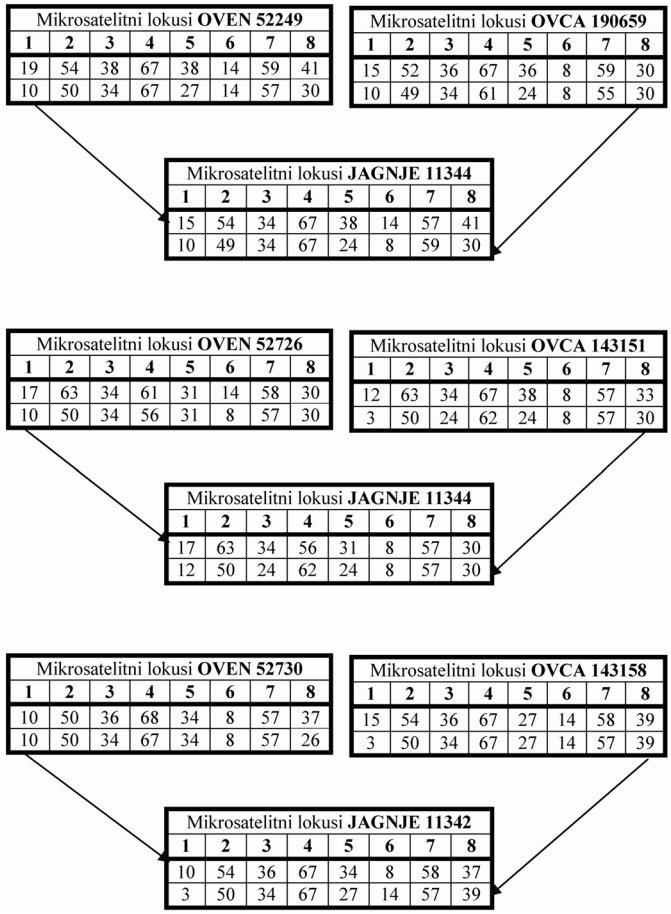
Analiza je temeljila na določanju očetovstva v čredi ovc istrske pramenke iz Centra za sonaravno rekultiviranje Vremščica, kjer v času paritev v čredo ovc pripuščajo dvajset plemenskih ovnov hkrati. Z namenom, da bi jagnjetom določili očete, smo genotipizirali dvajset plemenskih ovnov na osmih mikrosatelitnih lokusih ter rezultate primerjali z genotipi potomcev in njim pripisanih mater z uporabo programa ATLAS. Z osmimi mikrosateliti smo trem jagnjetom (št. 11344, 11345, 11342) lahko nedvoumno določili očeta izmed dvajset potencialnih očetov (slika 3).

Izbrani set osmih označevalcev ni bil dovolj informativen za določitev očeta potomcu 11346 (pregl. 5), saj je analiza s programom ATLAS od dvajsetih ovnov določila dva verjetna očeta. Da bi lahko jagnjetu 11346 kot očeta določili enega izmed dveh kandidatnih ovnov, smo v analizo dodali še štiri označevalce OarCP20, Oar116, Oar109 ter Oar50 (pregl. 6).

Zaradi podobnosti v genotipu na osmih lokusih med kandidatnima očetoma 1 in 2 (pregl. 5; 11211 in 151982) lahko domnevamo, da obstajajo sorodstvene povezave med njima, kar je za majhno populacijo istrske pramenke pričakovano (velika verjetnost je, da sta brata ali polbrata). Uporaba dodatnih štirih mikrosatelitnih lokusov je potomcu 11346 kot očeta nedvoumno določila kandidatnega ovna št. 151982 (pregl. 6). Mikrosatelitni lokus Oar109 je izločil ovna 11211 in se tako izkazal za odločilnega. Lokusa OarCP20 in Oar116 sta za to pasmo brez informativne vrednosti zaradi homozigotnosti na vseh alelih. Z določitvijo očetovstva potomcu 11346 smo tako lahko določili očetovstvo vsem preiskovanim jagnjetom istrskih pramenk.

3.3 ANALIZA FREKVENC ALELOV IN INFORMATIVNOSTI ZA POSAMEZNE MIKROSATELITNE LOKUSE PO PASMAH

V analizo frekvenc alelov smo vključili pasme jezersko-solčavska, oplemenjena JS in istrska pramenka



Slika 3: *Uspešno določanje očetovstva (od 20 možnih) z osmimi mikrosatelitnimi označevalci trem potomcem istrske pramenke.*
Figure 3: *Successfull assignment of the sire out of 20 potential fathers to three progeny.*

ter osem mikrosatelitnih lokusov. Število alelov, ki smo jih za posamezen mikrosatelitni lokus našli pri določeni pasmi ovc, je prikazano na sliki 4. Na vsakem od preučevanih lokusov smo pri vseh treh pasmah našli večino alelov. Poleg tega smo identificirali od dva do pet za pasmo specifičnih alelov (tako imenovani privatni aleli). Za istrsko pramenko je bilo za vseh osem lokusov ugotovljenih skupno trinajst privatnih alelov, osem za jezersko-solčavsko in šest za oplemenjeno jezersko-solčavsko ovco. Povprečno število alelov na mikrosatelitni lokus je za OPLEMENJENA JEZERSKO-SOLČAVSKA znašalo 7,5, prav toliko za JS, za IP pa 7,63 alelov na lokus.

Z namenom določitve stopnje polimorfnosti in s tem informativne moči posameznih lokusov znotraj preučevanih pasem smo izračunali delež heterozigotnosti. Glede na povprečno heterozigotnost smo označevalce (slika 4) razdelili v tri skupine, visoko polimorfne (heterozigotnost nad 70 %), srednje polimorfne (heterozigo-

Preglednica 5: *Rezultati genotipizacije družine z osmimi lokusi, ki prikazuje dva možna očeta*
Table 5: *Raw genotype data for eight markers that could not distinguish amongst two potential fathers*

ID		OarCP49		OarAE119		MAF65		MAF214		TGLA53		McM42		McM527		OarFCB11	
11346	SIN	2	19	50	50	36	40	67	70	27	27	7	8	57	58	26	30
143132	MATI	2	2	49	50	27	36	67	70	27	38	7	14	57	58	26	26
11211	Kandidatni oče 1	3	19	49	50	40	42	62	70	27	36	7	8	55	57	30	39
151982	Kandidatni oče 2	17	19	50	50	34	40	68	70	27	27	8	8	55	57	30	30

Preglednica 6: *Genotipi na dodatnih lokusih za potomca 11346*
Table 6: *Genotyping for four additional markers for progeny number 11346*

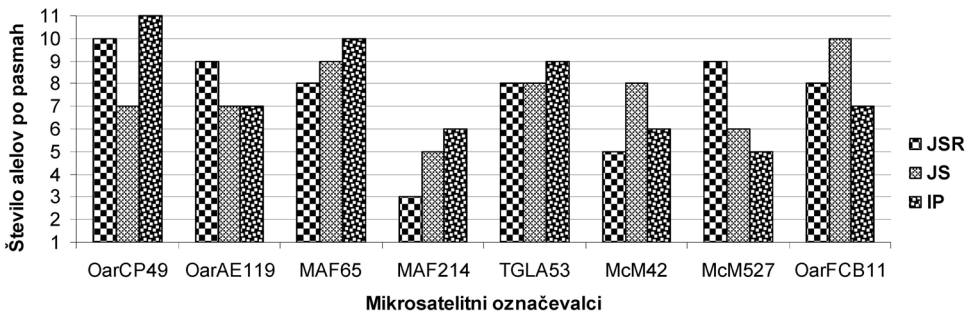
ID		OarCP20		Oar116		Oar109		Oar50	
11346	SIN	72	72	38	38	73	75	76	76
143132	MATI	72	72	38	38	73	73	74	76
11211	Kan. 1	72	72	38	38	73	78	76	77
151982	Kan. 2	72	72	38	38	74	75	76	76

tnost med 50–70 %) in nižje polimorfne (heterozigotnost od 0–50 %). Za vse tri pasme ovc smo opazili visoko polimorfnost označevalcev na lokusu MAF65, TGLA53 in OarFCB11 (slika 5). Pri posameznih pasmah je pri jezersko-solčavski izstopal lokus OarFCB11 (H= 0,86), pri oplemenjeni jezersko-solčavski lokus TGLA53 (0,84) in pri istrski pramenki lokus OarCP49 (0,86). V skupino srednje polimorfnih sta se pri jezersko-solčavski uvrstila lokusa OarCP49 (0,59) in McM42 (0,68), pri oplemenjeni jezersko-solčavski lokus OarAE119 (0,66), medtem ko smo pri istrski pramenki zabeležili tri lokuse OarAE119 (0,68), McM42 (0,63) in McM527 (0,51). V najmanj informativni skupini mikrosatelitnih označevalcev (heterozigotnost od 0–50 %) imamo en sam lokus, in sicer pri pri oplemenjeni jezersko-solčavski lokus MAF214 z deležem heterozigotnosti 0,32 – pri tej pasmi smo na tem lokusu zasledili le 3 alele, kar je najmanjše število alelov na vseh lokusih.

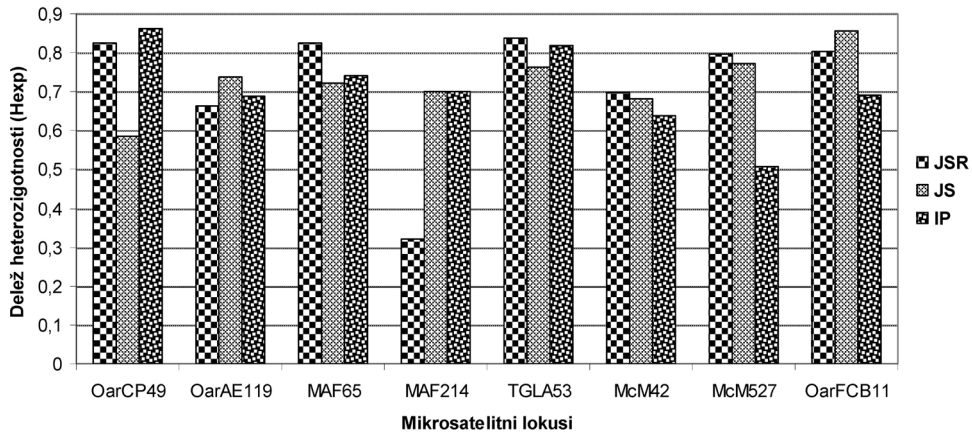
4 RAZPRAVA IN SKLEPI

4.1 ANALIZA RODOVNIKOV

Pri preverjanju rodovniških podatkov smo z analizo genotipov ugotovili, da prihaja pri vodenju rodovniških podatkov drobnice do napak. V štirih družinah od 44-



Slika 4: *Število alelov za posamezene mikrosatelitne lokuse pri jezersko-solčavski (JS), oplemenjeni jezersko-solčavski (JSR) in istrski pramenki (IP).*
Figure 4: *Number of alleles for genetic markers in Jezersko-Solčava (JS), Improved Jezersko-Solčava (JSR) and Bela krajina pramenka (IP) breeds.*

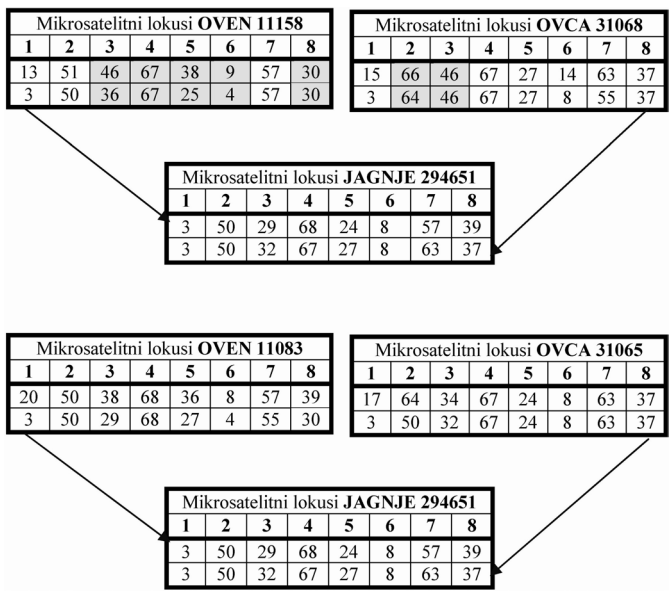


Slika 5: *Povprečna heterozigotnost na lokusih pri jezersko-solčavski (JS), oplemenjeni JS (JSR) in istrski pramenki (IP).*
Figure 5: *Average heterozygosity for analysed markers in Jezersko-solčava (JS), Improved Jezersko-solčava (JSR) and Bela Krajina pramenka (IP) breeds.*

ih (9,1 %) smo zasledili neskladja v rodovnikih pri štirih od skupno sedmih rejcev, vključenih v analizo. V dveh primerih (rejec 1 in 3) sta bila potomcu napačno pripisana oba starša. Pri primeru rejca 1 smo kasneje dodatno primerjali genotip jagnjeta (294651) z genotipi preostalih živali, ki smo jih pri tem rejcu imeli na razpolago. Rezultat primerjave je pokazal, da jagnje v resnici pripada materi (31065) in očetu (11083) in ne staršem, ki so mu bili ob rojstvu pripisani (slika 6). Poleg tega smo ugotovili, da ima jagnje št. 294651 brata (294649) z enakim genotipom, zato lahko predvidevamo, da sta enojajčna dvojčka. Podobno smo poskusili tudi za rejca 3, vendar zaradi majhnega števila živali (5), vključenih v analizo, pravih staršev nismo mogli določiti.

Razlogov, zaradi katerih prihaja pri vodenju rodovnika do napak, je lahko več. Po podatkih selekcijske službe za leto 2006 je v kontroliranih tropih v Sloveniji vključenih 106 tropov JS pasme, pri kateri je registriranih 2.969 ovc, ki so imele 3.784 jagnjitev in 116 tropov oplemenjene jezersko-solčavske pasme s 3.690 registriranimi ovcami s 4.762 jagnjitvami. Največ jagnjitev je bilo zabeleženih v spomladanskem obdobju z vrhoma v mesecu marcu in maju, z okrog 2500 rojenimi jagnjeti na mesec (Kompan s sod., 2006; Zajc in Komprej, 2007). V velikih tropih to lahko predstavlja problem, saj na isti dan lahko jagnji več ovc hkrati. Posledice tega so lahko zamenjave med materjo in novorojencem, kjer jagnje vzredi ovca, ki v resnici ni njegova mati. Do podobnih ugotovitev so prišli tudi Laughlin in sod. (2003). Lahko sklepamo, da se taki primeri največkrat dogajajo pri jagnjitvah na pašniku ali v neprimerno urejenih hlevih, kjer je nadzorovanje poteka jagnjitev omejeno. Za naša dva primera bi lahko predpostavili, da je šlo za samo napako pri zapisovanju rojstnih podatkov, ali pa je na isti dan jagnjilo več ovc, med katerimi je mati (31065) jagnjila dvojčka, eden od njiju (294651) pa se je pomešal z drugimi novorojenimi jagnjeti in ga je posvojila druga ovca. Rejec v tem primeru ni mogel predvideti, da jagnje pripada drugi materi in ga je avtomatsko pripisal materi, ki ni bila prava. Podobno bi lahko trdili tudi za rejca 2 (slika 2), ki je jagnjetu (294664) pripisal napačno mater (52848). Četrti primer neskladja genotipov smo ugotovili pri rejcu 5, ki ima v tropu stalno prisotnega ovna in ga zaradi preprečevanja parjenja v sorodstvu vsakih nekaj paritvenih obdobij menjuje. Problem se pojavi ravno v času menjave enega plemenskega ovna z drugim, ko sta v tropu prisotna oba ovna.

Možnosti, da je prišlo do napake pri določanju genotipov ali pri našem vzorčenju tkiv, kar je prispevalo k neskladju rezultatov, tudi ne moremo povsem izključiti. Ker smo določali genotipe na več lokusih, je možnost vpliva posamezne napake pri določanju genotipov majhna, saj neskladje navadno ugotovimo na več lokusih hkrati. Bolj verjetna bi bila napaka zaradi napačnega vzorčenja. Le ta je zaradi načina vzorčenja tudi malo verjetna, saj sta bila pri pregledu ušesne številke prisotna najmanj dva človeka – identifikaciji številke je takoj sledil odvzem vzorca. Obstaja sicer še možnost napačnega odčitavanja ušesne



Slika 6: *Določanje pravih staršev (oven 11083 in ovca 31065) za potomca 294651, ki je bil v rodovniku pripisan napačni družini (ovnu 11158 in ovci 31068).*
Figure 6: *Identification of correct parents (sire 11083 and dam 31065) for the progeny 294651 that was assigned to a wrong family (sire 11158 and dam 31068).*

številke in napačni pripis laboratorijske številke posameznemu vzorcu. Čeprav možnosti napak pri vzorčenju in obdelavi vzorcev v laboratoriju ne moremo povsem izključiti, menimo, da je večina neskladij (9,1 %) posledica napak pri vpisovanju in vodenju rodovnikov.

Zaključimo lahko z ugotovitvijo, da je natančnost rodovniških podatkov s strani rejcev in ob pomoči selekcijskih služb 90,9 %. Iz zgornje razprave sledi, da se nekatere napake s posegi v tehnologijo reje da odpraviti, nekatere pa bodo rejcem ostale prikrite. Zato se tu ponujajo alternativne metode ugotavljanja porekla, kot je opisana v tej študiji, ki bi omogočala, da bi odstotek točnosti rodovnikov približali 100 % in s tem dosegli vodenje rodovnikov brez napak.

4.2 DOLOČANJE OČETOVSTVA

V tropih, kjer se pri pripustih uporablja več plemenskih ovnov hkrati, so podatki o poreklu živali zaradi nezmožnosti nadzorovanja pripustov osiromašeni s podatkom očeta. Zato v teh tropih predstavlja velik pomen možnost določanja očetovstva in nadzora rodovniških podatkov s pomočjo tipizacije DNA. Poleg tega pa je za rejce, ki so vključeni v kontrolo porekla in proizvodnje, pogoj, da so vse živali predstavljene z znanim poreklom. Tako smo v tej študiji preverjali, če je možno z izbranih setom osmih mikrosatelitov in uporabo programa ATLAS jagnjetom istrske pramenke določiti enega od možnih dvajsetih očetov. Z izbranimi osmimi mikrosateliti smo lahko trem jagnjetom istrske pramenke nedvoumno določili očeta izmed potencialnih dvajsetih ovnov. V primeru enega jagnjeta smo lahko izločili 18 potencialnih očetov, dva očeta sta bila enako verjetna. Zato smo analizo razširili in vanjo vključili dodatne štiri mikrosatelitne označevalce ter s tem uspešno določili očetovstvo tudi temu potomcu.

4.3 INFORMATIVNOST

Najvišji nivo heterozigotnosti in s tem informativnosti lokusa smo pri vseh treh pasmah dosegli z označevalcem TGLA53, medtem ko se je za najmanj informativnega izkazal lokus MAF214. Lokusi OarCP49, OarAE119, MAF65 in OarFCB11 se pri teh treh pasmah uvrščajo v razred visoko polimorfnih mikrosatelitov, visok delež heterozigotnosti imata tudi McM42 in McM527, ki za najvišjim razredom ne zaostajata veliko. Rezultati deležev heterozigotnosti in števila alelov kažejo, da je najverjetneje stopnja inbridinga populacije istrske pramenke iz CSR Vremščice podobna kot pri jezersko-solčavski in oplemenjeni jezersko-solčavski pasmi. To ugotovitev utemeljujemo z rezultatom, da smo pri tej pasmi dobili v povprečju 7,6 alelov na lokus, kar je sploh največ od vseh pasem, vključenih v raziskavo, heterozigotnost alelov pa je znašala 71 %. Poudariti pa je potrebno, da je bilo v našo analizo zajetih za populacijsko-genetske študije še vedno relativno majhno število vzorcev in bi tako za bolj konkretne sklepe o inbridiranosti populacije istrske pramenke morali analizirati večje število živali. Vendarle smo v enem od štirih družin odkrili višjo stopnjo sorodstva, kar lahko kaže, da znotraj pasme istrske pramenke lahko obstajajo visoko inbridirane pod-populacije oziroma družine – v tem primeru je visoka stopnja sorodstva v tej družini istrske pramenke privedla k zmanjšanju stopnje polimorfnosti mikrosatelitnih lokusov in s tem njihove informativnosti. Za nadaljnje analize predlagamo, da se našemu uporabljenemu setu doda še označevalec Oar109 ali zamenja označevalec MAF512, ki je s petimi aleli in 51 % heterozigotnostjo na zelo nizkem informativnem nivoju.

Mikrosateliti, uporabljeni v tej študiji in analiza s programom ATLAS sta torej primerna pri preverjanju pravilnosti rodovniških podatkov in pri določanju očetovstva. V zadnjem času se v Sloveniji na področju selekcije v ovčereji vse bolj uveljavlja uporaba molekularno-genetskih metod. Molekularni podatki so dober pokazatelj variabilnosti v populacijah, kar nam omogoča zasnovo programov za ohranjanje genetskih raznovrstnosti v populacijah živali. Eden takih pristopov je prav gotovo tudi kontrola porekla z uporabo molekularnih markerjev, ki se je v Sloveniji že dobro uveljavila pri gospodarsko pomembnih domačih živalih (konji, govedo, prašiči itd.). Tudi pri drobnici se srečujemo s čedalje strožjim selekcijskim nadzorom, zato so analize te vrste preizkušene, v nekaterih primerih pa tudi uporabljene. Ker pa je vse več populacij ovc, vključenih v programe obveznega genotipiziranja za prionski gen, bi bilo zaželeno dodatno preverjanje porekla. To je pomembno tako z vidika ocenjevanja plemenske vrednosti jagnjeta kot z vidika kontrole inbridinga, torej izogibanja nadaljnjega parjenja v sorodstvu, ter z vidika zanesljivejše genotipizacije za lokus PRNP.

Ta študija je pokazala, da kljub skrbi za natančno vodenje rodovniških knjig pri teh še vedno prihaja do napak. Da bi se napakam izognili, se selekcijskim službam ponuja možnost nadzorovanja porekla z uporabo mikrosatelitnih označevalcev. V ta namen velja omeniti, da lahko postopek, s katerim smo izvedli analizo rodovništva, še nekoliko posodobimo s pomnoževanjem večjega števila mikrosatelitnih lokusov hkrati v eni PCR reakciji. To nam omogoči hitro, cenejšo ter enostavno izvedbo genotipizacije. Zaključimo lahko z dejstvom, da uporaba visoko polimorfnih DNA označevalcev omogoča učinkovito in objektivno preverjanje rodovniških po-

datkov, predvsem pomemben pa je podatek, da postajajo ta orodja vse pomembnejša pri projektih ohranjanja genetske raznovrstnosti.

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IN VITRO MAMMARY GLAND MODEL: ESTABLISHMENT AND CHARACTERIZATION OF A CAPRINE MAMMARY EPITHELIAL CELL LINE

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In vitro mammary gland model: establishment and characterization of a caprine mammary epithelial cell line

Demanding transcriptomic studies in combination with challenging experiments in livestock animal species could be replaced by good *in vitro* models mimicking the function of ruminant mammary gland. The objective of our study was to establish epithelial cell line obtained from primary cell culture of lactating goat mammary gland. Mammary tissue from lactating Saanen goat (*Capra hircus*) was digested in collagenase and hyaluronidase solution and plated on plastic flasks. When growing on plastic, typical cobblestone morphology of epithelial cells and larger irregularly shaped cells, corresponding to myoepithelial cells were observed. When growth medium was supplemented with lactogenic hormones (insulin, hydrocortisone, and prolactin) and cells were cultured on plastic for extended period of time at high density, dome-like structures appeared as a result of cell to cell contact induced differentiation. Immunofluorescence staining using antibodies against smooth muscle α-actin, vimentin and various cytokeratins were used to distinguish between different cell types. Cell types of epithelial and myoepithelial cells were confirmed. Complete differentiation of cells was achieved when growing them on a commercial basal membrane matrix preparation which contains laminin, collagen IV, and various growth factors. Cells grown on basal membrane matrix in growth medium supplemented with lactogenic hormones differentiated morphologically and functionally. Spherical structures that resembled the alveoli of lactating mammary gland were observed. Reverse transcription PCR (RT-PCR) was performed on the total RNA extracted from the cultured cells in order to detect the potentially present milk protein mRNAs.

Key words: goats / mammary gland / molecular genetics / cell culture / lactogenesis / caseins / expression / immunofluorescence

In vitro model mlečne žleze: vzpostavitev in določitev značilnosti epitelne celične linije iz kozje mlečne žleze

Zahtevne transkriptomske študije, ki vključujejo poskuse na živih živalih, je mogoče nadomestiti z ustreznimi *in vitro* modeli. Cilj naše raziskave je bil vzpostaviti celično linijo epitelnih celic, pridobljenih iz primarne celične kulture kozje mlečne žleze v laktaciji. Žlezno tkivo koze (*Capra hircus*) sanske pasme smo razgradili v raztopini kolagenaze in hialuronidaze in nacepili v plastične posodice. Pri rasti na plastični podlagi so se pojavile značilne epitelne strukture v obliki tlakovcev in večje celice nepravilnih oblik, ki so po zunanjosti ustrezale mioepitelnim celicam. Ob dodatku laktogenih hormonov (inzulin, hidrokortizon, prolaktin) v medij in po daljšem obdobju rasti ter ob visoki gostoti celic na plastični podlagi so se oblikovale kupolaste strukture, ki so posledica diferenciacije zaradi medceličnih interakcij. Za karakterizacijo različnih celičnih tipov smo uporabili imunofluorescenčno barvanje za α-aktin gladkih mišic, vimentin in različne citokeratine. Z barvanjem smo potrdili prisotnost epitelnih in mioepitelnih celic. Popolno diferenciacijo celic smo dosegli z gojenjem na komercialno pripravljenem matriksu, ki posnema bazalno membrano in vsebuje laminin, kolagen IV in različne rastne faktorje. Celice so se na podlagi iz ekstracelularnega matriksa in ob dodatku laktogenih hormonov morfološko in funkcionalno diferencirale. Nastale so sferične strukture, podobne alveolam mlečne žleze v laktaciji. Z reverzno verižno reakcijo s polimerazo (RT-PCR) smo na izolirani RNA preverili prisotnost mRNA za mlečne proteine.

Ključne besede: koze / mlečna žleza / molekularna genetika / celična kultura / laktogeneza / kazeini / ekspresija / imunofluorescenca

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1 INTRODUCTION

Because of the commercial value of milk there is a great interest in understanding mechanisms involved in milk protein expression and udder resistance to pathogens which cause infectious agalactia or secretion of abnormal milk. Demanding transcriptomic studies investigating mechanisms influencing mammary gland metabolism usually involve *in vivo* experiments. Additionally, treatments *in vivo* can have systemic effects which make controlling the environment of epithelial cells in a predictable way very difficult (Rose & McConoche, 2006). For this reasons adequate *in vitro* model mimicking the function of the mammary gland would be of great importance for the study of physiological, biochemical and immunologic functions of the mammary gland. Furthermore, there are almost no techniques that would allow the maintenance of organs *ex vivo* long enough to permit necessary molecular biological investigations. As such, an enormous potential exists in the use of three-dimensional (3-D) cell culture models as surrogates for tissues. In the recent years, mammary cell culture models were mainly used to study cell differentiation during lactation, innate immune response to infections and response to hormonal induction of lactogenesis in mammary epithelial cells (MECs).

Several ruminant immortalized cell lines such as MAC-T (Huyhn *et al*., 1991) and BME-UV (Zavizion *et al*., 1996) have been established by stable integration of the simian virus large T-antigen (SV40LTA). However, because of their low responsiveness to lactogenic hormones, transformed mammary cell lines were mainly used to study insulin growth factor 1 (IGF-1) metabolism (German & Barash, 2002). It is still not clear how modifications in immortalized cell lines alter physiological pathways of transformed cells, therefore the use of primary cell lines is much more representative of the *in vivo* system maintaining organ-specific functions and signal transduction pathways (Pantschenko *et al.,* 2000).

Growth of primary mammary cell cultures from lactating mammary gland on plastic usually results in loss of tissue specific functions. Cells in this state do not synthesize any of the milk components nor do they have the cellular response of *in vivo* cells (Blum *et al.,* 1989). On the other hand the growth of MECs on pre-formed extracellular matrices results in morphological differentiation as well as in synthesis of milk components (Rose *et al.,* 2002). Kabotyanski *et al.* (2009) studied transcription of β-casein (*CSN2*) and suggested that the expression of *CSN2* is induced synergistically by lactogenic hormones together with local growth factors, cell-cell and cell-substratum interactions.

The objective of our study was to establish goat MEC (GMEC) line, from primary cell culture, that is responsive to lactogenic hormonal induction and capable of expressing milk protein genes. The established GMEC line will be used for further studies of mammary gland differentiation, induction of lactation and infection response.

2 MATERIALS AND METHODS

2.1 ESTABLISHMENT OF CELL CULTURE

Mammary tissue was aseptically removed from the mammary gland of lactating Saanen goat (*Capra hircus*) immediately after slaughter. The gland was wiped with 70% ethanol and chopped up in chunks which were washed in HBSS (Hank's Buffered Salt Solution) medium containing penicillin (200 μg/ml), streptomycin (200 μg/ml), gentamicin (200 μg/ml), ampicilin (200 μg/ml) and amphotericin B (10 μg/ml). Tissue was further sliced in smaller pieces and digested in 100 ml of collagenase (Biochrom AG) and hyaluronidase (Sigma) solution (400 U/ml of each) prepared in HBSS with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) containing all of the above listed antibiotics in the same concentrations. The digestion was carried out at 37 °C with gentle shaking. The digesta were collected at 60, 120 and 180 minutes after the initiation of digestion and were filtered through a steel mesh. The filtrates were put in a 50 ml tube and washed several times with HBSS followed by centrifuging at 1200 rpm for 5 minutes. The cell suspension was filtered through 40 μm mesh, centrifuged and plated on plastic or further resuspended in 90% FBS and 10% DMSO for freezing in liquid nitrogen.

Aliquots of cell suspensions (fractions 1–3) were plated on plastic flasks in growth medium RPMI 1640 (Sigma) supplemented with lactogenic hormones insulin (1 μg/ml), hydrocortisone (1 μg/ml), and prolactin (1 μg/ml). Cells were incubated in a CO_2 incubator at 37 °C, 5% CO_2 and saturated humidity. The medium was changed every 2 to 3 days. For observing dome formation cells were maintained in culture for at least 20 days. When performing passaging the cells were treated with 0.05% trypsin-EDTA (Sigma) and incubated at 37 °C until the cells detached from the plastic dish. The cells were than resuspended in growth medium. Characteristics of the cell line were observed under light microscope.

2.2 IMMUNOFLUORESCENT STAINING

Cells were seeded in 6-well plates on cover glasses and cultured till they nearly reached confluence. They

were washed with cold phosphate buffered saline (PBS) and fixed in a mixture of cooled acetone and methanol (dilution 1:1) at –20 °C. Monoclonal antibodies against smooth muscle α-actin (sc-58669), vimentin (sc-73262) and cytokeratins (K) 14, 18, and 19 (sc-53253; sc-51582; sc-6278; all Santa Cruz Biotechnology) were used to distinguish between different cell types. The cover glasses were covered with solution of primary antibodies (dilution 1:200) in PBS with BSA (3%) and incubated overnight at room temperature. For unspecific binding control cover glass was incubated with PBS – BSA (3%). As a secondary antibody goat anti–mouse–FITC (F4143, Sigma) was used in dilution 1:500 in PBS – BSA (3%). Incubation was carried out in dark for 1 hour, and cells were observed under fluorescent microscope (Nikon Eclipse TE, 2000).

2.3 THIN LAYER METHOD AND 3D CULTURE METHOD

GMECs were grown on Geltrex Reduced Growth Factor Basement Membrane Matrix (Invitrogen) which is a soluble form of basement membrane purified from Engelbreth-Holm-Swarm tumor that gels at 37 °C forming a reconstituted basement membrane. The major components of Geltrex (GT) include laminin, collagen IV, entactin and various growth factors. In both methods GT was thawed at 2 to 8 °C overnight on ice in refrigerator. In thin layer method, used for culturing primary cell line, GT was diluted in cold serum-free RPMI 1640 medium in a concentration of 0.1 mg/ml and sufficient amount was used to cover the entire growth surface. Coated object was placed at 37 °C for 60 minutes or until dry. In 3D culture method 50 µl of GT was used per well of 24-well plate and left at 37 °C to promote gelling of matrix. GMECs were suspended in RPMI 1640 media containing 2% of GT and approximately 10^5 cells were plated per well. The cells were grown at 37 °C in humidified atmosphere of 5% CO2 in air and observed through microscope.

2.4 LACTATION INDUCTION (RT-PCR)

Total RNA was extracted from confluent second passage GMECs, grown on thin layer GT in lactogenic growth medium (as described previously), using TRI Reagent (Ambion) in accordance to manufacturer's instructions. In order to detect potentially present milk

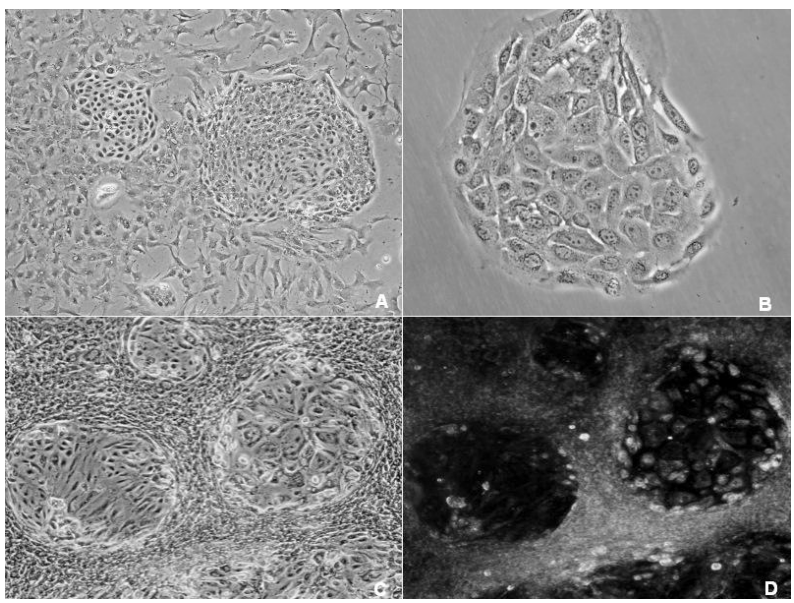

Figure 1: *Goat mammary cell line (second passage) growing as a monolayer on plastic.* ***A.*** *Colony morphology observed after 18 days in culture. Islands of epithelial cells surrounded with myoepithelial cells (magnification × 40).* ***B.*** *Island of densely packed epithelial cells (magnification × 200).* ***C*** *and* ***D.*** *Dome-like structures in 30 day old post-confluent cell line made by light microscopy using different light polarisation (magnification × 40).*

Slika 1: *Kozja celična linija (druga pasaža) v obliki monosloja na plastični podlagi.* ***A.*** *Morfologija kolonij v kulturi po 18. dneh. Otoki epitelnih celic obkroženi z mioepitelnim celicami (40 x povečava).* ***B.*** *Otoček epitelnih celic (200 x povečava).* ***C*** *in* ***D.*** *Kupolaste strukture v 30 dni stari postkonfluenti kulturi – svetlobna mikroskopija z uporabo različnih polarizacijskih filtrov (40 x povečava).*

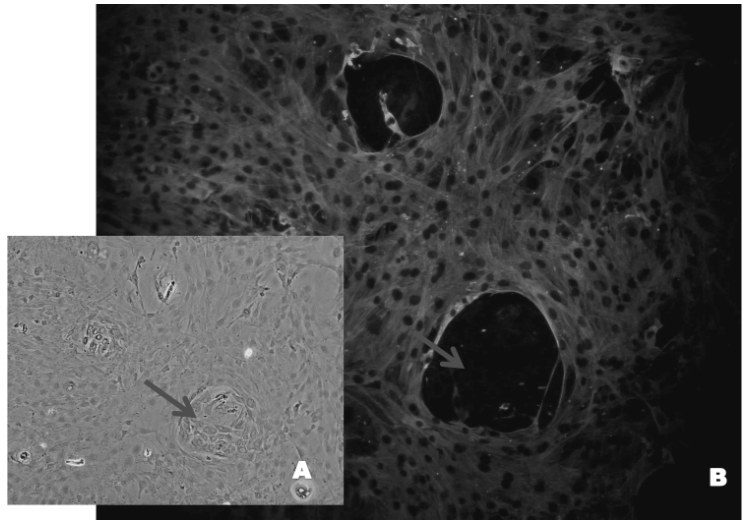

Figure 2: *Cell line labelled with antibodies for smooth muscle α-actin. Arrows indicate island of epithelial cells surrounded with myoepithelial cells.* ***A.*** *light microscopy (magnification ×100).* ***B.*** *fluorescent microscopy (magnification ×100).*
Slika 2: *Celična linija označena s protitelesi proti α-aktinu gladkih mišičnih vlaken. Puščici prikazujeta otok epitelnih celic obkrožen z mioepitelnimi celicami.* ***A.*** *svetlobna mikroskopija (100 x povečava).* ***B.*** *fluorescentna mikroskopija (100 x povečava).*

protein mRNAs reverse transcription-polymerase chain reaction (RT-PCR) was performed using OneStep RT-PCR kit (Qiagen). The PCR primers for amplification of β-casein (*CSN2)* and housekeeping gene β-actin (*ACTB)* were as follows: CSN2a-F: 5'-ACAGCCTC-CCACAAAACATC-3', CSN2a-R: 5'-AGGAAGGT-GCAGCTTTTCAA-3' with product length 206 bp; ACTBa-F: 5'-CCAACCGTGAGAAGATGACC-3', ACT-Ba-R: 5'-CGCTCCGTGAGAATCTTCAT-3' with product length 247 bp. RT-PCR products for β-casein and β-actin (*ACTB)* were isolated from agarose gel using gel extraction kit (Jetquick) and confirmed by sequencing.

3 RESULTS

Digestion of mammary tissue in collagenase and hyaluronidase solution resulted in isolation of heterogenous culture which contained mixed population of epithelial and myoepithelial (smooth muscle α-actin positive) cells. When grown on plastic, typical cobblestone morphology of epithelial cells and larger irregularly shaped cells corresponding to myoepithelial cells were observed (Figures 1 A and B). Dome-like structures appeared as a result of cell to cell contact induced differentiation, when cells were grown for extended period of time at high density (Figures 1 C and D).

The presence of cells representing epithelial and myoepithelial cell type was confirmed by immunostaining. Myoepithelial cells stained positively for smooth muscle α-actin whereas proposed luminal epithelial cells did not (Fig. 2). Cells of caprine cell line stained variously for cytokeratins (K14, K18, and K19) and negatively for mesenchymal intermediate filament protein vimentin.

Complete differentiation of cells was achieved when growing them on basal membrane (GT) matrix. GMECs grown on thin layer of GT matrix in growth medium supplemented with lactogenic hormones were able to express β-casein. Expression of β-casein was confirmed

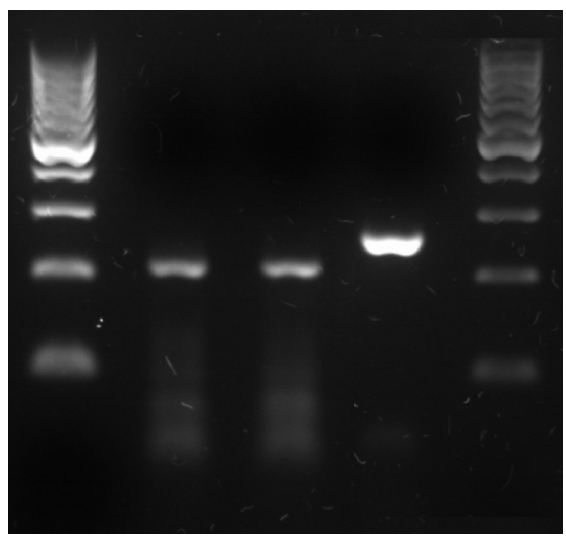

Figure 3: *Agarose gel electrophoresis of RT-PCR products for β-casein (lanes 2,3) and β-actin (lane 4) as control marker.*
Slika 3: *Agarozna gelska elektroforeza RT-PCR produktov za β-casein (stolpec 2,3) in β-aktin (stolpec 4), ki smo ga uporabili za kontrolo.*

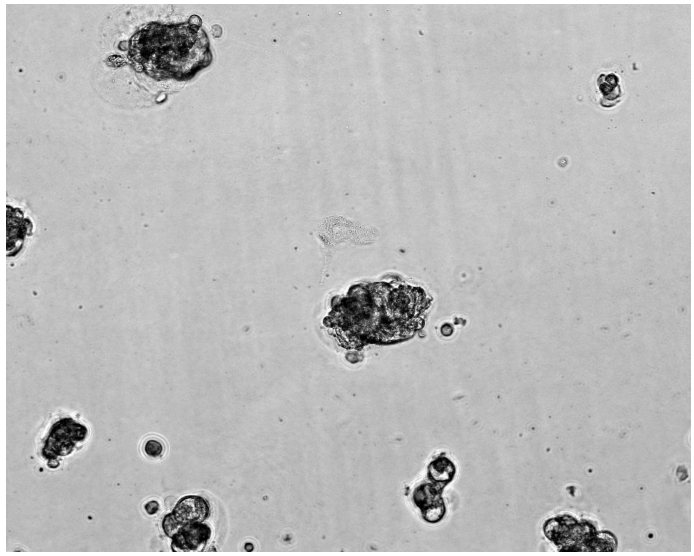

Figure 4: *3D culture of GMECs in GT matrix supplemented with growth medium containing lactogenic hormones (magnification ×100).*
Slika 4: *3D organizacija epitelne celične linije v GT matriksu, gojena v gojišču z dodanimi laktogenimi hormoni (100 x povečava).*

with reverse transcription PCR (Fig. 3). Products for β-casein (*CSN2)* and housekeeping gene β-actin (*ACTB)*, which we used as control, were confirmed by sequencing. This indicates that under lactogenic conditions milk proteins are being produced by GMECs.

When grown on GT matrix using 3-D culture method and supplemented with medium containing lactogenic hormones three-dimensional spheroids resembling acini of lactating mammary gland were formed (Fig. 4).

4 DISCUSSION

We describe the establishment of GMEC line focusing on growth morphology, expression of cytoskeletal proteins and evidence of differentiation. When the dissociated mammary gland cells were grown in vitro some of the cells formed island monolayer aggregates while others existed in free single-cell form. The GMECs were of different types. Epithelial type of cells depended closely on one another, were connected to each other and formed islands of similar densely packed cuboidal cells. Myoepithelial cell growth was observed in individual, random cell pattern at lover density compared to their epithelial counterparts.

Spontaneous domes are formed in post-confluent cell line which in a way is reminiscent of 3D organization of cells. It has been previously shown that formation of dome-like structures was connected with fluid under the epithelial cells that grew on plastic (Pickett *et al.*, 1975). Functional and structural changes that take place in dome-forming cells correspond to cellular changes occurring in vivo when tubules and alveoli are developed in the mammary gland at pregnancy (Zucchi & Dulbecco, 2002).

Specific smooth muscle α-actin monoclonal antibody reactivity was shown in myoepithelial cells. Actin is observed as sheets of filaments in the myoepithelial cell cytoplasm, whereas epithelial cells did not react with this antibody. Since vimentin is a marker of nonepithelial cells (i.e. cells of mesenchymal origin) non-staining of GMECs indicate that there are no fibroblasts in the cell line. Cytoskeletal protein expression is very much dependent on culture conditions and substrate of growth, thus we were not able to determine specific staining for cytokeratins.

The cells isolated from the goat mammary gland undergo three-dimensional organization in Geltrex. We observed formation of mammospheres or acinus-like structures, morphologically similar to those described as deriving from MECs (German & Barash, 2002, Rose *et al.*, 2002). Under this condition casein secretion by GMECs was dependent on the presence of lactogenic hormones. We were able to prove expression of β-casein, which is the major milk protein in goat milk, however we were not able to prove expression of other milk proteins.

When growing in culture, GMECs closely mimic the *in vivo* state of mammary gland, thus providing a suitable cell system model to study complex biological processes and pathways. Compared with monolayer cells in 2D culture, 3D cell culture provides physiologically much more relevant model for studying mammary cell

function. Our GMEC line will be exploited in transcriptomic studies focused on host response during infection, replacing challenging *in vivo* experiments.

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NEW PRIMER COMBINATIONS WITH COMPARABLE MELTING TEMPERATURES DETECTING HIGHEST NUMBERS OF *nosZ* SEQUENCES FROM SEQUENCE DATABASES

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New primer combinations with comparable melting temperatures detecting highest numbers of nosZ sequences from sequence databases

We explored existing primer sequences targeting nitrous oxide reductase (*nosZ*) gene in order to explore their capability to recognize variant *nosZ* sequences. Published *nosZ* sequences longer than 380 AA residues were obtained from FunctionalGene Database /Repository (http://flyingcloud.cme.msu.edu/fungene/) and used for explorations with PrimerChart program. The numbers of sequences recovered using all possible forward and reverse primer combinations were determined and the stringency of primer site recognition was further varied by allowing 1, 2, or 3 primer mismatches to DNA binding site. We identified novel primer combinations resulting in satisfactory amplicon length (> 500 bp) and increased sequence recognition capabilities at comparable forward and reverse primer melting temperatures. Overall, this study indicates that current state of the art molecular methods can be and should frequently be further refined by the use of targeted bioinformatic approaches.

Key words: microbiology / molecular biology / denitrification / nitrous oxide reductase / melting temperature / detection

Nove kombinacije začetnih oligonukleotidov s primerljivimi temperaturami taljenja zaznavajo najvišje število sekvenc nosZ v podatkovnih bazah

V tej študiji sva raziskala obstoječe sekvence začetnih oligonukleotidov, s katerimi se pomnožujejo fragmenti gena za reduktazo N_2O (*nosZ*), da bi proučila njihovo zmožnost prepoznavanja variant sekvenc *nosZ*. Objavljene sekvence gena *nosZ* daljše od 380 aminokislninskih ostankov sva pridobila od FunctionalGene Database /Repository (http://flyingcloud.cme.msu.edu/fungene/) in jih analizirala s programom PrimerChart. Raziskala sva število, ki ga prepoznajo posamične možne kombinacije začetnih oligonukleotidov. V nadaljevanju sva spreminjala natančnost prileganja začetnih oligonukleotidov na tarčno DNK tako, da sva dovolila 1, 2, or 3 napačna parjenja med začetnim oligonukleotidom in DNK. Tako sva identificirala nove kombinacije začetnih oligonukleotidov, ki ustvarijo ustrezno dolge fragmente (> 500 bp), s povišano sposobnostjo prepoznavanja sekvenc pri primerljivi temperaturi taljenja začetnih oligonukleotidov. Prav tako so se nakazale nove možnosti za izboljšanje začetnih oligonukleotidov z vnosom novih degeneriranih mest. Ta študija nakazuje, da je novejše molekularne metode možno in tudi potrebno pogosto nadgrajevati s ciljanimi bioinformatskimi pristopi.

Ključne besede: mikrobiologija / molekularna biologija / denitrifikacija / dušikov oksid / reduktaza / temperatura taljenja / zaznavanje

1 INTRODUCTION

Knowledge of abundances and kinds of organisms in an ecosystem is widely recognized as an important step towards understanding the ecology of the system (Prosser *et al.*, 2007). As most prokaryotic species cannot be readily studied by cultivation dependent approaches, culture-independent methods have been widely employed to explore microbial diversity and understand community dynamics. These methods are believed to provide a snapshot of the relative abundances of underlying microbial populations. However, as the number of

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sequences deposited to public databases increases and basic molecular approaches to studying microbial communities continue to differ, it is ever increasingly hard to grasp and contemplate the outcomes of numerous studies. A closer inspection of published literature (http://www.ncbi.nlm.nih.gov/PubMed or http://www.sciencdirect.com) reveals that a number of studies deployed unbalanced tools, the unbalance spanning from sampling, over DNA extraction to molecular tool development, their use and interpretation.

NosZ is the crucial enzyme in denitrification that is responsible for conversion of potent greenhouse gas N_2O to molecular dinitrogen. In an accompanying paper (Stres and Murovec, 2007) we explored the differences in predicted melting temperatures of available forward and reverse primers used in amplification of *nosZ* sequences. In this work we explored the capability of these primers to recognize *nosZ* sequences using 0, 1, 2, 3 primer mismatch thresholds in order to identify novel primer combinations resulting in increased sequence recognition capabilities at comparable melting temperatures. To achieve this in controlled manner, the aligned published sequences of nitrous oxide reductase (*nosZ*) gene of sufficient length were obtained from FunctionalGene Database /Repository (http://flyingcloud.cme.msu.edu/fungene/) and used as a model community.

2 MATERIALS AND METHODS

2.1 DATA SELECTION

Literature on the molecular methods used for amplification of target denitrification genes from environmental samples was explored as described in accompanying paper (Stres and Murovec, 2007). The primer sequences were extracted and organized into two dictionaries, containing forward and reverse primer sequences. Each primer sequence was characterized with a primer published name, first base binding location and its DNA binding sequence.

A selection of HMM aligned *nosZ* database currently containing 2025 sequences was downloaded from FunGene Repository / Pipeline (http://flyingcloud.cme.msu.edu/fungene/). The sequences were selected according to primary criterion length (L > 380 AA) and HMM score (s > 20) thus resulting in a dataset containing1985 sequences.

2.2 DATA ANALYSIS

Newly developed software PrimerChart (Murovec and Stres, unpublished) was used for analysis. The numbers of sequences recovered using all possible forward and reverse primer combinations were explored. The stringency of primer site recognition was varied by allowing 1, 2, or 3 primer mismatches to DNA binding site. The combinations of forward and reverse primer pairs were ranked according to the number of sequences detected.

3 RESULTS AND DISCUSSION

In the present study we used previously described primer sets to sample a model microbial community comprised of the longest available and aligned *nosZ* sequences. Figure 1 shows schematic distribution of primer binding sites to the gene of *nosZ* according to *Pseudomonas aeruginosa* 2192 complete genome full nitrous oxide reductase sequence under accession number NZ_AAKW01000028. As it can be seen, the primer binding sites are mainly designed and distributed in the center region of *nosZ* gene spanning between ~1000 bp and ~2000 bp covering roughly 1000 kb region between two conserved *CuA* and *CuZ* sites (Hoeren *et al.*, 1993). In this respect, this region contains the highest sequence coverage.

Figure 2 shows the distribution of hits as detected by different forward and reverse primer combinations used in this study. Each primer combination was assigned a number key after they were sorted according to the number of recognized sequences. As it can be seen, a small number of primer sets could be identified as potential candidates for primers with highest recognition capabilities of sequences from model *nosZ* community.

Figure 1: *The schematic representaion of primer-binding sites of primers used in this study. For more details on primers please see Stres and Murovec (2007).*
Slika 1: *Shematičen prikaz mest naleganja začetnih oligonukleotidov, uporabljenih v tej študiji. Za podrobnosti glede začetnih oligonukleotudov glej Stres and Murovec (2007).*

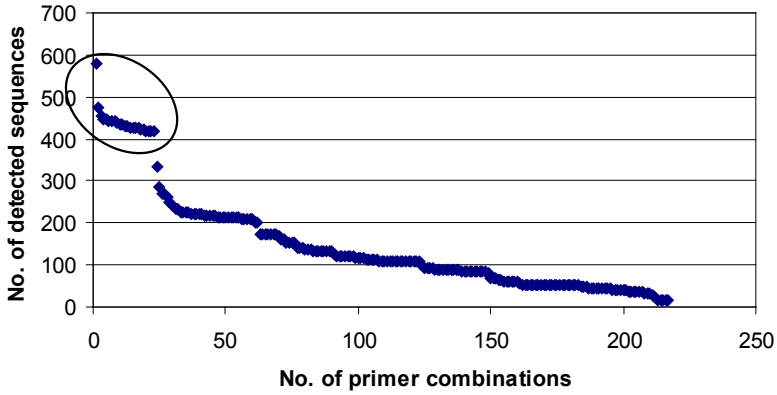

Figure 2: *The schematic representaion of primer-binding sites of primers used in this study. For more details on primers please see Stres and Murovec (2007).*
Slika 2: *Shematičen prikaz mest naleganja začetnih oligonukleotidov, uporabljenih v tej študiji. Za podrobnosti glede začetnih oligonukleotudov glej Stres and Murovec (2007).*

As primers were covering comparable gene region we explored the effect of additional primer mismatches to DNA primer binding site. Increasing the number of recognition sites from 0 to 3 resulted in roughly 50% more detected sequence (dat not shown). This indicates that the degeneracy of already degenerated primers should and could be further increased to incorporate unaccounted variations in primer binding sites. However, this was not the scope of our current research.

In theory, the melting temperature of forward and reverse primers used in a pair should be kept as comparable as possible (Ausubel *et al.*, 1999). Therefore the *in-silico* melting temperatures (Stres and Murovec, 2007) were taken as a measure of comparability of primers melting temperatures and a measure of their compatibility in order to be used as a primer pair in amplification. In this respect the following combination of forward and reverse primers can be suggested for further use in molecular studies (Table 1). However, differences in average melting temperatures of novel primer combinations should be

Table 1: *The combination of forward and reverse primers suggested for further use in molecular studies exploring nosZ diversity in complex samples. Designations ?, +, ++, +++ indicate > 10 °C, < 6 °C,< 4 °C and < 2 °C difference in average melting temperatures of paired oligonucleotides, respectively.*
Preglednica 1: *Predlagane kombinacije začetnih oligonukleotidov za uporabo v molekularnih študijah raznolikosti nosZ in complex samples. Oznake ?, +, ++, +++ kažejo > 10 °C, < 6 °C,< 4 °C in < 2 °C razlike v povprečnih temperaturah taljenja začetnih oligonukleotidov v paru.*

nameF	average FTm	sd	nameR	average RTm	sd	DNA Matches	Δ Tm	Δ Tm suitability	Fragment lenght
24nosZf436	48.52	2.59	1nos1319R	61.94	2.34	580	−13.42	?	883
24nosZf436	48.52	2.59	4nos1527R	59.33	1.97	474	−10.81	?	1 091
35PsNosZ175F	60.07	0.00	1nos1319R	61.94	2.34	454	−1.87	+++	1 144
2Nos1527F	64.65	1.87	17nosZ1773b	61.07	2.84	442	3.58	++	246
27nosZ-F-1181	66.01	1.83	19nosZ1R1421	65.42	2.31	441	0.58	+++	240
27nosZ-F-1181	66.01	1.83	17nosZ1773b	61.07	2.84	439	4.94	+	592
25nosZ-F1211	66.18	1.61	19nosZ1R1421	65.42	2.31	436	0.76	+++	210
25nosZ-F1211	66.18	1.61	17nosZ1773b	61.07	2.84	432	5.11	+	562
2Nos1527F	64.65	1.87	1773R	61.18	2.19	426	3.47	++	246
27nosZ-F-1181	66.01	1.83	1773R	61.18	2.19	423	4.83	+	592
25nosZ-F1211	66.18	1.61	1773R	61.18	2.19	418	5.00	+	562

noted before adopting these combinations for research. Two of the most promising primer combinations resulted in more than 10 °C difference in melting temperatures rendering them least suitable. Both have numerous degenerated sites as can be seen from accompanying standard deviations of their average melting temperatures. The most suitable primer pair satisfying (i) the need for sufficient amplicon length, (ii) comparable average melting temperatures, and (iii) sequence recognition capabilities appears to be NosZ175F and nosZ1319R.

However, the following problems still remain: (i) relatively low number of sequences deposited to public databases, (ii) low number of sequences of sufficient quality (containing only characters A, T, G, C), (iii) unequal melting temperatures of most suitable suggested primer pairs and (iv) relatively low resolution resulting from short sequences amplified by some of the suggested primers. Future studies, especially metagenomic studies and direct reconstructions of genomes from environment, are going to provide valuable data on the uncovered *nosZ* gene variants in environment.

4 CONCLUSIONS

The analysis of primer combinations revealed that existing primers sequence could be further modified to accommodate novel degenerated sites and thus be able to detect a broader sequence diversity. Further, some previously untested primer combinations were explored resulting in higher number of recognized sequences, sufficient length of amplicon (> 500 bp) and comparable melting temperatures, thus indicating their potential for future use in molecular studies. Future work is going to be directed towards detailed analysis of primer binding sites in order to generate combinations of primers targetting widest array of availabe sequences.

Overall, this study indicates that current state of the art molecular methods can be and should frequently be further refined by the use of targeted bioinformatic approaches.

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ISOLATION AND USE OF *Prevotella ruminicola* TC18 PLASMID pTC18 IN *Escherichia coli-P. ruminicola* SHUTTLE VECTOR CONSTRUCTION

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Isolation and use of Prevotella ruminicola TC18 plasmid pTC18 in Escherichia coli-P. ruminicola shuttle vector construction

A cryptic plasmid of approximately 3 kilobases named pTC18 was discovered in a ruminal *Prevotella ruminicola* TC18 strain and cloned into *Escherichia coli*. Based on pTC18, several shuttle vectors, containing *Prevotella*/*Bacteroides tetQ* selection marker and *E. coli* vector pUC19 inserted at two different positions in pTC18 were constructed. The shuttle vectors, protected with *Hae*III methylase against the *P. ruminicola* 23 restriction were electroporated into *P. ruminicola*. Despite numerous attempts a tetracycline resistant recombinant strain 23 was not obtained. The possible causes for electroporation failure are discussed.

Key words: microbiology / anaerobic bacteria / Prevotella ruminicola / shuttle vector / rumen

Osamitev plazmida pTC18 seva Prevotella ruminicola TC18 in njegova uporaba v razvoju prenosljivih vektorjev Escherichia coli-P. ruminicola

V vampnem sevu *Prevotella ruminicola* TC18 smo odkrili 3 kilobazne pare dolgo plazmidno DNA, jo poimenovali pTC18 in klonirali v *Escherichia coli*. Na njeni osnovi smo razvili več različic prenosljivega plazmida, ki je poleg pTC18 vseboval še selekcijski marker *tetQ* iz sevov rodu *Bacteroides* in plazmidni vektor *E. coli* pUC19. Prenosljive vektorje smo s *Hae*III metilazo zaščitili proti restrikciji v *P. ruminicola* 23 in jih nato poskusili vnesti v *P. ruminicola* 23 z elektrotransformacijo. Kljub mnogim poskusom nismo uspeli pridobiti proti tetraciklinu odpornih sevov *P. ruminicola* 23.

Ključne besede: mikrobiologija / anaerobne bakterije / Prevotella ruminicola / prenosljivi vektor / vamp

1 INTRODUCTION

Prevotella ruminicola is thought to be the most numerous among the strictly anaerobic gram negative rumen bacteria from the genus *Prevotella* which apparently play important roles in the rumen ecosystem (Tajima *et al*., 2001; Miyazaki *et al*., 2003). The genome of the *P. ruminicola* type strain 23 is currently being sequenced at former TIGR, now J. Craig Venter Institute (http://www.jcvi.org/rumenomics/). However, even the most basic genetic tools such as gene introduction system, which would enable verification of ideas that may originate from the genome data analysis, are undeveloped for this bacterial species. It was shown previously (Purdy *et al.*, 2002) in *Clostridium difficile* that plasmids, native to species to be genetically manipulated are needed and restriction barriers must be characterized and circumvented in order to develop a successful gene transfer system. To construct shuttle vectors for *P. ruminicola*, native *P. ruminicola* plasmids are therefore needed. Plasmids, however are surprisingly scarce in this bacterial genus (Peterka *et al*., 2003). One of the few reported *P. ruminicola* plasmids was found in *P. ruminicola* strain TC18 but was not characterized nor exploited as a shuttle vector (Avguštin, 1992). Recently, the type II restriction-modification system of *P. ruminicola* 23 was described as well as a procedure using *Hae*III methylase to protect DNA against it was developed (Accetto *et al*., 2005).

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2 MATERIAL AND METHODS

2.1 STRAIN, PLASMID, MEDIUM AND GROWTH

P. ruminicola TC18 (van Gylswyk, 1990) was grown anaerobically in M2 medium (Hobson, 1969) according to the Bryant's modification of the Hungate technique (Bryant, 1972). Source of *tetQ* alele was *E. coli-Bacteroides* shuttle plasmid pRH3 (Daniel *et al.*, 1995).

The plasmid DNA was extracted using standard alkaline lysis. Cleavage with restriction endonucleases, ligation and transformation of *Escherichia coli* were all done using standard molecular biology techniques (Sambrook, 2001). The DNA was protected against the *P. ruminicola* 23 restriction using *Hae*III methylase (NEB, USA) according to manufacturers instructions in reactions which contained S-adenosyl methionine as the methyl donor. The protected plasmid DNA was electroporated into *P. ruminicola* TC18 as described previously (Accetto *et al.*, 2005). Briefly: growth of *P. ruminicola* TC18 culture was stopped during exponential growth at OD_{600} = 0.5 by chilling on ice. The cells were then washed three times in anaerobic ice-cold 10% glycerol, electroporated at 12.5 kV/cm, resuspended in fresh M2 medium and left at 37 °C for an hour. Subsequently, the 0.1 ml portions of cells were transferred on tetracycline containing M2 agar plates in an anaerobic chamber.

3 RESULTS AND DISCUSSION

Plasmid DNA was isolated from *P. ruminicola* TC18 (Fig. 1A). Restriction enzymes *Hind*III, *Bam*HI, *Kpn*I in *Xho*I all convert plasmid DNA into a linear, approximately 3100 base pairs long DNA. The plasmid was named pTC18 and its restriction map is presented in Fig. 1B.

*Hind*III cleaved pTC18 was ligated into multiple cloning site of pUC19 and transformed into *E. coli* TOP10 (Invitrogen, USA). The resulting construct was cleaved using *Sst*I and ligated to *tetQ* allele. The latter was obtained by cleavage of pRH3 with *Sst*I and subsequent isolation of 2.6 kilobase pair fragment from the agarose gel. The ligation products were transformed into *E. coli* TOP10 and restriction analysis of plasmid DNA was performed on several recombinant strains to obtain strains harbouring both possible *tetQ* orientations (Fig. 2)

Since it is possible that *Hind*III site lies within the pTC18 replication region and thus cloning into this site would most likely inactivate replication in *Prevotella* hosts, we have also constructed shuttle vectors using the pTC18 *Kpn*I site. The procedures were essentially the same as above yielding constructs presented in figure 3.

All four shuttle vector constructs were subsequently protected against the *P. ruminicola* 23 restriction enzyme *Pru2*I using *Hae*III methylase and electroporated into *P. ruminicola* 23 cells. Despite numerous attempts we were unable to obtain a tetracycline resistant *P. ruminicola* 23 strain harbouring the shuttle vector. The electroporation parameters i.e. DNA concentration, electrocompetent cells density and electroporation time constant were essentially the same as in the previously described successful electroporation of plasmid pRH3 into *P. bryantii* TC1-1 strain (Accetto *et al.*, 2005). Several explanations for the failure of electroporation are possible: (i) both, *Hind*III and *Kpn*I site are placed within the region essential for pTC18 replication (ii) *P. ruminicola* 23 harbours

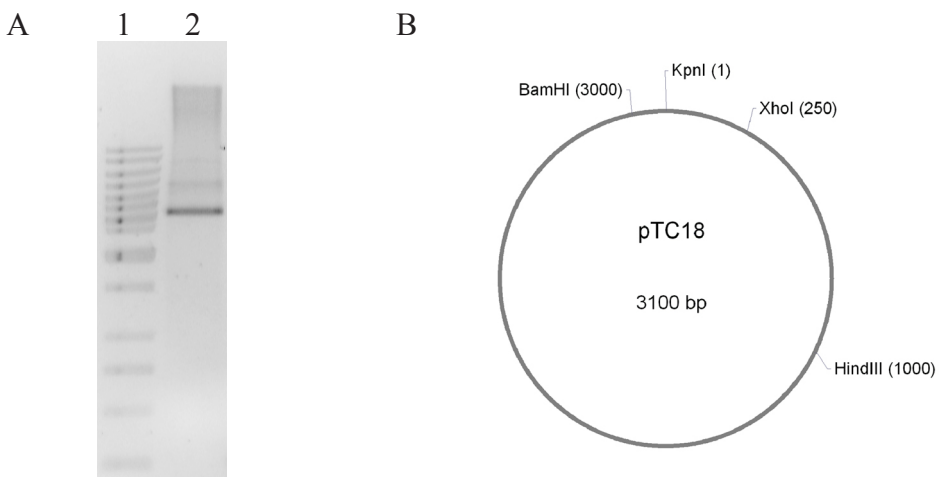

***Figure 1: A**: Plasmid DNA isolated from P. ruminicola TC18, agarose DNA electrophoresis. 1: marker generuler 1kb dna ladder (Fermentas); 2: plasmid DNA isolated from P. ruminicola TC18. **B**: Restriction map of pTC18.*
***Slika 1: A**: Plazmidna DNA iz P. ruminicola TC18, agarozna DNA elektroforeza. 1: velikostni standard generuler 1kb dna lestvica (Fermentas); 2: plazmidna DNA, osamljena iz P. ruminicola TC18. **B**: Restrikcijska mapa pTC18.*

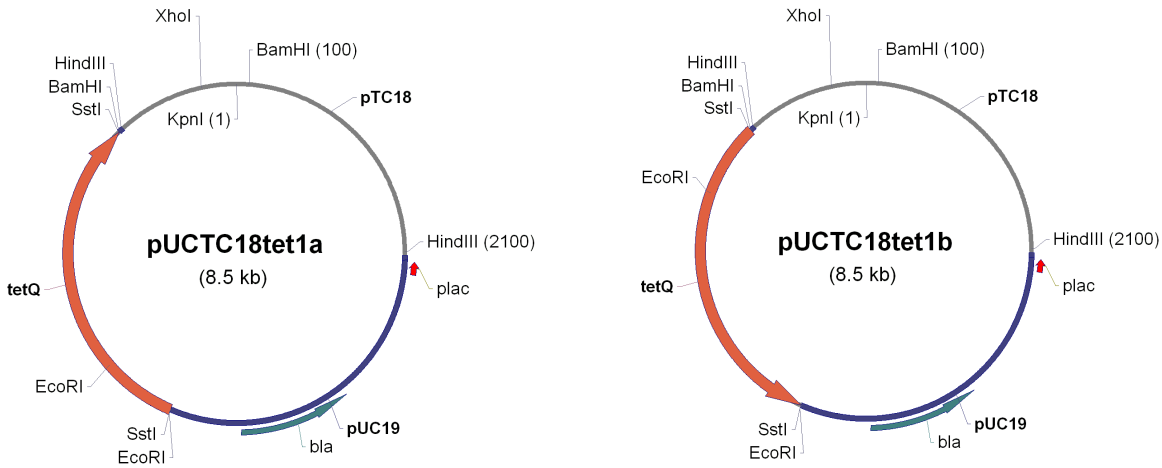

Figure 2: *Restriction maps of shuttle vectors based on pTC18 cleaved with HindIII and with different orientations of tetQ gene.*
Slika 2: *Restrikcijska mapa prenosljivih vektorjev osnovanih na pTC18 cepljenim s HindIII z različnima usmeritvama tetQ.*

another, non type II restriction system (iii) *P. ruminicola* 23 contains a cryptic plasmid that cannot be isolated by ordinary means or its relicts, but in both cases they belong to the same incompatibility group as pTC18 does and (iv) *tetQ* gene is lethal to or does not function in *P. ruminicola* 23.

4 CONCLUSIONS

The novel *Prevotella* plasmid pTC18 based shuttle vectors were unable to transform *P. ruminicola* 23. Several strategies to overcome this may be envisaged: transformation of other *P. ruminicola* strains preceded by protection of transforming DNA using cell free extract of strains to be transformed (Accetto *et al.*, 2005); the *tetQ* antibiotic resistance gene can be exchanged with *cfXA2* cephalosporinase resistance gene, known to reside in several oral *Prevotella* isolates (Giraud-Morin *et al.*, 2003) and finally, the other two unique restriction sites *BamH*I and *Xho*I can be exploited as cloning sites for antibiotic resistance gene and *E. coli* replicon in order to evade the pTC18 replication region supposedly inactivated by cloning into *Hind*III and *Kpn*I sites.

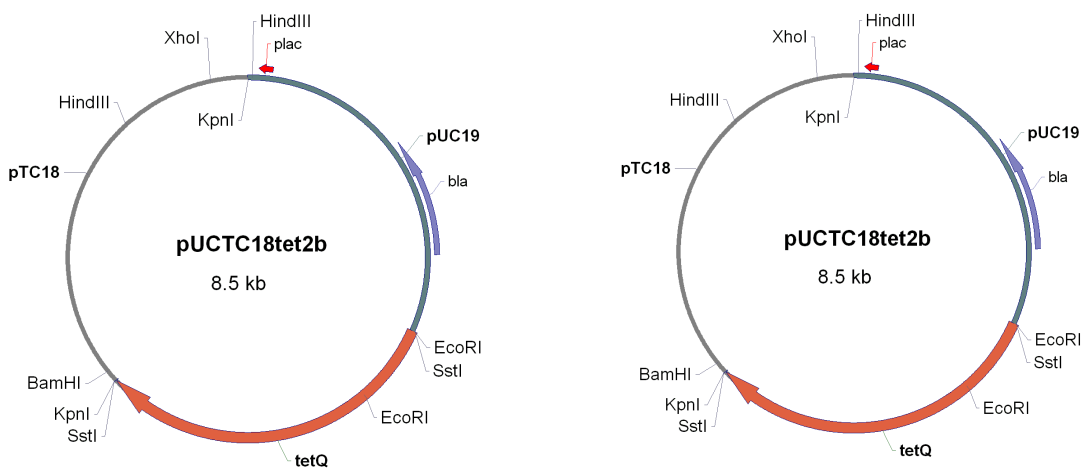

Figure 3: *Restriction map of shuttle vectors based on pTC18 cleaved with KpnI and with different orientation of tetQ gene.*
Slika 3: *Shema prenosljivih vektorjev osnovanih na pTC18 cepljenim s KpnI z različnima usmeritvama tetQ.*

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THE SEARCH FOR CONJUGATIVE TRANSPOSON IN RUMEN BACTERIUM *Prevotella bryantii* $B_1 4$

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The search for conjugative transposon in rumen bacterium Prevotella bryantii $B_1 4$

Only few plasmids and bacteriophages have been described to date in ruminal prevotella strains, therefore it appears plausible that the genetic exchange in these organisms must exploit other routes. Large conjugative transposons make possible the gene exchange process in bacteria from the genus *Bacteroides*, the phylogenetic relatives of ruminal prevotellas. The access to fully or partially finished genome sequences of *Bacteroides* and *Prevotella* representatives made possible the search for conserved regions within putative conjugative transposons. Multiple sequence alignment of known and putative conjugative transposon gene sequences of *Bacteroides thetaiotaomicron*, *Prevotella intermedia*, *Bacteroides fragilis* and *Tanerella sp.* was used to locate partially conserved regions within most preserved conjugative transposition genes, *traG*, and to construct appropriate degenerated oligonucleotide primers. These were used to amplify genome fragments from ruminal prevotella strains. Sequence analysis of the subcloned PCR products revealed the presence of a hypothetical gene in the genome of *Prevotella bryantii* $B_1 4$, similar to the ORF BF2880 from *B. fragilis* YCH46, which is a part of a large conjugative transposon. Inverse PCRs were designed and performed to confirm the initial findings. A partial map of *P. bryantii* $B_1 4$ putative conjugative transposon region was constructed, indicating an intergeneric horizontal gene transfer.

Key words: microbiology / molecular genetics / conjugative transposon / *Prevotella bryantii* / *Bacteroides fragilis*

Iskanje konjugativnega transpozona v vampni bakteriji Prevotella bryantii $B_1 4$

Ker sevi rodu *Prevotella* iz vampa le izjemoma posedujejo plazmide in je opisanih le nekaj bakteriofagov, je zelo verjetno, da izmenjava genov pri teh organizmih vključuje druge poti. Veliki konjugativni transpozoni omogočajo prenos genov pri rodu *Bacteroides*, filogenetskih sorodnikih vampnih prevotel. Dostop do delno ali v celoti sekvenciranih genomov predstavnikov *Bacteroides* in *Prevotella* je omogočil iskanje ohranjenih regij znotraj domnevnih konjugativnih transpozonov. S poravnavo več sekvenc znanih ali domnevnih genov konjugativnih transpozonov iz vrst *Bacteroides thetaiotaomicron*, *Prevotella intermedia*, *Bacteroides fragilis* in rodu *Tanerella* smo določili delno ohranjene regije v najbolj ohranjenem genu konjugativne transpozicije, *traG*, in jih uporabili za izdelavo primernih začetnih oligonukleotidov za pomnoževanje dela gena pri vampnih sevih iz rodu *Prevotella*. Analiza sekvenc subkloniranih pomnožkov je pri *P. bryantii* $B_1 4$ razkrila prisotnost hipotetičnega gena, podobnega odprtemu čitalnemu okvirju BF2880 seva *B. fragilis* YCH46, ki je del velikega konjugativnega transpozona. Z inverzno verižno reakcijo s polimerazo smo potrdili prvotne ugotovitve. Izdelali smo delno mapo regije domnevnega konjugativnega transpozona pri *P. bryantii* $B_1 4$, ki nakazuje, da je prišlo do medrodovnega horizontalnega prenosa genov.

Ključne besede: mikrobiologija / molekularna genetika / konjugativni transpozon / *Prevotella bryantii* / *Bacteroides fragilis*

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1 INTRODUCTION

Prevotella bryantii is a Gram-negative, strictly anaerobic member of the evolutionary distinct phylum *Bacteroidetes*, also known as the CFB group (Paster *et al.*, 1994; Shah and Collins, 1990). It is an important environmental commensal bacterium inhabiting the rumen (Avguštin, 1992; Hobson, 1997; Ramšak *et al.*, 2000). In this complex ecosystem some important functions have been assigned to ruminal prevotellas, *e.g.* degradation of proteins and certain plant cell polysaccharides (Daniel *et al.*, 1995; Hobson, 1997; Teather *et al.*, 1997). Due to its rather distinct capability to survive at lower pH values than other rumen bacteria, this organism was proposed as a suitable model organism for genetic manipulation studies in ruminal ecosystem (Russel and Dombrowski, 1980). The substantial evolutionary distance to well studied bacteria like *E.coli* is probably the main reason for poor understanding of prevotella genetics (Avguštin *et al.*, 1994, Accetto and Avguštin, 2007). The lack of suitable genetic tools hindered the progress and brought it almost to standstill in the mid 1990's. The recent progress in the genetic research of their relatives *i.e.* the prevotellas and the bacteroides from the human colon revived the interest in the genetics of ruminal prevotellas in last couple of years (Shoemaker *et al.*, 1992; Nikolich *et al.*, 1994; Vercoe and White, 1997; Flint and Scott, 2000; Accetto *et al.*, 2005, Accetto and Avguštin, 2007).

If an organism is to be a suitable genetic model, its horizontal gene transfer capacity should be known as well potential barriers for it. In addition to strong nonspecific deoxyribonuclease activity of *Prevotella bryantii* $B_1$4 (Flint and Thomson, 1990), only few plasmids and bacteriophages have been described from ruminal prevotella strains (Flint and Stewart, 1987; Ogata *et al.*, 1996; Accetto and Avguštin, 1997; Ambrožič *et al.*, 2001), therefore it appears plausible that the genetic exchange in these organisms must exploit other routes.

Conjugative transposons are distinct DNA segments that are normally integrated into bacterial chromosome and transfer by conjugation from donor to recipient bacterium. These genetic elements are integrated in the host genome except during transfer, therefore no method for their identification exists, analogous to plasmid isolation (Salyers *et al.*, 1995). All studies of conjugative transposition in ruminal *Prevotella* strains were linked to conjugative transposition elements in *Bacteroides spp.* In vitro experiments showed that bidirectional transfer of native conjugative transposition element Tc^r Em^r 12256 from *B. fragilis* clinical isolate can occur between closely related human commensal species *Bacteroides uniformis* and *B. thetaiotaomicron*, and ruminal *P. bryantii* strains (Shoemaker *et al.*, 1992). Additionally, mating experiments confirmed *in vitro* mobilization of Tc^r Em^r 7853 conjugative transposition element in *P. bryantii* $B_1$4 strain (Nikolich *et al.*, 1994). Nevertheless, to date the presence and identity of a conjugative transposon in any ruminal *Prevotella* species remains unproven.

2 MATERIAL AND METHODS

2.1 BACTERIAL STRAINS AND DNA ISOLATION

Two strictly anaerobic ruminal strains from genus *Prevotella* were used in this study: *P. bryantii* $B_1$4 (Russel, 1983) and *P. bryantii* TC 1-1 (Van Gylswyk, 1990). Strains were grown under strict anaerobic conditions in M2 medium (Hobson, 1969) by modification of the Hungate technique for cultivation of anaerobic bacteria, as described by Bryant (1972). Eight ml of M2 medium was inoculated and incubated under anaerobic conditions for 14–24 hrs at 37 °C, until optical density at 654 nm reached 0.9–1.4. Total genomic DNA was isolated by modification of the CTAB/NaCl isolation protocol, as described in "Current Protocols in Molecular Biology" (Ausubel *et al.*, 1987).

2.2 MULTIPLE SEQUENCE ALIGNMENT AND DEGENERATE PRIMER CONSTRUCTION

Multiple sequence alignment program tool Clustal X (Thompson *et al.*, 1997) was used to align known and putative conjugative transposon transfer gene sequences of *B. thetaiotaomicron*, *P. intermedia*, *B. fragilis* and *Tanerella sp.* Largest consensus regions within *traG* gene were located and, considering their appropriate length and reciprocal location, used to construct a pair of degenerate oligonucleotide primers CTf1310 and CTr2270 (see results).

2.3 DEGENERATE PCR, DNA CLONING, SEQUENCING AND SEQUENCE ANALYSIS

Degenerate PCR was used to amplify the putative homologs of the *traG* gene in *P. bryantii* $B_1$4 and *P. bryantii* TC 1-1 strains. 25 µl of reaction mixture contained 2.5 µl of the 10x Taq Buffer (Fermentas), 2 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate mixture (dATP, dGTP, dTTP, dCTP), 1.4 µM of each degenerate primer, 0.75 U Taq DNA polymerase (Fermentas) and 20 ng of genomic DNA, extracted from cultured ruminal prevotella strains. A series of PCR reactions was performed to determine the final amplification conditions: an ini-

tial denaturation step of 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 53 °C for 1 min and extension at 72 °C for 1 min. PCR was prolonged by final extension at 72 °C for 7 min. PCR products were separated by electrophoresis on 1% agarose gels, stained with ethidium bromide and visualized under UV light using GelDoc 1000 trans-illuminator (BioRad). Degenerate PCR products containing specific DNA bands were excised from the gel, purified with QIAQUICK Gel Extraction Kit (Qiagen) and subcloned with TOPO TA Cloning Kit (Invitrogen). DNA sequences of the pBAD/Thio-TOPO vector inserts were determined by Microsynth GmbH (Switzerland). Sequence data were analyzed using BLAST analysis and compared to sequences from the NCBI and TIGR CMR databanks.

2.4 INVERSE PCR AND PRIMER WALKING

Retrieved sequences were used as a template for construction of specific primer pair, oriented outwards of the retrieved sequence, and used for inverse PCR (Ochman *et al.*, 1988). 5 µg of genomic *P. bryantii* $B_1$4 DNA was partially digested using restriction endonucleases *EcoR*I (Gibco), *Hind*III (Promega), *Not*I or *Pst*I (Fermentas), followed by purification, agarose electrophoresis size selection and quantification of digested DNA. A series of self-ligation reactions was set, using 0.1, 0.25, 0.5, 0.75, 1.0; 5 and 10 ng/µl DNA with 5 U of T4 DNA ligase (Fermentas) per reaction. Self-ligation reaction was carried out at 22 °C for 1 h, again followed by purification, agarose electrophoresis size selection and DNA quantification.

A series of inverse PCRs with an annealing temperature span of 50–60 °C was set. 50 µl of reaction mixtures contained 5.0 µl of the 10x Long PCR Buffer+Mg (Fermentas), 0.5 mM deoxynucleoside triphosphate mixture (dATP, dGTP, dTTP, dCTP), 0.5 µM of each inverse primer, 2.5 U Long PCR Enzyme Mix (Fermentas) and 1.0 µl of purified self-ligation reactions containing 0,1 to 10 ng/µl DNA per reaction. Amplification conditions consisted of an initial denaturation step of 94 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 15 s, annealing for 30 s and extension at 68 °C for 10 min. 27 cycles followed, consisted of denaturation at 94 °C for 15 s, annealing for 30 s and extension at 68 °C for 10 min, with extra 5 s added each cycle. PCR was prolonged by final extension at 68 °C for 10 min. PCR products were examined by electrophoresis, purified and sequenced (Microsynth GmbH, Switzerland). The acquired sequences were analyzed and used to construct primers for next part of the sequence in primer walking procedure. Sequences were linked and compared to known genome sequences of related species.

3 RESULTS AND DISCUSSION

Multiple sequence alignment of known and putative conjugative transposition *traG* genes from sequenced members of the *Bacteroidetes* phylum showed that no conserved oligonucleotides larger than 5 nucleotides exist within the aligned 2.5 kbp region (complete alignment not shown). Thus two largest partially conserved regions were identified, spanning approx. 960 bp long region (Fig. 1). The mismatch positions were used as 2–4-base degeneracies for the primer construction. 19 bp forward primer CTf1310 (5'-CSA-AYM-GHA-ACA-ART-TYR-T-3') with 192-fold degeneracy and 17 bp reverse primer CTr2270 (5'-TCC-TTN-TCS-GTC-AGN-CC-3') with 32-fold degeneracy were constructed and subsequently used in degenerate PCR.

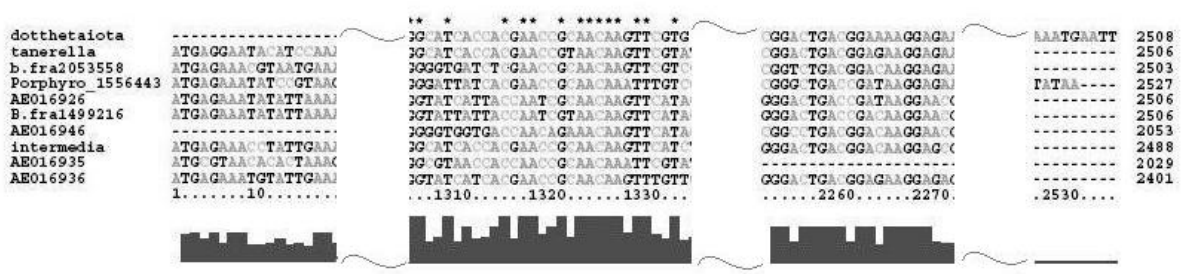

Figure 1: *Multiple sequence alignment of known and putative conjugative transposition transfer genes from sequenced members of the Bacteroidetes phylum. Partial display of the aligned 2.5 kbp region with the two largest partially conserved regions, used for construction of degenerated oligonucleotide primers CTf1310 and CTr2270, is shown.*

Slika 1: *Poravnava sekvenc znanih in domnevnih genov prenosa konjugativne transpozicije članov debla Bacteroidetes, pri katerih je že znana celotna sekvenca genoma. Prikazan je delni prikaz poravnave 2,5 kbp regije z dvema najobsežnejšima delno ohranjenima regijama, ki smo ju uporabili za izdelavo degeneriranih začetnih oligonukleotidov CTf1310 in CTr2270.*

In degenerate PCR the competitive inhibition due to high primer degeneracy may occur. Primers anneal to the correct template but are not extended by the polymerase due to unstable 3'-ends, which results in high inefficiency of the first few PCR cycles. This can be overcome by increasing PCR cycles, which in return may increase nonspecific background and decrease the yield of specific PCR product. Only P. bryantii B14 reaction showed the presence of expected fragment of approximately 1 kbp (Fig. 2), which was isolated and subcloned.

The analysis of the sequenced region revealed the presence of a complete open reading frame sharing 24% identity with *traI* gene from *P. intermedia* strain 17 cojugative transposon and 58% identity with the ORF BF2880 from the putative conjugative transposon of the *B. fragilis* strain YCH46, at the amino acid level. The 24% identity with the *traI* gene from *P. intermedia* 17 is on the border as far as assigning of the function or recognition of homology is concerned. However, the rather high degree of similarity with the ORF BF2880 from *B. fragilis* provides strong evidence for intergeneric horizontal gene transfer, especially if we bare in mind that the average DNA:DNA homology of total chromosomal DNA from *Bacteroides* species and ruminal *Prevotella* species is less than 5% (Johnson in Harich, 1986).

Determination of the complete sequence of the cloned *P. bryantii* B14 genome fragment made possible the construction of specific primer set, which was subsequently used for inverse PCR in order to extend the known sequence in both directions. Figure 3 shows a series of inverse PCR amplifications from self-ligation reactions under most suitable conditions.

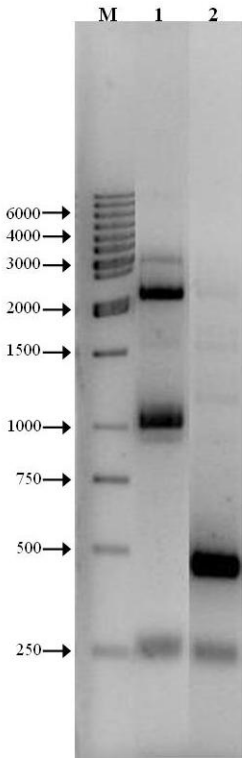

Figure 2: *Agarose gel electrophoresis of degenerate PCR multiplication of putative traG homologs in P. bryantii $B_1$4 and P. bryantii TC1-1. M – DNA size marker O'GeneRuler 1 kb DNA Ladder, 2 µl; 1 – Prevotella bryantii $B_1$4; 2 – Prevotella bryantii TC 1-1.*
Slika 2: *Agarozna gelska elektroforeza pomnoževanja domnevnega homologa gena traG z degenerirano verižno reakcijo s polimerazo pri P. bryantii $B_1$4 in P. bryantii TC1-1. M – velikostni standard O'GeneRuler 1 kb DNA Ladder, 2 µl; 1 – Prevotella bryantii $B_1$4; 2 – Prevotella bryantii TC 1-1.*

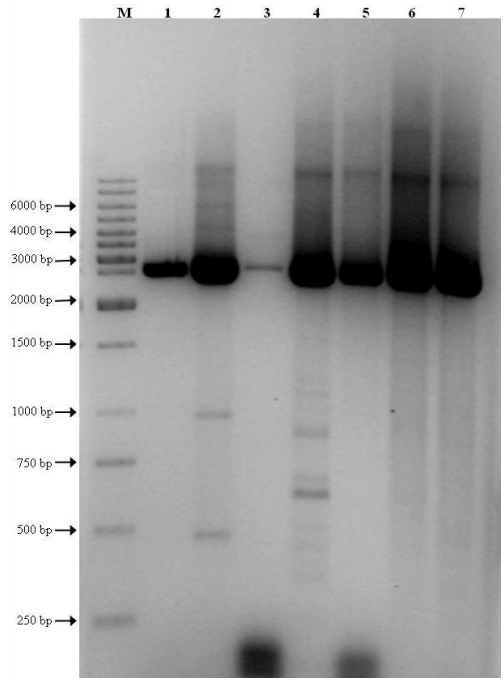

Figure 3: *Agarose gel electrophoresis of inverse PCR amplifications from self-ligation reactions of P. bryantii $B_1$4 genomic DNA flanking the targeted region. Reactions were performed at 54 °C annealing temperature, using 0,1 to 10 ng/µl DNA per reaction. M – DNA size marker O'GeneRuler 1 kb DNA Ladder, 2 µl; 10 µl reaction samples: 1 – 0,1 ng/µl DNA; 2 – 0,25 ng/µl DNA; 3 – 0,5 ng/µl DNA; 4 – 0,75 ng/µl DNA; 5 – 1,0 ng/µl DNA; 6 – 5 ng/µl DNA; 7 – 10 ng/µl DNA.*
Slika 3: *Agarozna gelska elektroforeza produktov inverzne verižne reakcije genomske DNK-P. bryantii $B_1$4. Reakcije so bile izvedene pri 54 °C z 0,1 do 10 ng/µl DNA na reakcijo. M – velikostni standard O'GeneRuler 1 kb DNA Ladder, 2 µl; vzorci po 10 µl na jamico: 1 – 0,1 ng/µl DNA; 2 – 0,25 ng/µl DNA; 3 – 0,5 ng/µl DNA; 4 – 0,75 ng/µl DNA; 5 – 1,0 ng/µl DNA; 6 – 5 ng/µl DNA; 7 – 10 ng/µl DNA.*

All inverse PCR reactions showed the presence of 2.5 kbp products. The products of 0.1 ng/µl DNA reaction without additional multiple or smeared bands were used for subsequent sequencing, primer construction and gene walking procedure. The retrieved sequence data was analyzed and used to construct a partial map of *P. bryantii* $B_1$4 genomic region homologous to the putative conjugative transposon region of the *B. fragilis* YCH46 . Its structure and organization is shown in Figure 4.

Five ORFs with 162, 231, 108, 102 and 164 amino-acid residues were identified, with the first two being completely sequenced. BLAST analysis at amino-acid

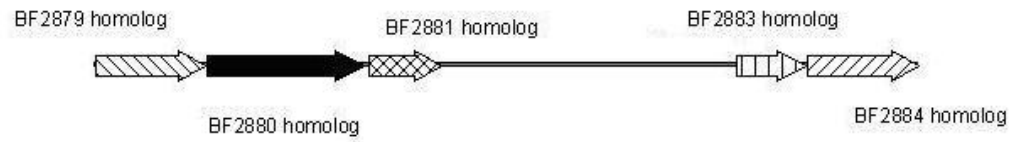

Figure 4: *Partial genetic map of P. bryantii $B_1$4 genomic region homologous to the putative conjugative transposon region of the B. fragilis YCH46. Arrows denote ORFs; gene homologs of Bacteroides fragilis YCH46 are indicated.*
Slika 4: *Delna genetska mapa genomske regije P. bryantii B14 homologne domnevnemu konjugativnemu transpozonu seva B. fragilis YCH46 . Puščice označujejo odprte bralne okvirje ORFs; prikazani so homologi genov pri Bacteroides fragilis YCH46.*

level (TIGR; http://tigrblast.tigr.org/cmr-blast/) showed the highest similarity with BF2879 – BF2884 ORFs (with 69%, 61%, 45%, 71% and 44% identities, respectively) in *B. fragilis* YCH46, which are placed within the larg, 120 kbp putative conjugative transposon CTn3Bf (Kuwahara *et al.*, 2004). Comparison of the mapped *P. bryantii* $B_1$4 region to the available genomic sequences of the related *Bacteroidetes* (TIGR CMR; http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi) shows a similar gene organization. These findings confirm that these genetic elements were likely transferred to or from *P. bryantii* $B_1$4. Nevertheless, additional work is needed to reveal the nature of conjugative transposition in ruminal *Prevotella* species, to determine the presence of an active conjugative transposition element and its complete sequence. We are currently optimizing the genomic DNA primer walking procedure and preparing a cosmid genomic library in order to reveal the sequence of this interesting region in *P. bryantii* $B_1$4.

4 CONCLUSIONS

Multiple sequence alignment of known and putative conjugative transposition *traG* genes from sequenced members of the *Bacteroidetes* phylum showed that no conserved oligonucleotides larger than 5 nucleotides exist within the aligned region, therefore appropriate degenerate oligonucleotide primers had to be constructed to amplify homologous gene fragments from ruminal prevotella strains. Sequence analysis of degenerate PCR products revealed the presence of an ORF homologous with ORF BF2880 from the putative conjugative transposon of *B. fragilis* YCH46, showing 58% identity at the amino acid level. Inverse PCRs making possible the amplification of the flanking regions in *P. bryantii* $B_1$4 were set and their products sequenced. A partial map of *P. bryantii* $B_1$4 genomic DNA homologous to the putative conjugative transposon region of *B. fragilis* YCH46 was constructed, showing the same gene organization and high gene similarity. The observed indices suggest that the conjugative transposition elements of *P. bryantii* $B_1$4 were introduced through an intergeneric horizontal gene transfer.

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STRAIN AND PLACEMENT DENSITY EFFECTS ON WELFARE, HAEMATOLOGICAL AND SERUM BIOCHEMICAL INDICES OF BROILERS IN NORTH CENTRAL NIGERIA

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Strain and placement density effects on welfare, haematological and serum biochemical indices of broilers in north central Nigeria

This study aimed at evaluating the influence of strain and stocking density on welfare, haematological and serum biochemical indices of broilers in a 28-day trial. Two hundred and seven 4-week old birds each of Anak Titan and Arbor Acre genetic provenience were randomly allocated to three housing densities of 8.3, 11.1 and 14.3 birds/m^2.These corresponded to 17, 22 and 30 birds per pen (2.01 × 1.00 m) in a 2x3 factorial experiment. Each treatment group was replicated three times. The welfare parameters estimated were gait score, feather score, foot and hock burns, pecking, pushes, chases, fights and mortality. Blood samples were tested for packed cell volume (PCV), red blood cells (RBC), white blood cells (WBC), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). Similarly, sera were utilized for the determination of total protein, albumin, globulin, glucose, cholesterol, and creatinine contents. There were no genotype-associated differences ($P > 0.05$) in the welfare indicators examined. However, placement density significantly ($P < 0.05$) influenced incidence of pushes, chases and fights, with higher values in most cases recorded for birds housed at the highest density. The strains and population densities were similar ($P > 0.05$) in haematological profile. Strain and stocking density also exerted no influence ($P > 0.05$) on serum biochemical components. Strain × stocking density interaction effects were not observed in all the parameters. Consequently, the two strains could be reared at a density of 14.3 birds/m^2 since density did not lead to a great degree of stress.

Key words: poultry / broilers / genotype / stocking density / blood parameters / animal welfare

Vpliv genotipa in gostote naselitve na počutje, serumske in biokemijske parametre pri brojlerjih v severni in osrednji Nigeriji

V raziskavi smo skušali oceniti vpliv genotipa in gostote naselitve na počutje živali, hematološke in biokemijske parametre seruma brojlerjev v 28 dnevnem poskusu. Dvesto sedem štiri tedne starih brojlerjev provinienc Anak Titan in Arbor Acre smo naključno porazdelili v tri oddelke z gostoto naselitve 8,3, 11,1 in 14,3 ptic/m^2, kar je ustrezalo 17, 22 in 30 pticam na oddelek (2,01 × 1,00 m) v faktorskem poskusu 2x3. Za vsako obravnavo smo imeli po tri ponovitve. Počutje živali smo ocenjevali z ocenami za držo telesa, perje, žulje na nogah, kljuvanje, odrivanje, preganjanje, spopade in smrtnost. V vzorcih krvi smo določili hematokrit (PCV), število rdečih krvniček (RBC), belih krvniček (WBC), hemoglobin (Hb), srednji volumen eritrocitov (MCV), srednjo maso hemoglobina na eritrocit (MCH) in srednjo koncentracijo hemoglobina v hematokritu (MCHC). Krvni serum smo uporabili za določitev vsebnosti skupnih beljakovin, albumina, globulina, glukoze, holesterola in kreatinina. V poskusu nismo opazili z genotipom povzročenih razlik v indikatorjih počutja ($P > 0,05$). Gostota naselitve je značilno ($P < 0,05$) vplivala na pogostnost odrivanja, lovljenja in bojev, ki smo jih najpogosteje opazovali pri najgosteje naseljenih živalih. Med različnimi genotipi in naselitvenimi različicami nismo opazili razlik v hematološkem profilu ($P > 0,05$). Genotip in gostota naselitve ravno tako nista vplivala na biokemijske parametre v krvnem serumu ($P > 0,05$). Pri nobenem od proučevanih parametrov nismo opazili interakcij med genotipom in gostoto naselitve. Zaključujemo, da tudi najvišja gostota naselitve,14,3 živali /m^2 ni povzročala značilnega stresa.

Ključne besede: perutnina / pitovni piščanci / genotip / gostota naselitve / krvni parametri / dobro počutje živali

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1 INTRODUCTION

Animal welfare has generated concerns from the domestic and global market sectors. According to English Federation for Humane Treatment of Animals (Jensens and Toates, 1997), animal welfare is accomplished if there are lack of hunger and thirst; lack of discomfort; lack of pain, injury or sickness; freedom for normal behavior; and lack of fear, anxiety and depression. Welfare of birds is to a large extent regulated by various intrinsic and extrinsic factors, among which stocking density plays a pivotal role. In the broiler industry, the major welfare concern is the effect of high stocking densities on the welfare of birds, especially during the final weeks of the growing period when body weight per unit area is high (Ravindran *et al.*,2006).

There are conflicting reports on the effects of high placement density on the welfare, performance and immune status of birds. Dozier *et al.* (2005) reported that body weight gain and feed consumption were adversely affected by increasing the housing density from 30 to 45kg of BW/m^2 of floor space. High rearing densities in broilers are associated with an increased incidence of leg problems (Sorensen *et al.*, 2000). However, Ravindran *et al.* (2006) reported that weight gain, feed intake, livability and carcass characteristics of broilers grown at densities of 16, 20 and 24 birds/m^2 were similar over the whole 35-day trial. Thaxton *et al.* (2006) reported that stocking density did not cause adaptive changes indicative of stress in birds.

The European Union is currently adopting standards for broilers aimed at a chief welfare concern, namely overcrowding by limiting maximum stocking density (Dawkins *et al.*, 2004). Focusing research on adequate space requirements may lead to management changes that could help diminish stress and subsequently lead to improved growth and survivability. In North Central Nigeria, there appeared to be virtually no documented evidence on the appropriate placement density for broilers. The current practice involves the indiscriminate allocation of birds to floor space based on the imagination (subjective evaluation) of the farmers. This tends to undermine animal welfare and hence, profitability of the enterprise.

Therefore, the present investigation set out to determine the effects of genotype and stocking density on welfare indicators, haematological and serum biochemical parameters of broilers. The result so obtained could contribute to the knowledge on optimal floor space of broilers in the semi-humid tropics characterized by high environmental temperature and relative humidity.

2 MATERIALS AND METHODS

2.1 STUDY LOCATION

The research was conducted in the Poultry Unit of the Teaching and Research Farm, Faculty of Agriculture, Nasarawa State University, Keffi, Shabu-Lafia Campus, located in the guinea savanna agro-ecological zone of northern Nigeria.The mean monthly environmental temperature during the study which lasted four weeks was 32.75^{0}C, while the monthly relative humidity, rainfall and evaporation were 79.00%, 207.45mm and 2.5ml respectively.

2.2 EXPERIMENTAL DESIGN

Four hundred and fourteen broiler chickens consisting of equal number of Anak Titan and Abor Acre strains were utilized for the investigation. Birds were raised on conventional starter ration (22.00% crude protein and 2800kcal/kgME) from day old to 4-week of age. Birds were randomly allocated to three stocking density treatments vis: 8.3, 11.1 and 14.3 birds /m^2. These corresponded to 17, 22 and 30 birds per pen in a 2 × 3 factorial arrangement. Each treatment group was replicated three times. The dimension of each pen made of wooden plank and wire netting was 2.01m^2 (2.01m × 1.00m), and was constructed in such a way as to permit straight-through ventilation. From week five to week eight, the birds were fed commercial broiler finisher ration (2900kcal/kg ME and 20.00% crude protein). Feed and fresh clean water were supplied *ad libitum*. The feeders and waterers were allotted proportionately depending on the number of birds in each pen. Vaccination schedule and other management practices were strictly adhered to.

2.3 DATA COLLECTION

Birds were assessed on a weekly basis for gait score, feather score and foot and hock burn as described by Ravindran *et al.* (2006). Number of pecking, pushes, fights and chases were recorded per pen per replicate during feeding and at 3-day interval, following the procedure adopted by Olukosi *et al.* (2001). Gait score was assessed for six randomly selected birds per pen. Birds were watched by two observers walking in the run within the poultry house, and their walking ability was scored on a three-point scale (0, normal gait, bird walks freely and has regular and even strides and is well balanced; 1, bird walks with irregular and uneven strides and appears unbalanced; 2, bird is reluctant to move and is unable to

walk many strides before sitting down). A score was ascribed only when there was consensus between the two observers.

Feather score or the degree of feather coverage over the breast was recorded for six birds per pen. Each bird was stroked over the keel with the palm of the hand in an anterior or posterior direction, and the amount of flesh showing was scored on a three -point scale (1, no visible skin, complete feather cover; 2, relatively small amount of skin showing; 3, relatively large amount of skin showing). Foot and hock burn was recorded for all the birds in a pen using a three -point scale (1, no burns; 2, mild burns; 3, severe burns).

2.4 HAEMATOLOGICAL AND BIOCHEMICAL ANALYSES

At the end of the experimental period, blood samples were collected from four randomly selected birds per treatment combination. Five-ml of blood was collected through the jugular veins in immobilized animals. Half of the sample was expelled gradually into vacutainer glass tubes containing ethylene diamine tetra acetic acid (EDTA) for the determination of haematological components following standard procedures described by Davice and Lewis (1991). The rest of the sample was collected in a second set of vacutainer glass tubes without EDTA for serum biochemical parameters. The haematological indices investigated were packed cell volume (PCV), red blood cells (RBC), white blood cells (WBC), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC).Similarly, sera were used for the determination of total protein, albumin, globulin, glucose, cholesterol and creatinine contents.

2.5 STATISTICAL ANALYSIS

Data collected were subjected to multivariate analysis of variance using SPSS (2001) statistical package. The separation of means was effected using least significant difference (LSD) method and tested at probability level of 5%.

3 RESULTS

The influence of genotype and stocking density on the welfare parameters of broilers is shown in Table 1. Genotype did not significantly ($P > 0.05$) affect the welfare parameters investigated.

Stocking density had no effect ($P > 0.05$) on gait score, foot and hock burns, feather score and pecking. However, housing density significantly ($P < 0.05$) influenced number of pushes, chases and fights, with higher mean values recorded for birds stocked at 14.3 birds/m^2.

Effect of genotype and population density on the haematological parameters of broilers is shown in Table 2. Strain and density effects on PCV, RBC, WBC, Hb, MCV, MCH and MCHC were not observed ($P > 0.05$).

Effect of strain and placement density on serum biochemical parameters of broilers is presented in Table 3. Genotype exerted no significant influence ($P > 0.05$) on the parameters estimated. There was also no placement density influence ($P > 0.05$) on total protein, albumin, globulin, glucose, cholesterol and creatinine contents. Strain × stocking density interaction effects were not observed in all the parameters investigated.

Table 1: *Effect of genotype and stocking density on welfare indices of broilers*
Preglednica 1: *Vpliv genotipa in gostote naselitve na počutje brojlerjev*

	Genotype				Stocking density (bird/m^2)				
Parameters	Anak Titan	Abor Acre	Prob.	S.E.M	8.3	11.1	14.3	Prob.	S.E.M
Gait score	1.19	1.17	0.85	0.14	1.21	1.04	1.29	0.36	0.13
Food and hock burns	1.47	1.39	0.59	0.15	1.33	1.33	1.63	0.28	0.13
Feather score	1.86	1.84	0.90	0.13	1.88	1.71	1.97	0.13	0.11
Pecking	1.77	1.52	0.32	0.24	1.72	1.53	1.71	0.56	0.30
Pushes	2.36	2.34	0.88	0.15	1.99	2.31	2.25	0.01	0.13
Chases	1.78	1.83	0.72	0.14	1.48	1.83	2.11	0.01	0.12
Fights	1.94	1.83	0.37	0.12	1.53	1.79	2.34	0.05	0.10
Mortality	0.01	0.01	0.85	0.006	0.01	0.01	0.008	0.65	0.007

S.E.M: Standard error of means

Table 2: *Effect of genotype and stocking density on haematological indices of broilers*
Preglednica 2: *Vpliv genotipa in gostote naselitve na hematološke parametre pri brojlerjih*

	Genotype				Stocking density (bird/m^2)				
Parameters	Anak Titan	Abor Acre	Prob.	S.E.M	8.3	11.1	14.3	Prob.	S.E.M
PCV (%)	31.25	31.17	0.92	0.85	31.50	31.13	31.00	0.64	1.03
RBC (x 106/µL)	2.46	2.47	0.84	0.05	2.48	2.47	2.45	0.76	0.07
WCB (x 103/µL)	24.61	24.48	0.64	2.76	24.64	24.61	24.37	0.45	3.38
Hb (g/dl)	9.42	9.89	0.23	0.38	9.73	9.64	9.59	0.85	0.47
MCV (fl)	128.87	130.06	0.45	1.52	129.95	130.50	127.93	0.18	1.86
MCH (pg)	38.86	39.58	0.25	0.62	39.38	39.40	38.87	0.50	0.76
MCHC (g/dl)	30.30	30.60	0.36	0.33	30.63	30.13	30.61	0.23	0.41

S.E.M: Standard error of means

4 DISCUSSION

Genotype-associated significant differences did not manifest in the welfare parameters investigated. This is an indication of similarity in the ranking of the two strains under consideration, although better numerical mean values were recorded for Abor Acres. The present result is in consonance with that of Albentosa *et al.* (2003), where strain of birds did not influence fearfulness and exploratory behaviour. Similarly, Anderson *et al.* (2004) reported that appetitive behaviours and feather pecking were not affected by strain, and that the patterns and number of aggressive acts did not increase to compromise the welfare status of the birds. In contrast to the present findings, Kjaer and Sorensen (2002) and Aerni *et al.* (2005) reported effect of genotype on feather pecking, mortality and cannibalism respectively. Gait score represents subjective method of evaluation of the walking ability of birds. Genetic differentials in gait score, foot and hock burns, feather pecking and cannibalism in Ross 208 and Labresse cross had been documented (Nielsen *et al.,* 2003). The performance of the birds in gait score, feather score, foot and hock burns and pecking was independent of placement density. The present findings are consistent with that of Ravindran *et al.* (2006) where placement density did not exert any influence on leg and feather scores. In contrast to the current observations, Sorensen *et al.* (2000) reported poorer walking ability in birds reared at higher densities and attributed this to constrained mobility and reduced opportunity for activity, especially as birds approach the end of the grow out phase.

Dozier *et al.* (2005) reported that foot pad lesson score increased progressively as placement density increased from 30 to 45kg of BW/m^2. The submission of Muniz *et al.* (2008) confirms this, as percentage foot-pad dermatitis in broilers increased linearly with increasing stocking density. The negative effect of high housing density on hock burns had also been reported (Thomas *et al.*, 2004), reflecting poorer litter quality and the increased time that the birds spend sitting, in contact with the litter.

The differential effects of stocking rate on gait score and foot and hock burn of the present investigation and others might be partly attributed to the length of rearing, environment and management practices. Under the con-

Table 3: *Effect of genotype and stocking density on serum biochemical indices of broilers*
Preglednica 3: *Vpliv genotipa in gostote naselitve na biokemijske parametre pri brojlerjih*

	Genotype				Stocking density (bird/m^2)				
Parameters	Anak Titan	Abor Acre	Prob.	S.E.M	8.3	11.1	14.3	Prob.	S.E.M
Total protein (g/dl)	5.76	5.91	0.54	0.26	5.98	5.89	5.63	0.29	0.32
Albumin (g/dl)	2.48	2.77	0.18	0.33	2.54	2.98	2.35	0.13	0.40
Globulin (gdl)	3.28	3.14	0.67	0.30	3.44	2.92	3.28	0.18	0.37
Glucose (mmol/L)	12.72	13.43	0.32	0.70	13.86	12.45	12.91	0.12	0.87
Cholesterol (mmol/L)	4.91	5.60	0 19	0.51	5.45	5.08	5.24	0.55	0.63
Creatinine (µmol/L)	71.83	71.33	0.90	4.02	70.25	75.00	69.50	0.28	4.92

S.E.M: Standard error of means

ditions of the current study, more of mild feather pecking and less of aggressive pecking which was mainly directed at the head was observed. The other agonistic behaviours such as pushes, chases and fights were not similar in the three stocking density treatments, as they were more associated with the highest housing density. The competition for feed could partly be responsible for the observed responses. Similar findings have been reported in broilers where increasing the feeder space reduced agonistic acts during the feeding period from 7.8 (at 2.4cm feeder space to 4.5 (at 3.6cm/bird) (Olukosi *et al.* 2001). According to Spinu *et al.* (2003), stereotyped pecking increased with an increase in density. Mortality is one of the most obvious measures of bird welfare. However, population density was not a significant explanatory factor in it in the current investigation. This concurs with the report of Thomas *et al.* (2004). In contrast, Imaeda (2000) found that mortality was markedly increased at higher animal densities.

Measurement of haematological indices provides valuable information on the immune status of animals. The literature provides varying evidence concerning the effect of stocking density on birds' physiological response and stress. The present findings are comparable to the report of Talebi *et al.* (2005) which revealed that the haematological values of four main broiler strains (Ross, Cobb, Abor Acres and Arian) showed slight but non-significant differences, indicating that the broiler strains are nearly similar to each other in haematological indices. Conversely, Manzoor *et al.,* (2003) reported genotype-associated differences in the haematological parameters of broiler lines. Placement density did not exert any influence on the haematological characteristics, as very similar values were recorded for the three stocking rates investigated.

The two strains under consideration appeared to be similar in their serum biochemical values. However, Abor Acres seemed to have better numerical mean values for total protein, albumin, glucose and creatinine. Serum biochemical parameters were also not density dependent. This is an indication that the body homeostasis, and hence health of the birds were not adversely disturbed as a result of housing the birds up to 14.3 birds/m^2. Using linear trend analysis, Thaxton *et al.* (2006) reported that stocking density did not cause physiological adaptive changes indicative of stress. Skomorucha and Muchacka (2007) submitted that the level of biochemical indicators was affected by animal density, although this manifested greatly in birds placed under housing density of 17 birds/m^2, which is quite higher than the 14.3 birds/m^2 reported in the present study.

5 CONCLUSIONS

The study has shown that genotype had no significant effect on gait score, foot and hock burns feather score, pecking, pushes, chases, fights and mortality of broilers. Conversely, stocking density significantly influenced incidence of pushes, chases and fights with higher mean values recorded for birds reared at 14.3 birds/m^2. There was no genotype and stocking density effects on the haemotological parameters. Serum biochemical indices were also not significantly affected by placement density. Genotype × stocking interaction effects were not observed in all the parameters investigated. It is concluded that the two strains could be reared at a stocking density of 14.3 birds/m^2, since density did not negatively affect the physiological adaptive responses of birds. This will eventually guarantee high yield per unit area, which could assist livestock farmers and the entire populace in poverty alleviation under the climatic and production conditions of this study.

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MINERALS MANAGEMENT IN SILVOPASTORAL SYSTEM OF KARST PASTURE

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Minerals management in silvopastoral system of karst pasture

A survey of mountain pasture topsoil was undertaken first to set up field experiment in karst region on effects of applied P on minerals concentration in herbage. Content of SOM, C, N, CEC of soil and its base saturation are presented in the article. Great variability in depth, pH value and K level was found in soil. Low base saturation and high deficit of P was more common for all soil samples. Six different plant species presenting a great portion of herbage available for grazing and browsing at different occasions during grazing season were sampled and analysed for macro- and some microminerals. Concentration of P was very low in perennial grasses (1.1 g P kg^{-1} of DM). In leaves of common hazel (*Corylus avellana* L.) and common beech (*Fagus silvatica* L.) the concentration of P was identical as in white clover (*Trifolium repens* L.) and the level was high enough to cover animal needs when intake of herbage was sufficient. Leaves of woody plants were high in Mn concentration, but still below the levels that reduced growth rate in lambs. Application of P fertilizer had only small effect on increase of P in herbage, but large one on decrease on concentration of Ca in herbage. There was not clear effect of added P on concentration of Zn, Mn, Fe and Cu. Higher yield of DM induced with added fertilizer had not have any dilution effect on concentration of those minor elements in herbage.

Key words: animal husbandry / animal nutrition / pasturing / karst pastures / soil / herbage / minerals / superphosphates

Drevesno pašna raba in rudnine na kraškem pašniku

Na območju planinskega pašnika je bil napravljen pregled rodovitnosti zemlje in nato izveden poljski poskus o vplivu gnojenja s P na vsebnost rudnin v zelinju kraške vegetacije. V prispevku so predstavljeni podatki o vsebnosti organske snovi v tleh, C in N, kapacitete sorpcije ter zasičenost z bazami sorptivnega dela tal. Ugotovljena je bila velika variabilnost v debelini vrhnje plasti zemlje, pH vrednosti in oskrbljenosti tal s K. Značilna je tudi nizka zasičenost z bazami sorptivnega dela tal in veliko pomanjkanje rastlinam dostopnega P v zemlji. Za določanje vsebnosti rudnin v zelinju razpoložljivem za pašo in smukanje, je bilo vzorčeno šest različnih vrst rastlin, ki predstavljajo znaten delež krme v različnih delih pašne sezone. V travah, ki predstavljajo ob koncu pomladi znaten delež razpoložljive krme je bila ugotovljena zelo nizka vsebnost P (1,1 g kg^{-1} SS). V listju navadne leske (*Corylus avellana* L.) in navadne bukve (*Fagus sylvatica* L.) je bila vsebnost P enaka kot v zelinju plazeče detelje (*Trifolium repens* L.) in je bila dovolj visoka za pokritje potreb pašnih živali po P, če je v obroku dovolj zaužitega zelinja. V listih lesnatih rastlin je bila ugotovljena visoka vsebnost Mn, toda še vedno pod vrednostjo, ki vpliva na zmanjšanje dnevnih prirastov pri jagnjetih. Gnojenje s fosfati ni imelo značilnega vpliva na povečanje vsebnosti P v zelinju, toda močno je vplivalo na zmanjšanje vsebnosti Ca v zelinju rastlin ruše. Uporabljena fosfatna gnojila niso značilno vplivala na spremembo vsebnosti Zn, Mn, Fe in Cu v zelinju razpoložljivem za pašo. Višji pridelek zelinja dosežen z uporabo fosfatnih gnojil ni učinkoval razredčitveno na vsebnost mikroelementov v zelinju kraškega pašnika.

Ključne besede: živinoreja / prehrana živali / paša / kraški pašniki / tla / zelinje / minerali / superfosfati

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1 INTRODUCTION

The grazing animals will play very important role in the process of farming restoration on hill and karst grassland of Slovenia. The land under consideration was mainly used as cut meadows (hand cut) in the past. The plant minerals such as phosphorus (P), calcium (Ca), magnesium (Mg), sodium (Na) and sulphur (S) were drained out from the grassland soils because the hay produced there was fed to animals in confinement to get large volume of yard manure, which was used then on arable land to keep their fertility at the level high enough for good crop production (Gruden, 1910). Seminatural grasslands have longer history than is often realized (Walter, 1973), and karst grassland was a subject to depletion of plant available minerals most of the time (Vidrih A, 2005). An additional reason for depletion of plant minerals from karst hay grassland is strong wind bora (Mihevc, 1997). Most of nutrients in plants are organic and recycled to grassland soils through the decomposition of herbage. That is why this material must come in good contact with the soil, and mineralized by soil microorganisms. This way dead leaves and tillers can not be blown away by wind. Trampling of grazing animals is most efficient method for to incorporate organic matter into top layer of soil. But this trampling was absent from hay grassland, and wind erosion took its share of minerals from karst grassland. Occasional burning (accidentally or purposely) of vegetation over abandoned karst grassland was an additional drawn out of minerals from karst soil. As a consequence of all this a large area of karst grassland has been abandoned and converted to woody plant types of ecosystem. It has become increasingly evident that region is loosing its typical landscape, and that important habitat of very large proportion of wild fauna and flora is decreasing in size very much (Grove and Rackham, 2001; Kaligarič *et al.*, 2006).

Very variable soil profile, stoniness as well as topography are serious limitations for levels of production and potential for improvement of karst pasture vegetation. Whilst it is recognised that these limitations exist, it is also clear that they are of a kind which cannot readily be changed by improvement measures used under more favourable conditions for farming. Thus greater attention must be given to the nature of chemical limitation of soil fertility and the methods for their correction (Vidrih M *et al.*, 2007). Sima *et al.* (2004) is suggesting that this soil factors are probably more important in limiting production of grazing animals on potentially improvable soils than are climatic factors. Concentration of minerals in both soil and plant, influences the mineral status of grazing animals, and considering all these facts, the soil and herbage available for grazing on karst pasture were analysed for concentration of minerals, and the effect of P fertilization on mineral status of herbage is reported.

2 MATERIAL AND METHODS

A study was conducted on a herbaceous community of the Festuco-Brometea Br.-Bl. (Kaligarič, 1997) in the Dinaric karst site of Slovenia (lat. 45.41 °N, long. 14.12 °E, alt. 820 m) with 1500 mm rainfall. The experimental site was part of the mountain pasture Vremščica for milking sheep. Field experiment was set in the farmlet that has North exposition, with moderate slope and was subdivided into 6 paddocks and lasted from 2002 to 2005. The herbage was utilized by grazing only in a 6 week rotation during 100 to 120 day-long grazing season. Soil samples were collected in spring before onset of the grazing season from sites appropriate to locate the planned field experiment. To determine the concentration of minerals in herbage available for grazing and browsing, samples were collected from formerly not fertilized area. The grass species: sheep's fescue (*Festuca ovina* L.), chalk false brome (*Brachypodium pinnatum* L.) and erect brome (*Bromus erectus* L.) were collected in spring, because of their high abundance for grazing during that period. Young branches and leaves of common hazel (*Corylus avellana* L.) and common beech (*Fagus sylvatica* L.) were sampled during summer grass dormant period, as leaves of shrubs represented supplemental feed at that time. Legume white clover (*Trifolium repens* L.) was sampled at beginning of second rotation from grazed but not fertilized area. As very valuable feed white clover is grazed soon after the sheep enter the paddock. All together six different plant species were collected separately (three grass species, two shrub species and a legume species). Within a paddock where lambs (sheep replacement) were grazed under regular rotation, an experiment of latin square design consisting of four treatments with four replicates was set. Four levels of P (0, 30, 90, 270 kg P_2O_5 ha^{-1}) were under investigation in the first year. Next year superphosphate was applied again only at treatment 30 and 90 kg P_2O_5 ha^{-1}. In spring of the third year the superphosphate was applied equally as in a first year; all three fertilized treatments received superphosphate at the same rate as the first year. Total amount of applied P during four years of investigation was 0, 90, 270 and 540 kg P_2O_5 ha^{-1} respectively for treatment 1, 2, 3 and 4.

Major soil physical and chemical properties were analysed in soil samples (Egner *et al.*, 1960; Janitzky, 1986; SIST ISO 13878, 1998; Soil Survey Staff, 2009). The concentration of minerals in herbage samples was determined by standard procedures for plant tissue (Ca, Mg, Na, Mn, Zn, Fe and Cu were determined by atomic

Table 1: *Chemical properties of soil (n=30) on mountain pasture Vremščica (SD – standard deviation, SE – standard error of average)*
Preglednica 1: *Fizikalne in kemijske lastnosti tal (n = 30) planinskega pašnika Vremščica (SD – standardna deviacija, SE – standardna napaka povprečja)*

	Soil depth cm	pH in KCl	Clay %	P_2O_5	K_2O	SOM %	C %	C:N ratio
				mg 100 g^{-1} of soil				
Average	12.6	4.5	21.1	2.7	14.4	9.9	5.7	13.2
SE	0.8	0.1	1.3	0.4	1.6	0.7	0.4	0.7

absorption spectrometry [Varian Model AA240, Palo Alto, CA], P by spectrophotometric determination [Fias, Perkin Elmer, USA], K by flame photometry [FP6410, Unicom Optics, PRC]). To determine concentration of minerals in herbage from fertilized experiment, samples were obtained immediately before grazing (pre-grazing). To represent the herbage grazed by the animals, samples were obtained by hand plucking at the end of the spring. Sampling units (n = 25) were randomly selected out of the population from each plot, and combined in one sample for each treatment. Herbage samples were oven dried for approximately 12h at 100 °C and ground in a hammer mill to pass a 1-mm sieve. These samples were chemically analysed and the results are assumed to relate closely to the composition of the herbage eaten. The data obtained from our study were subjected to analysis of variance for random block and latin square design with GenStat Release v7.1 (Genstat 7 Committee, 2003) and Duncan's post hoc test at $P < 0.05$ probability level to determine significant differences between the treatments.

3 RESULTS AND DISCUSSION

3.1 MINERALS IN SOIL

Most frequent type of soil found over pasture Vremščica is typically brown rendzina. This is less productive soil, where nutrient concentration and water holding capacity are low. Reasons for this are slow process of soil formation and very long history of utilisation of vegetation that existed there in the past (Lovrenčak, 1993). The top layer of soil – A1 horizon, where most roots of herbal vegetation can be found is in average 12.6 cm tick, has low pH, and is deficient in P available to plants (2.7 mg P_2O_5 100 g^{-1} of soil). Level of K in soil is low to moderate (Table 1). Content of soil organic matter (SOM) in top layer is high (10%). This and substantial content of clay in soil (> 20%), is the reason for the high cation exchange capacity (CEC) of the soils of karst pasture. Clay in rendzina is not only a source of nutrients, its also determine the degree to which organically bound P can accumulate in the soil (Table 2). Because of very high CEC the base saturation of this soil is low (32.3%). Ca and Mg together present most of this base saturation, which should be round 80% to assure balanced soil for good growth of white clover and efficient fixation on N by symbiotic bacteria. C to N ratio is too high (13.2 :1) for to accomplish efficient transformation of dead organic matter into humus. The very high saturation of soil with hydrogen ion (67.8% H^+) is in accordance with soil acidity (4.5 pH). The rank of saturation of exchange capacity with hydrogen ion should be 10 to 15% H^+ as stated by Kinsey and Walters (2006).

3.2 MINERALS IN HERBAGE

From results obtained in current research it is evident that great differences exist in concentration of minerals between three kinds of available herbage for animals grazing or browsing within vegetation of karst pasture. Native perennial grasses which are adapted to grow on P deficient soil with a pronounced dry season spell have very low concentration of minerals in total (49.4 g ash kg^{-1} of DM on average). In leaves of shrubs the concen-

Table 2: *Cation exchange capacity (CEC), base saturation and mineral composition in the soil (n = 30) on mountain pasture Vremščica (SD – standard deviation, SE – standard error of average)*
Preglednica 2: *Kapaciteta sorpcije, zasičenost z bazami in koncentracija mineralov v tleh (n = 30) planinskega pašnika Vremščica (SD – standardna deviacija, SE – standardna napaka povprečja)*

	Base	CEC	Base saturation	Ca	Mg	K	Na	H
	eq mmol H 100 g^{-1} soil		%					
Average	9.7	30.0	32.2	24.6	6.3	1.2	0.2	67.8
SE	1.2	1.3	3.4	2.9	0.8	0.1	0.07	3.4

Table 3: *Concentration of minerals (g kg^{-1} or mg kg^{-1} of DM) in six different plant species of sward available for grazing and browsing from vegetation most abundant on karst pasture. Means followed by the same letter in a row are not significantly different based on Duncan's test (P < 0.05)*

Preglednica 3: *Koncentracija mineralov (g kg^{-1} ali mg kg^{-1} SS) v šestih različnih vrstah rastlin ruše, ki so bile na razpolago za pašo in smukanje iz najbolj obilne vegetacije na kraškem pašniku. Povprečja z enako črko v vrstici se ne razlikujejo statistično značilno (p < 0,05)*

Herbage content	Sheep's fescue	Chalk false brome	Upright brome	Common hazel	Common beech	White clover
Ash (g kg^{-1})	42.8 a	55.8 c	49.7 b	60.0 c	46.4 b	104.8 d
Phosphorus (g kg^{-1})	0.98 a	0.97 a	1.25 b	2.65 c	2.63 c	2.64 d
Calcium (g kg^{-1})	2.74 a	5.33 b	4.11 b	13.2 d	8.1 c	15.9 d
Magnesium (g kg^{-1})	1.30 a	1.19 a	1.48 a	3.58 c	2.82 b	3.74 c
Potassium (g kg^{-1})	9.1 a	11.3 b	14.1 b	10.9 a	10.9 a	29.6c
Sodium (g kg^{-1})	0.04 a	0.16 b	0.24 b	0.17 b	0.15 b	0.89 c
Zink (mg kg^{-1})	15.8 a	22.6 b	21.2 b	32.9 c	26.7 b	27.9 b
Manganese (mg kg^{-1})	42.2 a	49.9 a	94.1 b	310.9 d	199.6 c	107.5 c
Iron (mg kg^{-1})	84.1 b	98.6 c	91.4 c	75.0 a	89.8 b	112.9 d
Copper (mg kg^{-1})	3.7 a	8.0 c	7.1 b	11.9 d	14.7 d	7.4 b

tration of minerals is similar (53.2 g ash kg^{-1} of DM) as in grasses. In the herbage of white clover was found 104.8 g ash kg^{-1} of DM, which is twice as much ash as have the formers (Table 3).

The average concentration of P in grasses under investigation was 1.1 g P kg^{-1} of DM. This is half of what is requirement for sheep and cattle in their diet. Only herbage containing at least 2.5 g P kg^{-1} of DM will ensure that the P requirement for all classes of sheep will be met if their DM intakes are adequate (Whitehead, 2000). For rapidly growing animals the herbage on offer should contain at least 3.0 g P kg^{-1} of DM (Ozanne and Howes, 1971).

Leaves of common hazel and common beech were much better source of P for animals grazing karst pasture vegetation than perennial grasses. On average the concentration of P in leaves of shrubs was 2.6 g P kg^{-1} of DM. This is very important to know, because during dry summer period when grasses are seed set and such a herbage has very low concentration of P, animals must have possibility to browse leaves of woody plants. Herbage of white clover had enough P (2.6 g kg^{-1} of DM) for the need of grazing animals. The problem is that white clover is very sparsely found and less abundant over karst pasture. During the period of improvement of karst pasture (control grazing, fertilizer or lime application) legumes are distributed as patches over area and their proportion in total in the sward is still very low. Only for a short period of time after entering the new paddock, the intake of legumes may be sufficient high by grazing animals.

Concentration of Ca was lowest in herbage of sheep's fescue (2.7 g Ca kg^{-1} of DM) and highest in white clover (15.9 g Ca kg^{-1} of DM). Leaves of common hazel had more Ca than leaves of common beech. Values for Ca in herbage of white clover were adequate for maintenance, growth and pregnancy of grazing animals. But when diet as a whole is deficient in P, as this is the case when perennial grasses present bulk of diet, than the concentration of Ca must be higher, because its absorption in animals is decreased due to P deficiency in herbage.

A K level in herbage under investigation was high and exceeded the requirements of grazing animals (Whitehead, 2000). High concentration of K in herbage of white clover (29.6 g K kg^{-1} of DM was indication that samples of this herbage were collected from the patches where animal dung was left formerly. Animal diet low in Na level, but high in K level could further reduce the Na intake of the grazing animals (Aspinall *et al.*, 2004). This was the case with grasses and leaves of shrubs in present research. Only herbage of white clover had adequate concentration of Na (0.89 g Na kg^{-1} of DM) to maintain full Na status of lactating ewes with lambs (Gillespie *et al.*, 2006).

Since the micronutrients in soils are derived almost entirely from the parent material, the soils on limestone are normally low in all micronutrients except Mn. Concentration of Zn, Mn, Fe and Cu elements in herbage were within the lower part of values reported elsewhere (Spears, 1994; Grace, 1983). There was less Zn in grasses and more in leaves of woody plants. Symptoms of Zn deficiency in sheep and cattle may occur when animals graze herbage with less than about 20 mg Zn kg^{-1} of DM.

Herbage of sheep's fescue had less than this and the values for Zn of other herbage was little above this.

Very high was the concentration of Mn (311 mg Mn kg^{-1} of DM) in leaves of common hazel and similar high was for common beech leaves. The average value for three grasses under survey was 62.1 mg Mn kg^{-1} of DM, and for herbage of white clover was 107.5 mg Mn kg^{-1} of DM. Limestone as a parent material where rendzina soil is formed has high content of Mn. With increasing acidity of top soil, leaching of Mn occurs and this element accumulates on clay particles in lower soil horizons. Because the roots of woody plants search for minerals deeper in the soil, the higher absorption of Mn is achieved by the roots of woody plants. Mn content was high in white clover too, because this valuable plant can be very easy introduced on places where woody plants were growing. When shrubs are thinned and open space with enough light is formed, white clover has an opportunity for fast establishment. Decomposing shrub leaves are additional source of Mn for white clover grown on shrub cleared areas.

Several studies, in which Mn cycling was researched, have indicated that 20 to 25 mg Mn kg^{-1} of DM is adequate for growth and reproduction (Grace, 1983). Concentration of Mn in leaves of shrubs exceeded 10 to 14 times the values required in animal diet for optimum skeletal development and to prevent reproductive problems. Variability of Mn concentration in different grass species grown at the same location can be very high as reported by Orešnik *et al.* (1999). But all these concentrations of Mn in herbage are still well below the levels that reduced growth rate observed in lambs grazing pastures which contained 400 mg Mn kg^{-1} of DM (Grace, 1983). Sheep may be somewhat more susceptible than cattle to excessive Mn intake.

As reported by Grace (1983) the animal growth rates were only significantly reduced where concentrations of Mn were above 1200 mg kg^{-1} of DM for the full 2 week grazing period. The soil pH strongly influences the Mn level in plants with the Mn uptake being greatest in acid soil. Liming tends to decrease herbage concentration of Mn through its effect on soil pH. Change from pH 5.2 to 6.2 reduced the concentration of Mn in the herbage of mixed sward from an average of 290 to 130 mg Mn kg^{-1} of DM. To lessen the amount of lime required to lower herbage Mn concentration, the improved grazing management which limits the dead material content appear to be effective as was found by Smith *et al.* (2006). Variability in concentrations of iron (Fe) between kinds of herbage under investigation is very small. In leaves of common hazel is the lowest concentration of Fe (75.0 mg Fe kg^{-1} of DM) and in white clover herbage is the highest (112.9 mg Fe kg^{-1} of DM). These concentrations in herbage are adequate to cover animal requirements upon Fe, and are not to high to interfere with the Cu metabolism in animals.

Leaves of common beech have highest concentration of Cu (14.7 mg Cu kg^{-1} of DM), lesser is in common hazel and much lower in white clover (7.4 mg Cu kg^{-1} of DM). Provided that the availability of the Cu is not greatly influenced by the presence of high S supply and the DM intakes are adequate, then herbage containing 5 to 6 mg Cu kg^{-1} of DM for sheep and 7 to 10 mg Cu kg^{-1} of DM for cattle requirements of Cu should meet (Spears, 1994).

3.3 P FERTILIZATION AND CONCENTRATION OF MINERALS IN HERBAGE

The regular application of different rate of superphosphate did not have any pronounced effect on ash content. It is normal with soils in the pH range from 4.5 to 5.5 which are deficient in P, that the application of P produces positive yield response. This is the reason that content of ash in herbage is not much increased because of dilution effect. But the application of superphosphate increased the concentration of P in herbage. On control treatment there was 3.4 g P kg^{-1} of DM of herbage. An increase of 23% for concentration of P in herbage was achieved in average on treatments where superphosphate was applied at the rates of 90 to 540 kg ha^{-1} P_2O_5 during three year time.

Contrary to the statement for P was found for the concentration of Ca in herbage. At the control treatment, there was 9.0 g Ca kg^{-1} of DM. Application of P fertilizer decreased the Ca concentration in herbage. With highest rate (540 kg ha^{-1} P_2O_5) of fertilizer use, the content of Ca in herbage was 6.1 g P kg^{-1} of DM. Changes in concentration of Mg in herbage through applied superphosphate are smaller than for Ca, but are in same direction; higher yield of DM, less Mg in herbage on weight basis.

Concentration of K is within the range found in grasses collected from grazed sward only (12.2 g K kg^{-1} of DM). Increased concentration of K in herbage from plots with higher rate of superphosphate fertilization can only be explained through effect of deposited sheep urine; more of it was left on plots with higher amount of herbage available for grazing and longer stay of animals on those plots.

Concentration of Na in the herbage, was in average 1.2 g Na kg^{-1} of DM. At low rate of applied P fertilizer the dilution effect through higher yield is more distinct. At heavier fertilization and higher yield the animals interfere with their excreta on concentration of Na in herbage. Sheep were all the time supplemented with common salt

Table 4: *Effect of superphosphate application on concentration of minerals (g kg^{-1} or mg kg^{-1} of DM) in herbage available for grazing (pre-grazing) on the karst pasture. Means followed by the same letter in a row are not significantly different based on Duncan's test (P < 0.05)*

Preglednica 4: *Vpliv gnojenja z superfosfatom na koncentracijo mineralov (g kg^{-1} ali mg kg^{-1} SS) v razpoložljivem zelinju za pašo na kraškem pašniku. Povprečja z enako črko v vrstici se ne razlikujejo statistično značilno (p < 0,05)*

	Fertilizer treatments – total applied kg P_2O_5 ha^{-1} in three years			
Herbage content	not fertilized	90 kg ha^{-1}	270 kg ha^{-1}	540 kg ha^{-1}
Ash (g kg^{-1})	87.2 a	84.2 a	85.4 a	84.9 a
Phosphorus (g kg^{-1})	3.40 a	3.38 a	4.93 b	4.18 b
Calcium (g kg^{-1})	8.97 b	6.84 a	6.03 a	6.08 a
Potassium (g kg^{-1})	12.22 b	9.85 a	10.53 a	12.53 b
Magnesium (g kg^{-1})	1.96 b	1.93 b	1.80 a	1.85 a
Sodium (g kg^{-1})	1.21 a	1.06 a	1.13 a	1.24 a
Zink (mg kg^{-1})	22.60 a	22.82 a	21.52 a	22.85 a
Manganese (mg kg^{-1})	60.2 b	60.4 b	50.1 a	62.6 b
Iron (mg kg^{-1})	371.8 b	503.7 c	274.4 a	379.1 b
Copper (mg kg^{-1})	13.06 b	10.18 a	10.93 a	10.90 a

and this might have an effect on concentration of Na in herbage.

There is not clear effect of added P on concentration of Zn, Mn, Fe and Cu (Table 4). Higher yield of DM induced with added fertilizer didn't have any dilution effect on concentration of those minor elements in herbage. Concentration of Fe is higher than found in different plant species at the onset of experiment. The reason for this might be the sampling of herbage closer to ground and possible contamination of sward with trampling of grazing animals.

The soils of karst grassland as such are very unsuitable for to improve their fertility and to increase the abundance of legumes in the sward on the short time. Slow decomposition of soil organic matter (SOM) is the limiting factor to the cycling nutrients in the soil-plant-animal system. As reported by Clark and Woodmansee (1992) the microorganisms have a substantial requirement for P. Net mineralization of SOM conducted by them, occurs only when their need on P has been met. The critical ratio of C to P for mineralization to occur must be less than 100:1 (Tate, 1985). In the soil of karst grassland dead SOM has the C to P ratio well above this. On addition the high content of SOM of karst grassland acts as big sink for P added with fertilization. As dead plant material of karst vegetation, largely present in the rooting zone, is insufficient in P, initially immobilisation of this element occurs when added to the soil, even in a large amount of P fertilizer.

Many different plant species can be found within the indigenous karst sward (Kaligarič, 1997; Vidrih M, 2003). Unfortunately, most of the grasses are of low feeding quality (Brachypodium spp., *Festuca ovina* L., Koeleria pyramidata L., *Bromus erectus* L., Carex spp.). Further obstacle for higher stocking rate or better production is low portion of legumes in existing sward. It almost never exceeds 6% (Vidrih and Kotnik, 1995; Batič *et al.*, 1999). Among different species of herbs 41 of them are medicinal plants, which can have distinct effect on animal health due to their higher concentration of minerals or any of secondary substances (Kotnik and Vidrih A, 1995).

As reported by Jones and Thomas (1987) there are several abiotic and biotic mechanisms that redistribute clay and nutrients in the landscape, similar to karst pastures, resulting in nutrient rich and nutrient poor patches which are differentially exploited by herbivores. Much of the spatial pattern of plant communities in karst pasture is similarly linked to variations in soil nutrient supply, which is in addition affected by deposition of excreta by grazing animals.

When a nutrient is extremely deficient, as a P in karst soil, the addition of a small amount of nutrient will increase the yield but actually cause a small decrease in concentration of minerals. Due to high Ca level, consumed leaves of woody plants may result in a diet with an excessively Ca to P ratio compared to grasses. Intake of leaves of woody plants would be advantageous and would balance a selected diet on pasture dominated by grasses, is a statement made by Garmo (1999). A large amount of forage for animals in former times comprised leaves of shrubs and trees. Thus herbivores are adapted to utilize the foliage of woody plants in amount not harmful to them. Patchy pattern of grazing even on ideal ryegrass -

white clover pasture is clear evidence that animals needs more than just an abundance of most palatable herbage for to satisfy their physiological requirements.

Mineral concentration in both soil and plant influences the mineral status of grazing animals and many other factors, such as selective grazing, interaction between minerals and production level. The important concept is that it is the concentration of the P in soil solution and not the total potentially available P that determines the rate at which the plant will grow (Mouat, 1984). A large amount of added P on small scale is achieved through the dung patch and at the site where the animals are camping or have drinking facilities. Since most nutrients ingested by grazing animals are returned to the soil in excreta, it is very important to achieve even distribution of it and to prevent nutrients transfer or accumulation on camp sites (Rigueiro-Rodríguez *et al.*, 2007). Nutrients in urine and dung patches within the main grazing area cause a mosaic of nutrient levels and may lead to nutrient losses, even from unfertilized soils. Leaching losses may be smaller when woody plants are present on pasture. Trees and shrubs can further sequester nutrients from deeper soil layers as reported by Lehmann *et al.* (1997).

Increased nutrient availability often causes or accelerates grass encroachment and a decrease in species diversity. However, grazing has been found to decrease grass encroachment and increase species diversity (Batič *et al.*, 1999), a finding attributed to improved light access to plant species of low growing habit. Such conditions favoured a number of plant species which had otherwise low competitive ability under conditions of higher nutrient availability and denser vegetation like the white clover has.

4 CONCLUSIONS

The mineral composition of plants collected from karst pasture vegetation vary widely, so any changes in botanical composition of the vegetation can lead to major changes in mineral intake of grazing animals. In karst environment seldom all necessary growth factors are adequate to meet the needs of all plants within a sward. The large amount of P needs to be applied to achieve better plants growth on karst pastures. This is often uneconomic in term of cost of input and value of current return. But the beneficial effect of P fertilization will still be felt in later years and if this residual effect is used as a base for continued application, more and more efficient systems may be developed. Even with higher rates of applied P initially there will be no much increase in the concentration of minerals in herbage available for grazing. To improve the efficiency of the system in shorter time, there is a need to exploit more efficiently all different groups of plants presented in vegetation of karst pasture and to enhance the speed of mineralisation of large amount of SOM.

Since most nutrients ingested by grazing animals are returned to the soil in excreta it is very important to know the content of the minerals in different plants the animals have on offer. And at the same time managing animal grazing the way to exploit most efficiently the available minerals in dung and urine and to increase the activity of soil microorganisms to achieve more efficient decomposition of dead organic matter. It is likely that the creation and maintenance of such patches is necessary for the survival of grazers in nutrient poor environment as the karst pasture is. The importance of nutrient return through dung and urine of grazing and browsing animals will have great value when feeding animals during the winter on grazing land.

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INTRODUCTION PILOT BIOGAS REACTORS AND APPLICATION TO DEFINE BIOGAS POTENTIAL OF BASIC SUBSTRAT, SWINE SLURRY

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Introduction pilot biogas reactors and application to define biogas potential of basic substrat, swine slurry

Cooperation between the 'Panvita Group' and the Biotechnical Faculty, University of Ljubljana, resulted in the construction of a pilot biogas reactor – a miniaturised version of the economical biogas reactor. The aim of construction was to support scientific research in the field of biogas generation, while, at the same time, optimising the processes conducted in economical reactors and testing the new substrates in the field of biogas generation. A 2500-litre reactor, containing a 500-litre gasholder, was built to this purpose. In the first operating period, biogas-generation potential of the raw and partially purified swine slurries was tested. The three repetitions allowed us to generate an average of 529 litres CH_4 per 1 kilogram of organic dry matter.

Key words: biogas reactor / pig slurry / biogas / methane / electric energy

Predstavitev pilotnega reaktorja in aplikativna določitev bioplinskega potenciala osnovenga substrata, prašičje gnojevke

Biotehniška fakulteta in Skupina Panvita smo v sodelovanju postavili preizkusni bioplinski reaktor, ki je pomanjšana različica gospodarskega bioplinskega reaktorja. Namen gradnje preizkusnega reaktorja je bil znanstveno raziskovalnem delo na področju pridobivanja bioplina in hkrati optimiziranje procesov v gospodarskih reaktorjih ter preizkušanja novih substratov v proizvodnji bioplina. Zgradili smo 2500 litrski reaktor, od celotnega volumna reaktorja je 500 litrov plinohrama. Kot cilj v prvem obratovalnem obdobju smo ovrednotili bioplinski potencial surove prašičje gnojevke in delno prečiščene prašičje gnojevke. Pravilno ovrednoten osnovni substrat je izhodišče za vse nadaljnje preizkuse, saj je to medij v katerem se bodo vrednotili vsi substrati ali mešanice substratov. S tremi ponovitvami smo dosegli v povprečju 524 litrov CH_4 na kilogram organske suhe snovi (OSS).

Ključne besede: bioplinski reaktor / prašičja gnojevka / bioplin / metan / električna energija

1 INTRODUCTION

The reduction of CO_2 values in the atmosphere is a world goal (UNFCCC, 1997). The EU member states have clear instructions to substitute the current fossil energy with renewable sources up to the value of 12% of the used gross energy by 2010. This means three times increasing the energy production from existent renewable sources in the EU.

In Slovenia there are already some existent biogas stations and some are being built. Biogas plants immensely help reducing greenhouse gas emissions and concurrently contribute a certain amount of renewable energy. The more biogas plants we have the bigger the effects of the test reactors will be, with which we can optimise the performance of biogas plants and test new substrates (Zver, 2005). Above all, the test reactors are intended for fast evaluation of a new organic mixture. This new organic mixture would be used in the actual reactor after the analysis of all the significant parameters for an economical and ecological performance of the reactor. The essential function of the test reactors is to enhance the economy of the real biogas plants.

Biogas is the mixture of gasses, which originate

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Table 1: *Efficacy of biogas production from swine slurry*
Preglednica 1: *Izpleni bioplina iz prašičje gnojevke po različnih avtorjih*

	Author	Extent of biogas production (l/kg organic substance)	Average biogas production (l/kg ODM)
Swine slurry	Beck, 1997	340–550	450
Swine slurry	Gaćeša, 1985		550
Swine slurry	Gobec, 2005	200–350	275
Swine slurry	Karpenstein-Machan, 2005	250–350	300

l/kg – litres of biogas per kilogram of organic substance; ODM organic dry matter

from anaerobic fermentation. Anaerobic fermentation is a biological process which is based on methanogenesis, where bacteria decompose organic material. The products of the process are mainly methane and carbon dioxide. The process can be simplified with the reaction: $2C + 2H_2O \rightarrow CH_4 + CO_2$ (Đulbić, 1986).

There are many factors to ensure optimal biogas production. They fall into three categories:

- Physical : imported heat in the reactor, mixing of the organic mixture in the reactor, density, structure of the organic mixture in the reactor, specific yield (1/kg DM)
- Chemical : C:N ratio, pH, content of macro and micro elements, toxins, heavy metals
- Biological: type of microorganisms, their quality and quantity

It is reasonable to search for and to optimise the mixture of different substrates in a test reactor. Thus we can ensure optimal exhaustion of substrates for biogas production and our findings can be applied in the economical devices.

KG Rakičan EKOTEH d.o.o. is a member of the Panvita Group. They already operate a 1.3 MW biogas plant and they are also currently building a new smaller biogas plant. The following substrates are imported in the existent biogas plant:

13t of raw slurry, 8.5 t of by-products of animal origin (BPOAO2 and BPOAO3), 35.6t of maize silage, and 60t of slurry.

The article focuses on the biogas yield produced from swine slurry. Therefore, the results of preceding research will be presented as well. Different authors quote various biogas yields. We could practically say that the span of results is too big and unrealistic. Furthermore, the reason for the span is not given and this is the key factor for future assessment and planning of the device.

2 METHODS

2.1 INTRODUCTION OF THE TEST REACTOR

A pilot reactor was built in the immediate vicinity of the existent biogas plant (Fig. 1). Its purpose is optimising the functioning of economical biogas plants and testing new substrates. The volume of the pilot reactor is 2500 litres, from which the working volume is 2000 litres and the remaining 500 litres is intended for the gasholder. It is a miniature version of the economical biogas reactor. In it we can test substrates processed in the same manner as in the economical biogas reactor.

2.2 COMPONENTS OF THE REACTOR

- The stirring device is placed horizontally and driven by a 500W electro engine.
- The heating appliance is placed inside on the walls of the reactor; it is heated by water from an outer heating source.
- The additional – auxiliary stirring device is adjustable in the spill-over edge, which prevents clogging of the exit; it is driven by a 350W engine.
- The swine slurry pump is driven by an electrical engine and is used to pump the purified slurry into the reactor. It pumps 2 litres of slurry per minute, taking into account the losses in the pipeline. We can set the intervals of the pumping of the slurry and the amount of it.
- The opening for adding additional substrates is a funnel-shaped opening into which, at certain intervals, substrates are added. Due to stirring the opening is being gradually emptied and thus it ensures a continuous filling.
- The biogas flow-metre is a mechanical counter. It notes the biogas flow on the basis of the turns of the spades. In our case the spades are being driven by the biogas.
- The analyser of the biogas quality is portable. It

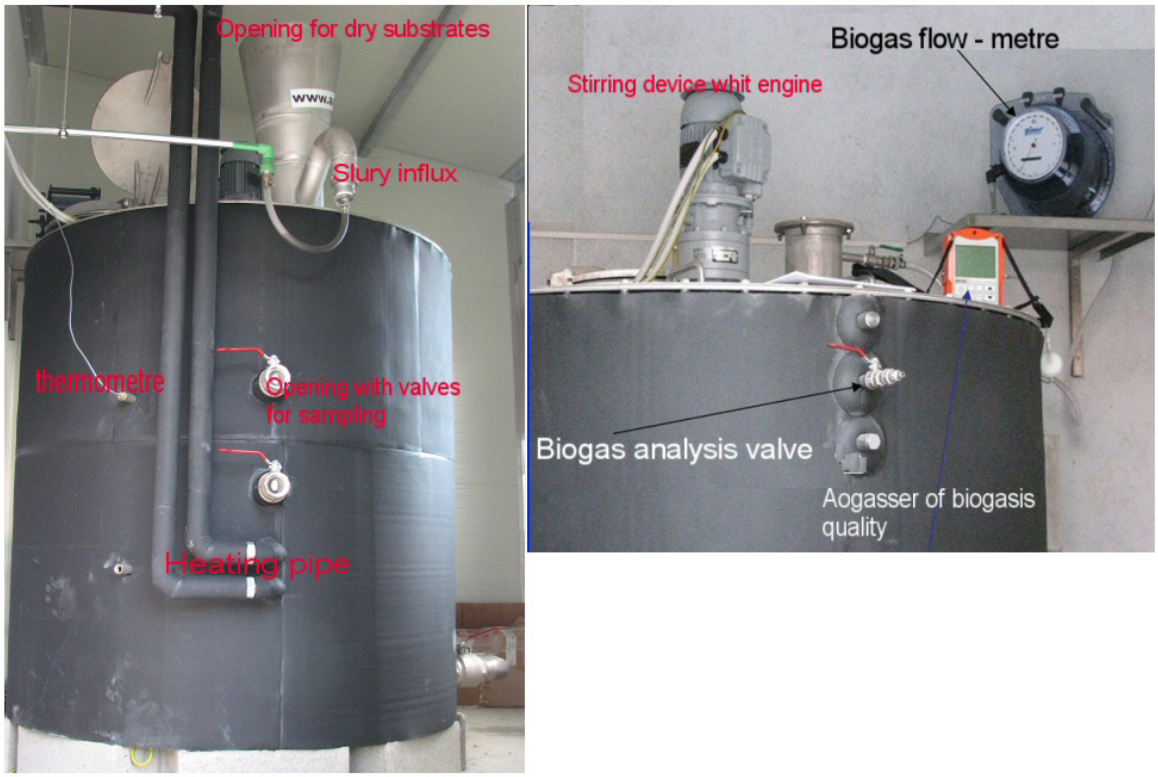

Figure 1: *Test reactor (on the left: view from the front, on the right: view from the back).*
Slika 1: *Preizkusni reaktor (levo: prednja stran, desno: zadnja stran).*

is used to analyse the biogas quality in certain intervals or when needed. We can measure % CH_4, % CO_2, % O_2, and ppm H_2S.

- The control box with a computer controls all the devices in the reactor except for the analyser of the biogas quality. The software enables the setting of the intervals for the stirring devices and the pump. It also provides a simultaneous register of the data during the intervals.
- Other equipment includes the laboratory with all the necessary equipment and other equipment for storage and handling of substrates.
- Sampling pints. The reactor has six exits with ball-bearing valves, which enable sampling for the analysis. Two of these are 80mm in diameter.
- The window is a glass door. We can observe the occurrence in the reactor from the top.

2.3 WORKING WITH THE REACTOR

The working volume of the reactor (2000 litres) is so big that the processes in it cannot be completely controlled and directed. Processes which are equal to those in the economical reactor are: complete stirring (usually a crust occurs on the substrates with a major proportion of dryness and fibres), emissions of biogas, comparable fluctuation of pH, structures of different microorganisms, etc.

The test reactor gives us more tangible results for a certain substrate in comparison to the results given in certain literatures, which usually contain average results gained in smaller reactor laboratories under optimal conditions. The results are then used directly in the economical reactor. Thus the fluctuation of the economical reactor is optimised. Concurrently, we can analyse the functioning of the test device.

2.4 INITIATION OF THE TEST REACTOR

The pilot reactor was filled with the mixture of bacteria from the anaerobic part of a purification device or an old biogas plant. The temperature was set to 37.5 °C with ±1.5 °C deviation. The stirring was adjusted to the economical devices. The swine slurry was gradually added.

After having established that the test reactor func-

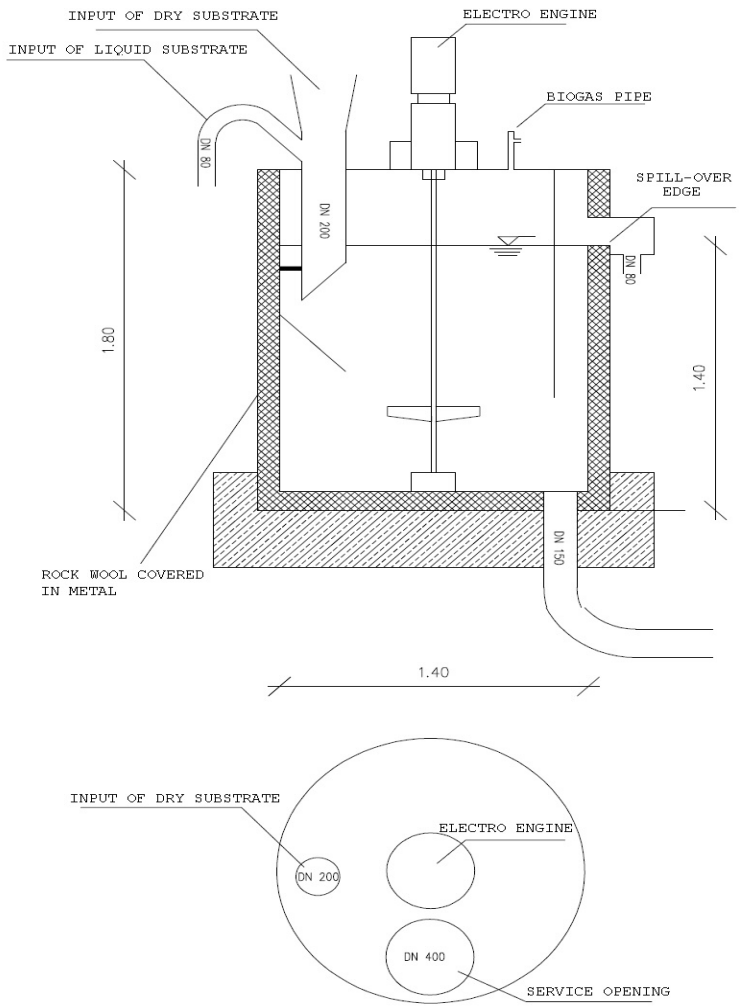

Figure 2: *Sketch of the test reactor (front view and ground plan).*
Slika 2: *Skica preizkusnega reaktorja (naris in tloris).*

tions perfectly, we started with the systematic research of the test reactor.

2.5 CHOOSING THE BASIC SUBSTRATE

During the first weeks of the operation we determined daily fluctuation of biogas production. We started researching the reasons for the unbalanced biogas production. The cause for this was the quality of the raw swine slurry and the changing dryness from 0.5% to 7%. The dryness of the swine slurry depended on the day of the week. During weekends the stables are not washed. During weekdays the stables are being washed, adding the water used for washing to the common reservoir of the slurry. From there the slurry is taken for the use in the experimental reactor. Another occurrence of unbalanced production was detected when the pipe for adding raw swine slurry was clogged by a ball of swine hair. The flow was lower than we expected. However, these are technical problems which can be solved by designing the device correctly. According to the above described technical problems we decided to use partially purified swine slurry with dryness between 1% and 1.5%. Thus we ensured that the input material is homogeneous and avoided technical stirring problems. The swine slurry under consideration has a very specific composition. On average it contains 41% of ash in dry matter.

2.6 EVALUATION OF SWINE SLURRY

The purified swine slurry will in future be the basic medium in the reactor. Thus we needed to assess its en-

Table 2: *Basic statistics of biogas produced from partially purified swine slurry*
Preglednica 2: *Osnovna statistika bioplina iz surove in delno prečiščene prašičje gnojevke*

	Biogas production in 10 days					
Substrate	Min.	Max.	Average	Median	Standard deviation	Produced biogas (l)
Slurry 1	70	510	240	200	141	2405
Slurry 2	280	420	379	398	53	3790
Partially purified slurry 3	250	320	284	298	28	2840
Partially purified slurry 4	225	320	267	265	33	2665

ergy potential at a certain residence time. We decided on a 30-day residence time. The purified swine slurry had 41% of ash in dry matter. After months of evaluation we gained the sought data for this swine slurry, biogas yield per kilogram of dry matter, and biogas yield per kilogram of organic dry matter. However, we did not seek for non-degradable organic dry matter.

3 RESULTS AND DISCUSSION

During the first three months of production, we were evaluating biogas potential of the raw swine slurry and partially purified swine slurry. Figure 3 and Table 2 show biogas productions in time periods 1, 2, 3 and 4. In the time periods 1 and 2 we used raw swine slurry. However, due to its physical characteristics it was not suitable to include it in further experiments. The main problem was the unbalanced amount of dry matter. In the time periods 3 and 4 we evaluated partially purified swine slurry. This is shown in Figure 3. Curve 1 shows that raw swine slurry is not suitable as the basic substrate because of dryness volatility. Curve 2 shows operation with raw slurry. However, in this case the slurry was left in a container for the thick part to sediment. We only used the cleaner upper part of the slurry, but the volatility of the dryness was still too high. Thus it is not suitable to be used as the basic substrate.

Table 2 shows data about the biogas yield from slurry tested during certain time periods. The standard deviation with raw slurry is immense. Thus it is not suitable to be used as the basic substrate in further experiments.

3.1 BIOGAS POTENTIAL OF PARTIALLY PURIFIED SWINE SLURRY

Due to technical problems and homogeneity of the basic substrate we decided to use partially purified swine slurry. In Table 3 we see the average biogas yield for a

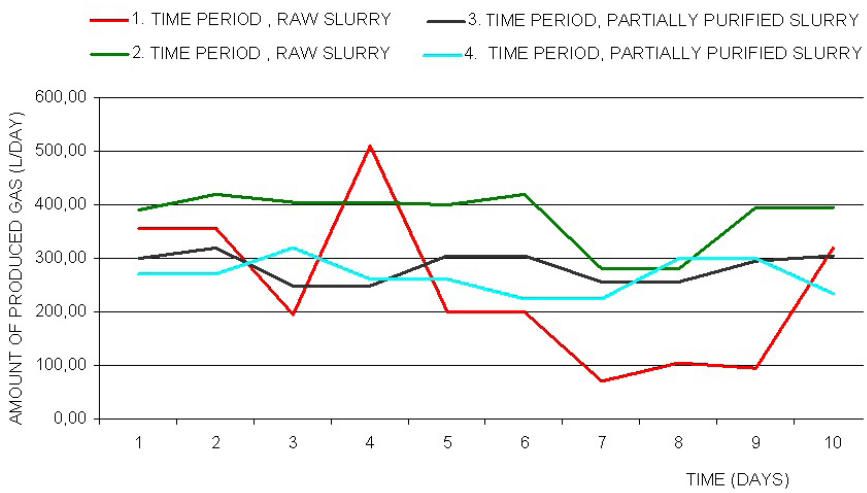

Figure 3: *Biogas production per day during the evaluation of biogas potential of raw swine slurry (1 and 2) and partially purified swine slurry (3 and 4).*
Slika 3: *Produkcija bioplina po dnevih pri vrednotenju bioplinskega potenciala surove gnojevke (1 in 2) in delno prečiščene prašičje gnojevke (3 in 4).*

Table 3: *Chemical characteristics of partially purified swine slurry and biogas yield*
Preglednica 3: *Kemične lastnosti delno prečiščene prašičje gnojevke in izplen bioplina*

	Substrate
	Partially purified swine slurry
% DM	1.0–1.5
% CA* in DM	44
C : N	36
Biogas / litre of slurry (l)	5.29
Biogas / kg DM (l)	423
Biogas / kg ODM (l)	756
CH_4 / litre of slurry (l)	3.71
CH_4 / kg DM (l)	296
CH_4 / kg ODM (l)	529

*CA = crude ashes, BG; We gained comparable results with previous researchers on the test biogas reactor.

20-day period. We used a total of 1040 litres of slurry and produced 5505 litres of biogas or 3854 litres of methane. We reached the average biogas yield of 275 litres per day, or 756 litres of biogas per kilogram of organic dry matter, or 423 litres per dry matter. The average biogas composition is: CH_4 70%, CO_2 30%, O_2 0% in 1500 ppm H_2S.

4 CONCLUSION

We evaluated the biogas potential of specific swine slurry. Partially purified swine slurry has specific characteristics in comparison with other slurries mentioned by other authors. It was reasonable to use partially purified slurry. It gave us valuable information concerning its biogas potential.

In future, partially purified swine slurry will be used as the basic medium for testing and determining the biogas potential of different substrates. One of the examples is the mixture of partially purified slurry and silage. From the gross produced biogas, gained from this mixture, we mathematically subtract the produce of partially purified swine slurry. Thus we gain the net biogas yield from certain substrate. Partially purified slurry will also hold the function of a means of transportation into the biogas reactor for different substrates. Thus, with the help of the fluid partially purified slurry, for which the energy potential is already known, we can import into the reactor organic material with bigger content of dry matter. This enables us to define the common or partial energy potential, or we can anticipate the energy utility of a certain organic material.

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ODDAJA

Avtorji prispevke oddajo v natisnjenem in elektronskem izvodu. Priložijo tudi izjavo s podpisi vseh avtorjev, da avtorske pravice v celoti odstopajo reviji.

Prispevke recenziramo in lektoriramo. Praviloma pošljemo mnenje prvemu avtorju, po želji lahko tudi drugače. Če urednik ali recenzenti predlagajo spremembe oz. izboljšave, vrne avtor popravljeno besedilo v 10 dneh v natisnjenem in elektronskem izvodu. Ko prvi avtor vnese še lektorjeve pripombe, odda popravljeno besedilo v natisnjenem in elektronskem izvodu.

Pri oddaji končne verzije avtor priloži jasno označene izvirnike slik (ločene grafične datoteke ali fotografije). Datoteke slik poimenuje enako kot v tekstu (npr. Slika1.jpg, Slika2.eps, Slika3.bmp). Originalne fotografije na avtorjevo željo vrnemo. Vektorske slike sprejemamo samo v eps (Encapsulated Postscript) formatu, s tekstom, ki je spremenjen v krivulje. Rasterske slike morajo biti v enem od običajnih formatov (npr. tiff, jpg, bmp). Ločljivost naj bo vsaj 300 dpi.

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The papers should be condensed, short and should not exceed 12 pages, editted with word processor and submitted as doc or rtf file (Windows). Text formatting should be as simple as possible, without headers and footers. Font Times New Roman, size 12 should be used for text and tables (in large tables size 10 is allowed), Ariel should be used for graphs and figures (letter size at least 8) and Courier for nucleic- and amino acid sequence alignments. Right margin is 2.0 cm, left margin 2.5 cm

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Under the address of the authors some space for dates of arrival and acceptance for publishing should be left. A comprehensive and explicit abstract up to 250 words follows indicating the objective and methods of work, results, discussion and conclusions. Key words follow the abstract.

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Simončič M., Horvat S., Stevenson P.L., Bünger L., Holmes M.C., Kenyon C.J., Speakman J.R., Morton N.M. 2008. Divergent physical activity and novel alternative responses to high fat feeding in polygenic fat and lean mice. Behavior Genetics, 38, 3: 292–300

Fraser A.F., Broom D.M. 1990. Farm animal behaviour and welfare. London, Bailliere Tindall: 437 p.

Hvelplund T. 1989. Protein evaluation of treated straws. In: Evaluation of straws in ruminant feeding. Chenost M., Reiniger, A. (eds.). London, Elsevier Applied Science: 66–74

Žgajnar J., Kermauner A., Kavčič S. 2007. Model za ocenjevanje prehranskih potreb prežvekovalcev in optimiranje krmnih obrokov. In: Slovensko kmetijstvo in podeželje v Evropi, ki se širi in spreminja. 4. konferenca DAES, Ljubljana, 8–9 sep. 2007. Kavčič S. (ed.). Domžale, Društvo agrarnih ekonomistov Slovenije: 279–288

ISO 5534 / IDF 4. Cheese and processed cheese – Determination of the total solids content – Reference method. 2004: 1–7

Frajman P., Dovč P. 2004. Milk production in the post-genomic era. *Acta agriculturae Slovenica*, 84, 2: 109–119. http://aas.bf.uni-lj.si/zootehnika/84-2004/PDF/84-2004-2-109-119.pdf (15. mar. 2009)

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