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IN MEMORIAM: Professor Remzi I. BAKALLI, Ph.D.

On December 16., 2007, Prof. Remzi I. Bakalli, a distinguished member of the Editorial board of *Acta agriculturae Slovenica,* passed away at the age of 63 in Athens, Georgia, USA. Prof. Bakalli has served as a member of the Editorial board for more than two decades. We will remember Remzi as a competent scientist, exceptional person, and a good friend.

Remzi I. Bakalli obtained his BSc degree in Animal Science at the Faculty of Agriculture in Belgrade in 1967 and in 1971 he completed MSc training in the field of animal nutrition and food technology at the Faculty of Agricultural Sciences in Zagreb. In the academic year 1974-1975 he was research fellow at the Department of Animal nutrition at the University of Florida, Gainesville. He received his PhD in poultry nutrition from the Veterinary Faculty, University of Zagreb in 1976. After returning from the research visit at the University of Georgia in Athens, he became Assistant Professor of Animal Nutrition at the Faculty of Agriculture, University of Prishtina in 1976. In the period from 1977 to 1979 he was a Dean of the Faculty of Agriculture, University of Prishtina, and in 1988 he became a full professor of Animal nutrition at the same University. Since 1992 he has been a visiting professor at the University of Georgia in Athens, first as Associate Immunologist at the College of Veterinary Medicine and from 1994 as Research Coordinator at Department of Poultry Science, University of Georgia, Athens, GA.

Professor Remzi I. Bakalli was a member of Academy of Sciences and Arts of Kosovo. In 1979 he received The December Prize of Kosovo for research work in the field of animal nutrition. He was Editor of "*Bioteknika*" – the official journal of the Association of Agriculture of Kosovo, member of the Editorial Board of *Acta Biologiae et Medicinae Experimentalis*, Head of the Editorial Board of "*Peradarstvo*" and member of the Editorial Board of *AAS*.

Throughout his career Prof. Remzi I. Bakalli published five books in the field of animal nutrition, over hundred peer-reviewed articles in international journals, and numerous congress contributions. He had teaching appointments at the University of Prishtina and at the University of Georgia in Athens and worked as consultant for the industry and government institutions.

Prof. dr. Remziju I. BAKALLIJU V SPOMIN

Šestnajstega decembra 2007 se je od nas v 63. letu starosti v Athensu, zvezna država Georgija, ZDA, poslovil član uredniškega odbora *Acte agriculturae Slovenice* prof. dr. Remzi I. Bakalli, Prof. Bakalli je bil več kot dve desetletji član uredniškega odbora *AAS*. V spominu ga bomo ohranili kot kompetentnega znanstvenika, izjemno osebnost in dobrega prijatelja.

Remzi I. Bakalli je študiral živinorejo na Kmetijski fakulteti v Beogradu, kjer je leta 1967 diplomiral, iz prehrane živali pa je magistriral leta 1971 na Kmetijski fakulteti Univeze v Zagrebu. V akademskem letu 1974–1975 je deloval kot raziskovalec na Department of Animal nutrition, University of Florida, Gainesville. Leta 1976 je na Veterinarski fakulteti Univerze v Zagrebu doktoriral s področja perutninarstva. Po krajšem raziskovalnem obisku na University of Georgia, Athens, ZDA je postal docent za prehrano živali na Kmetijski fakulteti Univerze v Prištini. Med leti 1977 in 1979 je bil dekan Kmetijske fakultete v Prištini in od leta 1988 redni profesor za področje prehrane živali. Od leta 1992 je bil gostujoči profesor na University of Georgia, Athens, najprej kot imunolog na College of Veterinary Medicine in od 1994 koordinator raziskovalne dejavnosti na Department of Poultry Science, University of Georgia v Athensu.

Profesor Remzi I. Bakalli je bil od leta 1979 član kosovske Akademije znanosti in umetnosti, leta 1979 je prejel Decembersko nagrado za raziskovalno delo na področju prehrane živali. Bil je urednik revije *Bioteknika*, uradnega glasila kosovske kmetijske organizacije, član uredniškega odbora *Acta Biologiae et Medicinae Experimentalis*, Predsednik uredniškega odbora revije *Peradarstvo* in član uredniškega odbora *AAS*.

Remzi I. Bakalli je napisal pet knjig s področja prehrane živali in objavil preko sto znanstvenih člankov v mednarodnih revijah ter številne kongresne prispevke: Predaval je na Univerzi v Prištini in na University of Georgia, Athens ter deloval kot svetovalec za industrijo in različne vladne inštitutcije.

Prof. dr. Peter DOVČ, urednik AAS

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NAČIN DELOVANJA IN UČINKI PROBIOTIKOV V PREHRANI ŽIVALI

Maša VODOVNIK [a] in Romana MARINŠEK-LOGAR [b]

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IZVLEČEK

Probiotiki so živi mikroorganizmi, ki zaužiti v ustreznem številu, ugodno vplivajo na zdravje gostitelja. Njihovi učinki so praviloma povezani z vzpostavitvijo ugodnega mikrobnega ravnovesja v prebavilih gostitelja ter uravnavanjem njegovega imunskega odziva. Pri domačih živalih so ključni učinki probiotikov povezani z izboljšano učinkovitostjo prireje. Poleg ugodnega vpliva na zdravstveno stanje (predvsem mladih) živali, slednje obsega tudi izboljšano konverzijo krme, povečano hitrost rasti in nekatere druge. Probiotični krmni dodatki registrirani v EU vsebujejo predvsem Gram-pozitivne bakterije iz rodov *Bacillus*, *Enterococcus, Lactobacillus*, *Pediococcus*, *Streptococcus* ter kvasovke *Saccharomyces cerevisiae* in *Kluyveromyces sp*. Medtem, ko je večina omenjenih mikroorganizmov načeloma varnih, imajo nekateri lastnosti, ki so lahko škodljive tako za živali, kot tudi za ljudi. Tak primer so enterokoki, pri katerih pogosto opažajo zapise za prenosljive determinante odpornosti proti antibiotikom. Slednji tako predstavljajo potencialno nevarnost za širjenje odpornosti v patogenih mikrobnih populacijah ljudi in živali. Hiter napredek na področju sintetične in sistemske biologije združen s podporo bioinformatike in novimi orodji genskega inženirstva v prihodnosti obeta skoraj neskončne možnosti za pripravo probiotičnih sevov s poljubnimi lastnostmi, vendar pa bodo le-ti lahko prestopili meje laboratorijev šele ob ustrezni spremembi zakonodaje in javnega mnenja.

Ključne besede: živinoreja / prehrana živali / mikrobiologija / probiotiki / krma / rekombinantni probiotiki / zakonodaja / EU

PROBIOTICS IN ANIMAL NUTRITION

ABSTRACT

Probiotics are defined as living micro-organisms, that upon ingestion in certain numbers, exert health benefits to the host. Their use is linked to proven efficacy on the gastrointestinal microbial equilibrium as well as immunomodulation. The positive effect in animals exerts not only in an improved health status, especially in young animals, but also in improved animal performance, including growth rate and feed conversion efficiency. Microorganisms that are used in animal feeds in the EU are mainly Gram-positive bacteria belonging to genera *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus* and *Streptococcus* and yeasts, such as *Saccharomyces cerevisiae* or *Kluyveromyces* species. While most of the species are apparently safe, certain microorganisms may exert harmful properties for animals as well as humans. Enterococci, for example, might harbour transmissible antibiotic resistance determinants, which have the potential to spread in animal and human-associated pathogenic microbial populations. Recent developments in synthetic and systems biology, coupled with bioinformatics and novel tools for genetic engineering, will soon enable the construction of 'artificial' probiotic microorganisms with virtually any combination of properties. Whether and when these 'designer probiotics' will reach out of the labs depends on legislation as well as public opinion.

Key words: animal production / animal nutrition / microbiology / probiotics / feed / recombinant probiotics / legislation / EU

UVOD

Koncept probiotikov se je začel razvijati v zgodnjih letih 20. stoletja, ko je Nobelov nagrajenec Elie Metchnikoff predpostavil povezavo med dolgoživostjo bolgarskih kmetov in njihovim uživanjem velikih količin fermentiranih mlečnih izdelkov. Verjel je, da se z uživanjem ustreznih količin tovrstnih izdelkov v prebavila vnesejo mlečnokislinske bakterije, ki se tam razmnožijo in preprečijo rast škodljivim mikroorganizmom (Tannock, 2003). V 50. letih prejšnjega stoletja so pri USDA (United States Department of Agriculture) registrirali prvi probiotični izdelek, ki naj bi pomagal pri prašičji griži, kot posledici okužbe z *E. coli* (Orrhage in sod, 1994). Število probiotikov, namenjenih preventivi in lajšanju različnih bolezenskih stanj pri ljudeh in živalih, od tedaj strmo narašča. Medtem, ko se probiotični pripravki, namenjeni ljudem, uvrščajo med prehranske izdelke oziroma prehranska dopolnila (redkeje tudi zdravila), probiotični krmni dodatki veljajo za alternativne rastne pospeševalce. Njihov prodor na tržišče je v Evropi podvržen strogi regulativi z leta 2003 (EC 1831/2003). Kljub vsemu probiotiki zasedajo pomembno mesto med krmnimi dodatki, njihova vloga v živalski produkciji pa se je v Evropi še dodatno okrepila ob prepovedi uporabe krmnih antibiotikov, leta 2006 (Anadon, 2006a).

POMEN PROBIOTIKOV PRI VZDRŽEVANJU MIKROBNEGA RAVNOVESJA V PREBAVILIH ŽIVALI

"Probiotiki so živi mikroorganizmi, ki zaužiti v ustreznem številu ugodno vplivajo na zdravje gostitelja" se glasi definicija, ki sta jo leta 2001 družno predlagala FAO in WHO (Khan in Ansari, 2007; Maldonado Galdeano in sod., 2007). V splošnem velja, da probiotiki na fiziologijo gostitelja vplivajo s spreminjanjem oziroma vzpostavljanjem ugodnega mikrobnega ravnovesja v prebavnem traktu oziroma preko zmernega spodbujanja njegovega imunskega sistema. Ključna tarča delovanja probiotikov je prebavni trakt (GIT) gostitelja, še posebno predeli, ki so že naravno najgosteje poseljeni z mikrobi. Pri monogastričnih živalih in ljudeh so to različni deli črevesja, kjer poteka mikrobna razgradnja vlaknin in drugih snovi, ki jih gostitelj ni mogel razgraditi in absorbirati v prednjih delih prebavil. Poleg vloge pri prebavi in absorpciji hranil, predstavlja črevesna sluznica tudi največji telesni imunski organ in prvo obrambno linijo proti vdoru patogenov (Gilmore in Faretti, 2003). V nasprotju z monogastričnimi živalmi, pri prežvekovalcih večji del mikrobne aktivnosti poteka v delu prebavnega trakta pred želodcem. Avtohtona mikrobna združba v vampu, ki je sestavljena iz množice bakterij ter nekaterih gliv in praživali, igra ključno vlogo pri pridobivanju energije iz rastlinskih strukturnih polisaharidov, poleg tega pa predstavlja za gostitelja tudi vir beljakovin. Tako pri monogastričnih živalih kot pri prežvekovalcih lahko pride do porušenja ustreznega mikrobnega ravnovesja, kar se odraža na zdravju in proizvodnosti živali. Najpomembnejši razlogi za to so stres (ob brejosti, odstavitvi, transportu...), drastične spremembe v sestavi ali slaba kakovost krme ter neustrezna higiena krmljenja. Eden najpogostejših primerov porušenja mikrobne homeostaze zaradi neustrezne sestave krme je vampna acidoza. Do omenjenega stanja lahko pride, če prežvekovalec zaužije prevelik delež lahko razgradljivih ogljikovih hidratov (škroba, sladkorjev). Posledično pride v vampu do hitrega porasta skupine mikroorganizmov, ki fermentirajo omenjene substrate (predvsem nekaterih vrst iz rodov *Streptococcus*, *Lactobacillus, Butyrivibrio, Eubacterium, Selenomonas, Ruminobacter, Prevotella, Bifidobacterium, Succinimonas in Succinivibrio*) na račun zmanjšanja preostalih. Spremembe v fermentacijskih vzorcih vodijo do povečane akumulacije organskih kislin, predvsem laktata (ključno vlogo pri tem pripisujejo predvsem vrsti *S. bovis* ter homofermentativnim vrstam rodu *Lactobacillus*). Posledica je pretirano znižanje pH vampa ter popolno porušenje mikrobnega ravnotežja v vampu, kar pri gostitelju povzroči različne negativne fiziološke spremembe, ki vplivajo na zdravje in počutje živali (Nagaraja in Titgemeyer, 2007; Chaucheyras-Durand, 2008; Chiquette in sod., 2008). Stresni pogoji

najpogosteje vplivajo na povečano tveganje za infekcije s patogenimi mikroorganizmi. Ob stresu namreč pride poleg oslabitve imunskega sistema tudi do sprememb v izločanju prebavnih sokov ter peristaltiki črevesja (Berezina in Ovsyannikov, 2001), kar vodi v spremembe fizikalno-kemijskih parametrov, kot sta pH in redoks potencial, znotraj različnih delov prebavnega trakta. Spremenjeni pogoji favorizirajo drugačno sestavo mikrobne združbe, v kateri se lahko uveljavijo tudi patogeni.

SPLOŠNE LASTNOSTI PROBIOTIKOV

Probiotični sevi morajo biti za gostitelja varni, hkrati pa izkazovati tudi ustrezno mero učinkovitosti, torej merljivih učinkov na zdravje oziroma proizvodnost živali. Zahteva po varnosti vključuje odsotnost nevarnosti za sistemske infekcije, poleg tega pa mikroorganizmi oziroma njihovi metaboliti ne smejo kazati učinkov toksičnosti za gostitelja, ne smejo proizvajati toksinov ter drugih virulenčnih dejavnikov. Eden pomembnejših vidikov varnosti je v zadnjem času postala tudi odsotnost prenosljivih determinant antibiotične rezistence, torej zapisov za odpornost proti antibiotikom, ki se nahajajo na plazmidih, transpozonih ali drugih vrstah mobilnih genetskih elementov (Anadon in sod., 2006b; Becquet, 2003).

Da bi probiotiki lahko dosegli ustrezno aktivnost na tarčnem mestu morajo posedovati specifične fiziološke lastnosti, ki jim omogočajo preživetje ob prehodu skozi vse dele prebavil. Takšne lastnosti so na primer odpornost proti kislini (želodčni sok), žolčnim solem in proteazam, ter zmožnost metabolne aktivnosti v odsotnosti kisika. Poleg tega je pri probiotikih, namenjenih živalim, zelo pomembna sposobnost preživetja različnih (agresivnih) tehnoloških postopkov priprave krme (briketiranje, peletiranje, ekspandiranje) in ohranitev živosti tudi po daljšem času skladiščenja (Simon, 2005).

VRSTE PROBIOTIKOV

V splošnem lahko probiotične mikroorganizme glede na izvorni ekosistem razdelimo v dve skupini. Prvo skupino sestavljajo vrste, katerih naravno življenjsko okolje je prebavni trakt ljudi ali živali (predstavljajo del avtohtone ali indigene mikrobne populacije GIT). Takšne so na primer mnoge vrste mlečnokislinskih bakterij iz rodov *Lactobacillus* (*L. acidophilus, L. casei, L. gasseri, L. rhamnosus, L. farciminis*) in *Bifidobacterium* (*B. bifidum, B. thermophila, B. longum, B. breve, B. infantis*), pa tudi *Enterococcus (E. fecalis, E. faecium), Pediococcus (P. acidilactici) in Streptococcus (S. infantarius)*. Prednost te skupine probiotičnih mikroorganizmov je, da so že naravno prilagojeni na fizikalno-kemijske razmere, ki vladajo znotraj prebavnega trakta gostitelja, medtem ko njihovo uporabo v živalski prehrani omejuje nestabilnost med pripravo in skladiščenjem krme (Anadon in sod., 2006b; Simon, 2005). Pri rodu *Enterococcus* se pojavlja tudi težava pogoste prisotnosti mobilnih determinant antibiotične rezistence. Gre predvsem za transpozicijske elemente z zapisom za odpornost proti glikopeptidnemu antibiotiku vankomicinu, ki so vključeni v konjugativne plazmide (Garcia-Migura in sod., 2007).

V zadnjem času se kot probiotični kandidati preizkušajo tudi nekateri anaerobni izolati iz vampa, kot sta *Prevotella bryantii* 25A in *Pseudobutyrivibrio xylanivorans* Mz5. Medtem, ko prvi kaže potencial za preprečevanje vampne acidoze, so pri drugem opazili več lastnosti, ki bi lahko bile koristne tako za prežvekovalce, kot za monogastrične živali. Širšo uporabo omenjenih mikroorganizmov omejuje predvsem občutljivost na prisotnost kisika, vdor katerega je med postopki priprave, shranjevanja in uporabe probiotikov težko preprečiti (Chiquette in sod., 2008; Čepeljnik in sod., 2003; 2006).

V probiotičnih dodatkih živalski krmi zelo pogosto najdemo mikroorganizme, ki niso del avtohtone mikrobiote prebavnega trakta. V to skupino spadajo različne vrste rodu *Bacillus* (*B. subtilis, B. cereus, B. licheniformis, B. pumilus, B. clausii, B. coagulans*), dva tesna sorodnika omenjenega rodu, *Paenybacillus polymyxa* in *Brevibacillus laterosporus* ter nekatere kvasovke (*S. cerevisiae, Kluyveromyces* spp.). Najpogosteje uporabljeni probiotiki omenjene skupine so endospore gram-pozitivnih bakterij iz rodu *Bacillus*, ki se odlikujejo po izjemni odpornosti na zunanje pogoje, zaradi česar ohranjajo visoko viabilnost tako med procesi priprave krme, skladiščenjem in prehodom skozi različne dele prebavnega trakta. Neugodna lastnost nekaterih sevov *B. cereus* in *B. licheniformis* je zmožnost proizvodnje toksinov, zato je pred uporabo v živalski prehrani potrebno seve testirati na prisotnost omenjenih virulenčnih dejavnikov (Simon, 2005; Anadon in sod., 2006b; Hong in sod., 2005).

MEHANIZMI DELOVANJA PROBIOTIKOV

Mehanizmi delovanja probiotikov še niso najbolje preučeni, kar je predvsem posledica pomanjkanja neposrednih dokazov. Sklepanje o večini izmed njih namreč izhaja iz *"in vitro"* poskusov, kjer pogoji nikoli niso enaki tistim v kompleksnem naravnem ekosistemu, kot so prebavila. Kljub vsemu se je v znanstveni literaturi utrdilo mnenje, da sta za delovanje probiotikov ključna dva osnovna mehanizma: kompetitivno izločanje patogenov ter za gostitelja ugodno uravnavanje imunskega sistema (imunomodulacija).

Koncept kompetitivnega izločanja zaobjema širok spekter različnih potencialnih mehanizmov. Eden od splošnejših je spreminjanje fizioloških pogojev (pH, redoks potenciala), ki se odraža kot posledica spreminjanja ravnotežja produktov mikrobnega metabolizma. Ugodne učinke, ki so jih pri prežvekovalcih opazili ob dodajanju kvasovke *S. cerevisiae,* na primer, med drugim povezujejo s povečano porabo kisika v vampu, kot posledico respiratorne aktivnosti tega fakultativno anaerobnega mikroorganizma. Ustrezno nizek redoks potencial v tem ekosistemu je namreč poglavitnega pomena za mnoge celulolitične bakterije in druge striktno anaerobne predstavnike vampne mikrobne združbe (Fonty, 2006; Newbold, 1996). Poleg tega naj bi probiotične kvasovke preko preprečevanja akumulacije laktata uravnavale tudi pH vampa, zaradi česar se uporabljajo za preprečevanje vampne acidoze (Fonty, 2006; Marden in sod., 2008). Po drugi strani pa je prav izločanje kratkoverižnih karboksilnih kislin (SCCA) eden od mehanizmov, s katerimi mlečnokislinske bakterije inhibirajo patogene v prebavnem traktu monogastričnih živali (De Vuyst in Leroy, 2007).

Poleg nespecifičnega spreminjanja fizikalno-kemijskih pogojev med predpostavljene mehanizme spada tudi tekmovanje za hranila in vezavna mesta, običajno sluznične receptorje (Hong in sod., 2005; Tuohy in sod., 2003). Kompeticija za vezavna mesta pa ni edini način onemogočanja vezave patogenov, saj lahko probiotični mikroorganizmi vplivajo na ta proces tudi posredno, preko regulacije izražanja gostiteljevih genov. Tako so na primer dokazali, da probiotična seva *L. plantarum* 299v in *L. rhamnosus* GG *'in vitro'* kvantitativno inhibirata pritrjanje enteropatogene *E. coli* na epitelijske celice črevesja preko stimulacije izražanja genov z zapisi za mucina MUC-2 in MUC-3 (Mack in sod., 1999).

Eden najpomembnejših mehanizmov zaviranja patogenov s strani probiotičnih mikroorganizmov je proizvodnja protimikrobnih snovi. Protimikrobno delovanje mlečnokislinskih bakterij pripisujejo širokemu spektru bioaktivnih spojin, med katere sodijo poleg produktov primarnega metabolizma, kot so organske kisline, etanol in maščobne kisline, tudi vodikov peroksid, bakteriocini ter bakteriocinom podobne inhibitorne spojine (BLIS). Med slednjimi so pogoste peptidne molekule s protiglivnim delovanjem, nekateri sevi pa naj bi proizvajali celo protivirusne beljakovine (De Vuyst in Leroy, 2007; Saito, 2003; Tannock, 2006). Pri različnih vrstah rodu *Bacillus* so prav tako dokazali proizvodnjo različnih bakteriocinov, kot

tudi bakteriocinom podobnih inhibitornih spojin in antibiotikov. Nekateri sevi *B. subtilis* na primer proizvajajo toplotno stabilen in na razgradnjo s proteazami odporen antibiotik (aminocoumacin A), ki je aktiven proti vrstam *Staphylococcus aureus*, *Enterococcus faecium*, *Shigella flexneri*, *Campylobacter jejuni* in *Helicobacter pylori*, medtem ko probiotični sevi vrste *B. coagulans* proizvajajo za proteaze občutljive peptidne molekule, z aktivnostjo proti Gram-pozitivnim bakterijam (koagulin) (Hong in sod., 2005). Proizvodnjo bakteriocinov, ki inhibirajo nekatere potencialno patogene enterobakterije, so dokazali tudi pri vampnem probiotičnem kandidatu *Pseudobutyrivibrio xylanovorans* (Čepeljnik in sod., 2003).

Spodbujanje različnih komponent imunskega sistema oziroma imunomodulacija velja za pomemben, a zelo kompleksen mehanizem probiotičnega delovanja. Vloga probiotikov pri slednjem naj bi bila dvojna – po eni strani naj bi spodbujali nekatere obrambne mehanizme naravne in pridobljene imunosti, po drugi strani pa naj bi bili pomembni tudi pri zaviranju prekomernega vnetnega odziva, ki je pogosto povezan z različnimi alergijami in t.i. avtoimunskimi boleznimi (Chronova bolezen, diabetes tipa 1, multipla skleroza) (Maldonado Galdeano in sod., 2007; Rook in Brunet, 2005). Probiotični mikroorganizmi lahko vplivajo tako na lokalni, kot na sistemski imunski odziv. Eden od pomembnejših načinov delovanja na lokalni ravni je preko spodbujanja nastajanja celic, ki proizvajajo sluznična protitelesa, IgA. Nekatere probiotične bakterije delujejo kot adjuvansi, torej spodbujajo intenziteto sluzničnega in sistemskega imunskega odziva ob stiku s patogeni. Analiza citokinskih profilov je pokazala, da nekatere mlečnokislinske bakterije pomembno spodbujajo proizvodnjo tumor nekrotizirajočega faktorja α (TNF-α), interferona γ (IFN-γ) in regulatornega citokina interlevkina-10 (IL-10) (Maldonado Galdeano in sod., 2007).

Preostali mehanizmi, ki lahko pripomorejo k ugodnim učinkom probiotikov vključujejo predvsem aktivnost različnih mikrobnih encimov, ki se odraža na različnih nivojih. Slednji, predvsem pri rastlinojedih živalih, pomembno prispevajo k učinkovitejši razgradnji in izkoriščanju krme. Pri tem so ključnega pomena predvsem celulaze, ksilanaze in nekateri drugi fibrolitični encimi, ki jih običajno posedujejo vampni mikrobi (Čepeljnik in sod., 2003; Liu in sod., 2005). Drugi mehanizem, ki vključuje aktivnost mikrobnih encimov je pretvarjanje genotoksičnih spojin v nereaktivne produkte (detoksikacija), posledica česar je zmanjšano tveganje za, na primer, rakava obolenja prebavil. Poleg biokonverzije, predlagani mehanizmi zmanjševanja genotoksičnosti ksenobiotikov predpostavljajo še neke vrste imobilizacijo preko vezave na strukturne komponente mikrobnih celic (peptidoglikan, polisaharide) ter reagiranje z bakterijskimi metaboliti (Cenci in sod., 2008; Cenci in sod., 2005; Orrhage in sod., 2002). Učinki zmanjšanja genotoksičnosti različnih mutagenov so opisani tako pri nekaterih predstavnikih mlečnokislinskih bakterij kot tudi pri nekaterih probiotičnih sevih iz rodu *Bacillus* (Cenci in sod., 2008). Protirakavi učinki probiotičnih sevov pa niso povezani le z razgradnjo ali nevtralizacijo kancerogenih spojin, temveč tudi s proizvodnjo nekaterih zaščitnih molekul, kot sta butirat in konjugirana linolna kislina (CLA) (Barcenilla in sod., 2000; Kritchevsky in sod., 2000).

Eden od zanimivejših mehanizmov, povezanih z encimatskim delovanjem mikroorganizmov je tudi razgradnja toksinov ali receptorjev, na katere se ti vežejo. Tako na primer ugodne učinke kvasovke *Saccharomyces boulardii* ob zdravljenju okužb s patogenom *Clostridium difficile* povezujejo z aktivno proteolitično razgradnjo molekul toksina A ter receptorjev za ta toksin na celicah črevesnega epitela (Castagliuolo in sod., 1996).

UČINKI PROBIOTIKOV

V splošnem velja, da so učinki specifični za sev probiotičnega mikroorganizma, odvisni pa so tudi od vrste, starosti in zdravstvenega oziroma imunskega stanja gostitelja (Casey in sod., 2007). Prav specifičnost pogojev, ki vplivajo na delovanje probiotičnih pripravkov je

najverjetneje tudi razlog za neenotnost rezultatov, pridobljenih v različnih študijah. Slednje najpogosteje poročajo o pozitivnih učinkih aplikacije probiotikov takoj po rojstvu živali, po antibiotičnem zdravljenju, ob okužbah z enteričnimi patogeni ter v stresnih obdobjih. Splošne učinki, ki naj bi jih imeli probiotiki na pitovne živali so: povečana odpornost na infekcijske bolezni ter posledično manjša pogostost poginov, hitrejša rast, ugodnejša konverzija krme, izboljšana prebava in absorpcija hranil, večja produktivnost ter izboljšana kakovost mleka in jajc. Študije o učinkih probiotikov na živalih so najpogosteje osredotočene na prašiče, prežvekovalce in perutnino, zanimivi pa so tudi izsledki uporabe probiotičnih pripravkov v akvakulturi. V nadaljevanju so povzete ugotovitve nekaterih novejših raziskav, ki vključujejo te kategorije.

Prašiči

Driske, ki jih povzročajo patogene bakterije, predstavljajo pomemben vzrok smrti pri odstavljenih pujskih. Probiotiki so se v več študijah pokazali kot nekoliko manj učinkovita, a kljub vsemu potencialno uporabna alternativa nutritivni antibiotični profilaksi ter dobrodošla pomoč pri zdravljenju. Casey in sod. (2007) so na primer pokazali, da 30-dnevno uživanje mešanice petih sevov mlečnokislinskih bakterij (dva seva vrste *L. murinus*, *L. salivarius* subsp. *salivarius*, *L. pentosus*, *P. pentosaceus*) bistveno izboljša izid okužbe z bakterijo *Salmonella enterica* (serovar Typhimurium), enemu ključnih povzročiteljev drisk pri prašičih. Podobna študija (Schroeder in sod., 2006) poroča tudi o učinkovitosti probiotičnega seva *E. coli* Nissle 1917 pri preprečevanju in lajšanju simptomov driske, kot posledice okužbe z enterotoksigenimi sevi *E. coli* (ETEC). Zaščitna vloga probiotikov pri okužbi z ETEC naj bi bila povezana z različnimi možnimi mehanizmi, od vpliva na adhezijo patogena, ohranjanje integritete tesnih stikov med celicami epitela, povečano izločanje mucina in nekatere druge (Lalles in sod., 2007). O zmanjšani pojavnosti drisk po odstavitvi poročajo tudi ob uporabi t.i. neavtohtonih probiotičnih sevov *Enterococcus faecium* NCIMB 10415 in *Bacillus cereus* var. Toyi. Študija, ki so jo naredili Siggers in sod. (2008) kaže na možnost učinkovitega preprečevanja atrofije sluznice in lajšanju simptomov nekrotizirajočega enterokolitisa (močnega vnetja črevesne sluznice) pri prezgodaj rojenih pujskih, če so ti krmljeni s probiotično mešanico mlečnokislinskih bakterij rodov *Bifidobacterium* in *Lactobacillus*.

Prežvekovalci

Mnoge študije kažejo pozitivne učinke kvasovke *S. cerevisiea* na produktivnost prežvekovalcev. Med slednjimi so najpomembnejši: (1) ugoden vpliv na 'dozorevanje' vampne mikrobne združbe pri teletih, (2) stabilizacija vampnega pH ter (3) povečanje razgradnje vlaknin (Chaucheyras-Durand, 2008).

Galvao in sod.(2005) na primer poročajo o ugodnem učinku *S. cerevisiae* na hitrost rasti in zdravje takoj ob rojstvu odstavljenih telet, ki so jih krmili z žiti. O stabilizaciji vampnega pH ter s tem povezanim učinkom preprečevanja pojava vampne acidoze ob dodajanju živih kvasovk visoko koncentrirani krmi poročajo tako pri ovcah (Chaucheyras-Durand in Fonty, 2006), kot pri kravah (Williams in sod, 1991; Bach in sod., 2007). Učinek povezujejo z različnimi možnimi mehanizmi, od interakcij kvasovke z bakterijami, ki metabolizirajo laktat, do kompeticije pri fermentaciji sladkorjev in spodbujanju amilolitičnih praživali (Chaucheyras-Durand, 2008).

Zelvyte in sod. (2006) poročajo o izboljšani prebavljivosti krme in večji mlečnosti, če so krave 58 dni uživale probiotični izdelek LEVUCELL SC (vsebuje *S. cerevisiae*). Slednje povezujejo s povečanjem števila celulolitičnih mikroorganizmov in izboljšano fermentacijo sladkorjev. Poleg kvasovk preizkušajo tudi morebitne ugodne učinke nekaterih bakterij. Poskus, v katerem so kravam molznicam (2 tedna pred telitvijo in 5 tednov po njej) v vamp dodajali

pripravek z bakterijskim probiotičnim kandidatom *Prevotella bryantii* 25A, je pokazal povečano proizvodnjo hlapnih maščobnih kislin v vampu ter večjo vsebnost maščob v mleku (Chiquette in sod., 2008). Karitas in sod. (2006) poročajo o večji mlečnosti in izboljšani kakovosti mleka ter zmanjšanem poginu jagenjčkov zaradi driske ob krmljenju ovc s probiotičnim pripravkom BioPlus 2B (vsebuje spore bakterij *B. licheniformis* in *B. subtilis*).

Preglednica 1. Izvor in učinki nekaterih probiotikov na izbrane vrste vodnih živali (prirejeno po Brunt in Austin, 2008; Kesarcodi-Watson, 2007)
Table 1. Sources and effects of selected probiotics on aquatic host species (modified from Brunt and Austen, 2008; Kesarcodi-Watson, 2007)

Probiotični mikroorganizem	Izvor probiotičnega mikroorganizma	Tarčni gostitelj	Učinek probiotičnega mikroorganizma na gostitelja
Aeromonas hydrophila, Vibrio fluvialis, Carnobacterium spp., *Micrococcus luteus*	Prebavila *O. mykiss*	Šarenka (*Oncorhyncus mykiss*)	Omejevanje furunkuloze (infekcijske bolezni, ki jo povzroča bakterija *Aeromonas salmonicida*).
Aeromonas media (A199)	Neznan	Ostrige (*Crassostrea gigas*)	Zmanjšana smrtnost ob okužbi z *Vibrio tubiashii.*
Carnobacterium spp.	Prebavila *S. salar* L.	Atlantski losos (*Salmo salar* L.)	Ob sočasni inokulaciji zmanjšanje obsega bolezni, ki jih povzročajo *A. salmoncida*, *V.ordalii* in *Y.ruckeri.*
Enterococcus faecium (SF68)	Neznan	Jegulja (*Angulla anguilla*)	Omejevanje edvardsieloze (infekcijske bolezni, ki jo povzroča bakterija. *Edwardsiella tarda*)
Lactobacillus fructivorans (AS17B)[1], *Lactobacillus plantarum* (906)[2]	[1] Prebavila *S. auratu*, [2] človeški feces	Zlata orada (*Sparus auratu*)	Zmanjšana smrtnost pri larvah starih 39–66 dni.
Lactobacillus spp. in *Carnobacterium* spp.	*B. plicatilis*	Larve romba (*Scophthalmus rombus*)	Zmanjšana smrtnost ob infekciji z *Vibrio* spp.
Pseudomonas fluorescens	*L. niloticus*	Šarenka (*Oncorhyncus mykiss*)	Zmanjšana smrtnost ob infekciji z *Vibrio angullarum.*
Roseobacter spp.	Ribogojnica z larvami *S. rombus*	Romb (*Scophthalmus rombus*)	"*In vitro*" antagonizem proti *V. angullarum.*
Vibrio alginolyticus	Komercialna gojilnica morskih rakov	Atlantski losos (*Salmo salar* L.)	Učinkovito omejevanje bolezni, ki jih povzročajo *A.salmonicida*, *V. anguillarum* in *V.ordalii,*

Perutnina

Zmanjšana smrtnost zaradi okužb z enteričnimi patogeni, izboljšana konverzija krme in hitrejša rast so najpogosteje opisani učinki dodajanja probiotikov v krmo perutnine. Vicente in sod. (2007) so vse omenjene učinke opazili pri brojlerjih ob uporabi probiotičnega pripravka na

osnovi več sevov rodu *Lactobacillus*. Do podobnih rezultatov so ob uporabi tekočega probiotičnega pripravka s sedmimi, iz piščančjega prebavnega trakta izoliranimi sevi rodu *Lactobacillus*, prišli tudi Timmerman in sod. (2006). Nekatere študije s probiotičnimi pripravki, ki vsebujejo endospore nekaterih sevov *B. subtilis*, *B. cereus*, *B. licheniformis* prav tako kažejo na pozitivne učinke v smislu izboljšane konverzije krme oziroma preprečevanja kolonizacije in perzistence nekaterih enteričnih patogenov (Hong in sod., 2005; Midilli in sod., 2008).

Vodni organizmi

Uporaba probiotikov se je uveljavila tudi na področju gojenja vodnih živali (predvsem rib, rakov in školjk), kjer infekcijske bolezni predstavljajo poglavitni vzrok finančnih izgub. Probiotični pripravki, ki se uporabljajo v akvakulturi poleg mlečnokislinskih bakterij in endospor rodu *Bacillus*, vsebujejo tudi bakterije iz rodov *Pseudomonas*, *Vibrio*, *Aeromonas*, *Carnobacterium* in nekatere druge (Brunt in Austin, 2008; Kesarcodi-Watson, 2008). Preglednica 1 prikazuje izvor in učinke nekaterih probiotičnih mikroorganizmov na gostitelje.

PROBIOTIČNI KRMNI DODATKI NA TRŽIŠČU

Registracija probiotičnih proizvodov za uporabo v živalski prehrani je v Evropi podvržena strogi Regulativi (EC) No. 1831/2003, ki ureja celotno področje krmnih dodatkov (avtorizacijo, uporabo, označevanje in pakiranje). Vsi odobreni krmni dodatki morajo v splošnem zadoščati trem pogojem: a) ne smejo biti škodljivi za zdravje živali in ljudi ter okolju, (b) ne smejo biti predstavljeni zavajujoče za kupca in (c) ne smejo škodljivo vplivati na značilne lastnosti živalskih proizvodov ali zavajati kupca glede vpliva na omenjene lastnosti. Registracija probiotičnih krmnih pripravkov v Evropi je pogojena z natančnim testiranjem varnosti in učinkovitosti le teh, kar pa za proizvajalce predstavlja precejšnje finančno breme (Anadon, 2006a; Anadon in sod., 2006b). Na tržišču so se zato, do pred kratkim, poleg registriranih, vedno znova pojavljali tudi nepreverjeni proizvodi brez ustreznih dovoljenj. Da bi izboljšala prepoznavnost svojih proizvodov in pripomogla k hitrejšemu izključevanju pripravkov, ki ne ustrezajo evropskim standardom, se je večina pomembnejših evropskih podjetij za proizvodnjo in distribucijo probiotičnih krmnih pripravkov že leta 1999 povezala v t.i. Evropsko združenje za probiotike (EPA). V letu 2001 je EPA na trgu prepoznala 21 registriranih probiotičnih proizvodov, namenjenih živalim (Ziggers, 2008). Nekatere med njimi prikazuje Preglednica 2.

PROBIOTIKI PRIHODNOSTI?

Razvoj tehnologije genskega inženirstva je prinesel na področje probiotikov nove možnosti za izboljšave na različnih ravneh. Kot učinkovitejša alternativa prilagajanja probiotičnih sevov na razmere med proizvodnjo in prehodom prebavil s predhodno izpostavitvijo blagim oblikam stresa (nespecifična indukcija genov tolerančnega odziva), se je pojavila možnost kloniranja genov odpornosti oz. tolerance na različne oblike stresa, iz drugih mikroorganizmov (Sleator in Hill, 2008). Tako so na primer s kloniranjem gena *betL*, ki kodira transporter za kompatibilni topljenec betain pri bakteriji *Listeria monocytogenes*, dosegli povečanje tolerance probiotičnega seva *L. salivarius* UCC118 na soli ter preživetje pri nizkih temperaturah (Sheehan in sod., 2006). Kloniranje istega gena v sev *B. breve* UCC2003, pa se je odrazilo v obliki izboljšane odpornosti proti želodčnemu soku (Sheehan in sod., 2007). Učinek povečane odpornosti na želodčno kislino so dosegli tudi s prenosom genov za sintezo trehaloze (kompatibilni topljenec *E. coli*) *ostAB* v probiotični sev *L. lactis,* poleg tega pa je omenjeni rekombinantni sev kazal tudi boljšo preživljivost ob izpostavitvi žolčnim solem in pogojem med postopkom liofilizacije (Termont in sod., 2006).

Preglednica 2. Nekateri v Evropi registrirani probiotični pripravki in skupine živali, za katere je bil dokazan njihov učinek in varnost (prirejeno po Hong in sod., 2005 ; EFSA, 2004a; 2004b; 2006a; 2005a; 2005b; 2006b; 2007a;2007b; 2007c; 2008a; 2008b; 2008 c)

Table 2. Selected commercial probiotic products and groups of animals for which beneficial effects and safety of these products were shown (modified from Hong *et al*., 2005; EFSA, 2004a; 2004b; 2006a; 2005a; 2005b; 2006b; 2007a; 2007b; 2007c; 2008a; 2008b; 2008 c)

Blagovna znamka	Probiotični mikroorganizem	Tarčni gostitelji
Toyocerin®	Endospore *B. cereus* var Toyoi (NCIMB 40112/CNCM I -1012)	Govedo (pitanci, teleta), kokoši (nesnice, brojlerji), purani, kunci, prašiči (pitanci, svinje)
Bioplus 2B®	Endospore *B. licheniformis* (DSM5749) in *B. subtilis* (DSM5750)	Prašiči (pujski po odstavitvi, svinje, pitanci), kokoši (brojlerji), purani, govedo (teleta)
Bactocell®/ Fermaid®	*P. acidilactici* (CNCM MA 18/5 M)	Kokoši (brojlerji), prašiči (pitanci)
Biosaf® SC 47	*S. cerevisiae* (NCYC Sc47)	Govedo (pitanci, krave molznice), kunci, prašiči (pujski po odstavitvi, svinje), konji, ovce (jagenjčki, molznice), koze (molznice)
Biosprint®	*S. cerevisiae* (BCCM/MUCL39885)	Prašiči (pitanci), govedo (pitanci, krave molznice)
Levucell® SC 20/ SC 10ME	*S. cerevisiae* (CNCM I-1077)	Govedo (pitanci, krave molznice), koze, ovce (molznice, jagenjčki)
Yea Sacc®	*S. cerevisiae* (CBS 493.94)	Govedo (pitanci, krave molznice, teleta), konji
Oralin®	*E. faecium* (DSM 10663 / NCIMB 10415)	Govedo (teleta), prašiči (pujski po odstavitvi), perutnina (brojlerji, purani), psi
Bonvital®	*E. faecium* (DSM 7134)	Prašiči (pujski po odstavitvi, pitanci, svinje), perutnina (brojlerji)
Fecinor®	*E. faecium* (CECT 4515)	Prašiči (pujski po odstavitvi), govedo (teleta), perutnina (brojlerji)
Lactiferm®	*E. faecium* (NCIMB 11181 (M74))	Govedo (teleta), prašiči (pujski po odstavitvi), perutnina (brojlerji)
Biacton®	*L. farciminis* (CNCM MA 67/4R)	Prašiči (pujski po odstavitvi), purani, kokoši (brojlerji, nesnice)

Poleg novih možnosti za izboljšanje tolerance na stresne razmere, so tehnologije genskega inženirstva skupaj z vse boljšim poznavanjem mehanizmov delovanja patogenov omogočile tudi pripravo sevov, ki specifično prepoznavajo patogene oziroma njihove toksine. T.i. strategija interference vezave na receptorje temeljijo na molekulskem posnemanju gostiteljskih tarč za patogene (toksine), ki jih izražajo probiotični sevi. Omenjene pristope so že uspešno preizkusili za lajšanje nekaterih enteričnih okužb. Eden takšnih konstruktov, rekombinantni sev *E. coli*, ki je na površini izražal himerno molekulo lipopolisaharida (LPS) z receptorjem za šiga-toksin (Stx), je izjemno učinkovito nevtraliziral omenjeni toksin (1 mg rekombinantne bakterije je vezal več kot 100 μg molekul Stx1 in Stx2) (Paton in sod., 2000). Podobno so pripravili tudi rekombinantni probiotični sev *E. coli* CWG308, ki nevtralizira toksin Stx2e, ključni virulenčni

dejavnik povzročitelja edemske bolezni (pomemben vzrok smrti pri odstavljenih pujskih) ter probiotična seva s potencialom za nevralizacijo temperaturno občutljivega toksina (LT) enterotoksigene *E. coli* (ETEC) in toksina kolere (Ctx) (Paton in sod., 2006). Možnost uporabe probiotikov pa se ne kaže le v terapiji, temveč tudi v preprečevanju nekaterih bolezni. Probiotiki s potencialom za usmerjeno spodbujanje imunskega sistema predstavljajo obetavno alternativo klasičnim cepivom (Sleator in Hill, 2008).

Nenavsezadnje tehnologija genskega inženirstva omogoča tudi pripravo probiotičnih sevov z izboljšanimi ali novimi lastnostmi, ki gostitelju koristijo v smislu izboljšanja razgradnje in izkoriščanja krme. Liu in sod. (2005) so tako v probiotični sev *L. reuteri* klonirali gene za razgradnjo vlaknin treh različnih vampnih mikroorganizmov (zapis za ksilanazo glive *Neocallimastix patriciarum*, zapis za beta-glukanazo bakterije *Fibrobacter succinogenes* in zapis za celulazo glive *Pyromyces rhizinflata*). Novi sevi so tako pridobili sposobnost razgradnje karboksimetil celuloze, beta-glukana ali ksilana, poleg tega pa so se še vedno učinkovito pritrjali na sluznico ter obdržali odpornost proti žolčnim solem in kislini. Rekombinantni sevi tako lahko preživijo in aktivno razgrajujejo vlaknine tudi v prebavilih monogastričnih živali.

Hitro naraščajoče znanje o funkcijah genskih lokusov ter razvoj novih orodij na področju genskega inženirstva obeta v končni fazi skoraj brezmejne možnosti konstrukcije probiotičnih sevov s poljubnimi kombinacijami želenih lastnosti (Lartigue, 2007), vendar pa bo prestop slednjih iz laboratorijev v prakso možen le ob ustrezni zakonodaji, podprti s pozitivnim strokovnim in javnim mnenjem.

SUMMARY

Probiotics are defined as living micro-organisms that upon ingestion in certain numbers, exert health benefits to the host. Their use is linked to proven efficacy on the gastrointestinal microbial equilibrium as well as immunomodulation, even though the exact mechanisms of action are not yet fully elucidated. The positive effect in animals exerts not only in an improved health status, especially in young animals, but also in improved animal performance, including growth rate and feed conversion efficiency. Microorganisms that are used in animal feeds in the EU are mainly Gram-positive bacteria belonging to genera *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus* and *Streptococcus* and yeasts, such as *Saccharomyces cerevisiae* or *Kluyveromyces* species. While most of the species are apparently safe, certain microorganisms may exert harmful properties for animals as well as humans. Enterococci, for example, might harbour transmissible antibiotic resistance determinants, which have the potential to spread in animal and human-associated pathogenic microbial populations. The advantage of spore-forming bacteria is their natural resistance to harsh environmental conditions, which allows them to survive feed manufacturing processes as well as low pH and bile salts inside animal gastrointestinal tract. *S. cerevisiae* is most frequently used probiotic species used in ruminant nutrition, as it helps to establish primary microbial community in rumen of calves, prevent rumen acidosis by stabilizing rumen pH and improve fiber digestion by stimulating the growth and activity of cellulolytic bacteria. Probiotics are also widely used in aquaculture. Besides lactic acid bacteria and spore-forming *Bacilli*, some other genera, including *Aeromonas, Vibrio, Edwardsiella* are also in use, usually in order to prevent infective disease in fish, shellfish and shrimps.

The rules for the authorisation, use, monitoring, labeling and packaging of probiotic feed additives are set by new Regulation (EC) No. 1831/2003 of the European Parliament and of the Council of September 2003 on additives for use in animal nutrition. Recent developments in synthetic and systems biology, coupled with bioinformatics and novel tools for genetic engineering, will soon enable the construction of 'artificial' probiotic microorganisms with

virtually any combination of properties. Whether and when these 'designer probiotics' will reach out of the labs depends on legislation as well as public opinion.

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NEKATERE PREHRANSKE MOŽNOSTI ZA PREPREČITEV ŠKODLJIVEGA VPLIVA FUZARIJSKIH TOKSINOV (T-2 IN DON) NA PROIZVODNE LASTNOSTI IN LIPIDNO PEROKSIDACIJO PRI PIŠČANCIH

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IZVLEČEK

Mikotoksini, sekundarni metaboliti gliv, so strupeni tako za ljudi, kot tudi za živali. Prisotnost fuzarijskih toksinov (T-2 toksina in deoksinivalenola (DON-a)) v krmi, predvsem v višjih koncentracijah, vpliva na zmanjšanje zauživanja krme, posledično pa tudi na prirast živali in zmanjšano težo živali, kar lahko povzroča večje ekonomske izgube. Novejše raziskave so pokazale, da toksini v krmi vplivajo tudi na imunsko odpornost organizma in povzročajo oksidacijski stres oz. povečano lipidno peroksidacijo v organizmu. Namen predstavljenih raziskav je bil ugotoviti vpliv stopnjevane koncentracije T-2 toksina v krmi (od 0,5 do 13,5 mg/kg) in koncentracije 10 mg/kg T-2 toksina in DON-a na proizvodne lastnosti in oksidacijski stres, pri rastočih piščancih, ter ugotoviti potencialni prehranski vpliv mikotoksinskega vezalca in nukleotidov na zmanjšanje negativnih vplivov fuzarijskih toksinov. Rezultati so pokazali, da koncentraciji 10 in 13,5 mg/kg T-2 toksina vplivata na zmanjšanje zauživanja krme in slabše priraste. Deset in 13,5 mg/kg T-2 toksina in 10 mg/kg DON-a v krmi povzroči poškodbe DNA v levkocitih, merjene s kometnim testom. Rezultati dokazujejo, da lahko z dodajanjem mikotoksinskega vezalca v krmo vsaj delno preprečimo absorpcijo mikotoksinov iz črevesja in tako zmanjšamo njihov negativni vpliv na oksidacijski stres (primerjava med skupinama z in brez dodatka s 13,5 mg T-2 toksina/kg krme), medtem ko z dodajanjem nukleotidov lahko vplivamo na mehanizme popravljanja poškodb DNA imunskih celic, ki so jih povzročili fuzarijski toksini.

Ključne besede: perutnina / pitovni piščanci / prehrana živali / krma / mikotoksini / fuzarijski toksini / lipidna peroksidacija / proizvodne lastnosti

SOME NUTRITIONAL STARTEGIES IN PREVENTION OF DETRIMENTAL INFLUENCE OF *Fusarium* TOXINS (T-2 AND DON) ON PRODUCTION PARAMETERS AND LIPID PEROXIDATION IN CHICKENS

ABSTRACT

Mycotoxins, a group of secondary fungal metabolites, are toxic for people and animals. Presence of *Fusarium* toxins (T-2 toxin and deoxinivalenol (DON)) in feed, especially in higher concentrations, reduces feed consumption and consequently live weight gain, which can cause higher economic losses. Recent studies showed that toxins in feed influence the action of the immune system and cause oxidative stress. The objective of the present studies was to: (i) establish the dose dependant effect of T-2 toxin (from 0.5 to 13.5 mg/kg) on production parameters and oxidative stress in broiler chickens, (ii) test the effect of commercial mycotoxin

binder at the highest used concentration of T-2 toxin (13.5 mg/kg), and (iii) test the protective effect of nucleotides at high T-2 toxin and DON intoxication (10 mg/kg). Results showed that concentrations of 10 and 13.5 mg/kg of T-2 toxin reduced the feed consumption and live weight gain. T-2 toxin at 10 and 13.5 mg/kg and DON at 10 mg/kg caused DNA damage in leucocytes measured by comet assay. Results proved that supplementation with mycotoxin binder can partly reduce the absorption of mycotoxins from intestine and thus decrease their negative influence on oxidative stress. The crucial role of nucleotide supplementation in feed is to repair DNA damage in immune cells, which are highly sensitive to mycotoxin action.

Key words: poultry / broiler chickens / animal nutrition / feed / mycotoxins / *Fusarium* toxins / lipid peroxidation / production parameters

UVOD

V zadnjih desetletjih intenzivnega razvoja živinoreje namenjamo vse večjo pozornost higienski kakovosti krme in živilom živalskega izvora. Okužbam z mikotoksini se praktično ne moremo izogniti in v svetu predstavljajo zelo velik problem (Wood, 1992; Eriksen in Pettersson, 2004). Mikotoksini, sekundarni metaboliti gliv, so strupeni tako za ljudi, kot tudi za živali. Do danes je poznanih že več kot 300 mikotoksinov, njihovo število pa še kar naprej narašča. Po ocenah strokovnjakov naj bi bilo z mikotoksini okuženega 25 % svetovnega pridelka žit (Fink-Gremmels, 1999). Iz toksikološkega in ekonomskega vidika so za onesnaženje krme najpomembnejši rodovi gliv *Aspergillus, Fusarium* in *Penicillinum*. V evropskih podnebnih razmerah najpogosteje prihaja do onesnaženja krme, ki ga povzročajo glive iz rodu *Fusarium* in sicer z mikotoksini iz skupine trihotecenov (deoksinivalenol, nivalenol, T-2 toksin) in zearalenonom (ZON) (Dänicke, 2001). S toksikološkega in ekonomskega vidika najpomembnejši mikotoksini so prikazani v pregl. 1.

Preglednica 1. Najpogostejši rodovi gliv in toksini, ki kontaminirajo krmo (Dänicke, 2001)
Table 1. Frequent mycotoxin producing fungi and their mycotoxins (Dänicke, 2001)

Rod	Mikotoksini
Fusarium-species *(F. gramineum, F. culmorum, F. avenaceum, F. poae, F. sporotrichioides, F. moniliforme)*	– Zearalenon – Trihoteceni (T-2 toksin, HT-2 toksin, deoksinivalenol (DON), nivalenol…) – Moniliformin – Fumonizini B_1, B_2, B_3
Aspergilus flavus, A. parasiticus	Aflatoksini, posebej Aflatoksin B_1
A. alutaceus, Penicillium verrucosum	Ohratoksini, posebej Ohratoksin A
P.citrinum, P. verrucosum	Citrinin
Claviceps purpurea	Ergot-Alkaloidi
Alternaria alternata	Tenuazonična kislina

Mikotoksine v krmi je v praktičnih razmerah zelo težko odkriti in nadzorovati. Koncentracije mikotoksinov v krmi so običajno dovolj nizke, tako da ne povzročajo kliničnih znakov zastrupitev pri živalih, vseeno pa zmanjšujejo učinkovitost prireje, povečujejo možnost pojava kužnih bolezni (Surai, 2002) in s tem povzročajo veliko gospodarsko in ekonomsko škodo.

Prisotnost fuzarijskih toksinov (T-2 toksina in DON-a) v krmi je škodljiva tako za perutnino kot tudi za prašiče, predvsem v višjih koncentracijah, kar so pokazale tudi naše raziskave (Frankič in sod., 2006, Frankič in sod., 2008). Značilni učinki prisotnosti trihotecenov v krmi perutnine so izguba apetita in odklanjanje krme, rane na koži in sluznicah, slabše operjanje,

poškodbe jeter in ledvic, živčne motnje, pešanje imunskega sistema, nekroze v prebavnem traktu, nižja relativna masa nekaterih organov (želodca, F. burse…), anemija in pogin (Lesson in sod., 1995; Raju in Devegowda, 2000; Garcia in sod., 2003). Opazne so tudi spremembe na kljunu in manjša nesnost (Veldman, 2004).

Pri delovanju mikotoksinov prihaja v celicah do številnih biokemičnih sprememb. Trihoteceni so najbolj toksični za hitro deleče se celice imunskega sistema, jeter in črevesja. Na celični ravni stimulirajo nastajanje prostih radikalov in posledično povzročajo poškodbe na celičnih membranah in DNA. Poleg tega, da toksini inhibitorno vplivajo na sintezo beljakovin v telesu, nekateri znanstveniki trdijo, da je zelo pomemben mehanizem delovanja tudi reakcija lipidne peroksidacije (Surai, 2002), pri kateri se sproščajo prosti radikali in nastajajo toksični produkti npr. malondialdehid (MDA). Ravnotežje med prooksidanti in antioksidanti v celotnem telesu in še posebej v celici, je pomembno za regulacijo številnih metabolnih poti, ki vzdržujejo imunski sistem, nadzirajo rast in razvoj ter ščitijo pred stresnimi dejavniki, značilnimi za sodobno komercialno rejo perutnine (Surai in Dvorska, 2005).

Zaradi vseh naštetih težav in posledic, ki jih povzročajo mikotoksini, je izredno pomembno zagotavljanje krme z majhno vsebnostjo mikotoksinov. Najboljša rešitev, v primeru za živali onesnažene krme z mikotoksini, so učinkoviti prehranski dodatki, ki detoksicirajo krmo, med pasažo skozi prebavni trakt (Pasteiner, 1997) in tako preprečijo ali vsaj omilijo njihovo škodljivo delovanje. Ti prehranski dodatki delujejo večinoma po principu absorpcije toksinov na druge molekule, nekateri pa vsebujejo tudi encime, ki cepijo kemijske vezi v molekulah toksinov do neškodljivih produktov (Dänicke, 2001).

Mikotoksinski vezalec, ki smo ga uporabili v raziskavi, je vseboval vezalce (sintetične polimere-polivinilpolipirolidone) in encime. Na hidratizirane polimere se vežejo mikotoksini, ki imajo polarne funkcionalne skupine. Mikotoksinski vezalec deluje kot adsorbent, ki selektivno veže in imobilizira mikotoksine v prebavnem traktu živali (vezava se začne v ustih in se nadaljuje v želodcu in črevesju). Dodatno encimi razgrajujejo epoksidni obroč fuzarijskih mikotoksinov in jih tako deaktivirajo. Nukleotidi so pogojno esencialno hranilo, ki lahko postane esencialno v primeru bolezenskih stanj, ki zahtevajo večjo sintezo nukleinskih kislin in beljakovin za pospešeno rast in obnovo nekaterih tkiv: sluznice prebavnega trakta (Holen in Jansson, 2004), celic imunskega sistema (Cameron in sod., 2001) ter jetrnega in možganskega tkiva (Perez in sod., 2004).

Namen predstavljenih raziskav je bil ugotoviti vpliv stopnjevane koncentracije T-2 toksina in koncentracije 10 mg/kg T-2 toksina in deoksinivalenola (DON) na oksidacijski stres, poškodbe DNA imunskih celic in poškodbe jeter pri rastočih piščancih (Ross 308) ter ugotoviti potencialni prehranski vpliv mikotoksinskega vezalca in nukleotidov na zmanjšanje negativnih posledic, ki jih povzročajo toksini.

MATERIAL IN METODE DELA

Dvaindvajset oz. dvajset dni stare komercialne pitovne linijske križance (Ross 308) smo uhlevili v individualne kletke. Krmo in vodo so imele živali na voljo. Krmljene so bile s krmno mešanico, ki je temeljila na koruzi in sojinih tropinah (pregl. 2). Krma je bila sestavljena glede na potrebe živali v starosti od 3 do 6 tednov (NRC, 1994). Za izvajanje poskusa smo pridobili dovoljenje Veterinarske uprave Republike Slovenije.

V prvem poskusu smo na začetku poskusa živali razdelili v 6 skupin (10 živali na skupino), ki smo jih krmili z osnovno krmo z različnimi koncentracijami T-2 toksina: Kont (0,0 mg T-2), T-0,5 (0,5 mg T-2/kg krme), T-1,5 (1,5 mg T-2/kg krme), T-4,5 (4,5 mg T-2/kg krme), T-13,5 (13,5 mg T-2/kg krme) in T-13,5+ (13,5 mg T-2/kg krme + 1,5 g/kg krme mikotoksinskega vezalca). V drugem poskusu smo živali razdelili v 5 poskusnih skupin (10 živali na skupino), ki

smo jih krmili z osnovno krmo in dodatkom T-2 toksina in DON-a, glede na skupino: Kont (osnovna krma brez dodatkov), DON (10 mg DON/kg krme, DON+ (10 mg DON/kg krme + 2 g nukleotidov/kg krme, T-2 (10 mg T-2 toksina/kg krme, T-2+ (10 mg T-2 toksina/kg krme + 2 g nukleotidov/kg krme).

Kot vir T-2 toksina in DON-a smo uporabili kontaminirano pšenico avstrijskega proizvajalca Biopure, ki je vsebovala 0,49 % (w/w) T-2 toksina in 1,04 % DON-a (w/w) na kg pšenice. Mikotoksinski vezalec in nukleotide, smo uporabili z namenom deaktivacije mikotoksinov v kontaminirani krmi. Dodatka sta bila uporabljena v koncentracijah priporočenih s strani proizvajalca.

Preglednica 2. Sestava osnovne krme
Table 2. Composition of basic feed mixture

Količina krmila	%	Količina krmila	%
Koruza	61,10	Apnenec	1,24
Koruzni gluten	6,00	Mono kalcije fosfat	1,59
Sojine tropine	24,03	L-lizin-HCl 78.8 %	0,19
Sončnično olje	4,90	DL-metionin 98 %	0,09
Sol	0,36	Premiks	0,50

Spremljali smo vpliv T-2 toksina in DON-a na proizvodne lastnosti (telesno maso, dnevne priraste in zauživanje krme) in oksidacijski stres. Vpliv na proizvodne lastnosti smo ugotavljali 6., 11. dan poskusa ter ob zakolu (17. dan). Ob koncu poskusa smo živali žrtvovali, odvzeli smo jim vzorce krvi.

Za ugotavljanje oksidacijskega stresa smo določili vsebnost MDA v krvni plazmi, skupni antioksidativni status krvne plazme (TAS) in glutation peroksidazno aktivnost v eritrocitih (GPx). Poškodbe DNA, ki so lahko posledica oksidacijskega stresa ali pa direktnega učinka toksinov, smo merili v levkocitih krvi s kometnim testom.

Za določitev MDA v krvni plazmi smo v osnovi uporabili metodo, kot jo navajajo Wong in sod. (1987), z modifikacijami po Chirico (1994), Fukunaga in sod. (1995). Skupno antioksidativno kapaciteto (TAS) plazme smo izmerili z železo/metmioglobin absorpcijsko Randox metodo (Randox, Crumlin, UK). Aktivnost glutation peroksidaze (GPx) v eritrocitih smo določili s pomočjo testih kitov Randox (Randox, Crumlin, UK).

Postopek kometnega testa, ki smo ga uporabili, z manjšimi modifikacijami (več slojev agaroze, daljši čas elektroforeze, manjša koncentracija etidijevega bromida) v glavnem sledi postopku po Singh-u in sod. (1988). Izolirane levkocite smo vključili v agarozne gele. Pri pripravi vzorcev smo upoštevali negativno kontrolo (kontrolna skupina) in pozitivno kontrolo (minigele smo pred alkalno celično lizo potopili v raztopino 500 μM H_2O_2). Sledila je alkalna celična liza, elektroforeza, nevtralizacija in barvanje z etidijevim bromidom (Et-Br), ter ponovno spiranje.

Podatki so bili statistično obdelani s programskim paketom SAS/STAT. Uporabili smo proceduro GLM (General Linear Model) (SAS 8e, 2000; SAS Inc., Cary, NC, USA). Statistično značilno razliko smo ocenili pri $p < 0.05$.

REZULTATI IN RAZPRAVA

Rezultati so pokazali, da koncentracija 10 in 13,5 mg/kg T-2 toksina vpliva na zmanjšanje zauživanja krme in slabše priraste (pregl. 3).

Preglednica 3. Telesna masa, zauživanje krme in prirast pri 1) piščancih, krmljenih z različnimi koncentracijami T-2 toksina in mikotoksinskega vezalca ter 2) pri piščancih krmljenih z dodatkom T-2 toksina, DON-a in nukleotidov

Table 3. Body mass, feed consumption, and live weight gain of 1) chickens exposed to different concentrations of T-2 toxin and mycotoxin binder and 2) chickens fed T-2 toxin, DON and nucleotides

	Telesna masa začetek poskusa, g	Telesna masa konec poskusa, g	Zauživanje krme, g/dan	Prirast, g/dan
1) Piščanci krmljeni z dodatkom T-2 toksina in mikotoksinskega vezalca				
Kont	760,6	2207^{a}	145,9^{a}	88,3^{a}
T 0,5	721,7	2181^{a}	146,4^{a}	88,7^{a}
T 1,5	766,2	2160^{a}	141,0^{a}	86,2^{ac}
T 4,5	752,4	2025^{a}	129,9^{b}	77,6^{c}
T 13,5	752,4	1589^{b}	100,9^{c}	51,0^{b}
T 13,5+	764,0	1482^{b}	99,1^{c}	44,5^{b}
p	0,6282	< 0,0001	< 0,0001	< 0,0001
2) Piščanci krmljeni z dodatkom T-2 toksina, DON-a in nukleotidov				
Kont	746,8	1655^{a}	108,3^{a}	63,6^{a}
DON	739,8	1528^{a}	96,8^{a}	52,6^{a}
DON+	747,8	1510^{a}	98,3^{a}	53,4^{a}
T-2	745,4	1158^{b}	70,7^{b}	33,5^{b}
T-2+	748,9	1242^{b}	71,9^{b}	34,1^{b}
p	0,814	< 0,0001	< 0,0001	< 0,0001

$^{a, b, c}$ LS- vrednosti, ki so označene z različnimi črkami, se znotraj poskusa statistično značilno razlikujejo; $p < 0,05$.

Številne raziskave potrjujejo naše ugotovitve vpliva T-2 toksina na proizvodne lastnosti. Smith in sod. (2000) so ugotovili, da sta značilna simptoma pri živalih, ki zauživajo krmo kontaminirano s toksini iz skupine trihotecenov, zmanjšana konzumacija in slabša rast. Tudi Hoehler in Marquardt (1996) sta v raziskavi na piščancih prišla do podobnih ugotovitev, da je dodatek T-2 toksina v krmo (v koncentracijah 4 in 5 mg/kg krme), v primerjavi s kontrolno skupino, zmanjšal zauživanje krme in negativno vplival na telesno maso živali. DON ima prav tako škodljive vplive na proizvodne lastnosti, vendar, kot kažejo nekatere raziskave, le pri koncentracijah višjih od 12,6 mg/kg krme (Yegani in sod., 2006). Kubena in sod. (1988) so ugotovili, da so se teža živali in prirasti pri piščancih zmanjšali, če je bilo v krmi 16 mg/kg DON-a, medtem ko so v raziskavi Kubena in sod. (1997), ugotovili, da 15 mg DON-a/kg pri piščancih skoraj ni imelo vpliva na telesno maso živali. Tudi naša raziskava kaže, da 10 mg DON-a/kg krme ne zmanjša telesne mase živali in tudi ne zauživanja krme v primerjavi s kontrolno skupino.

Mikotoksinski vezalec uporabljen v naši raziskavi ni izboljšal proizvodnih lastnosti pri piščancih izpostavljenih 13,5 mg T-2 toksina na kilogram krme. Najverjetneje je količina vezalca, uporabljena v poskusu po priporočilih proizvajalca, premajhna, da bi lahko preprečila negativen učinke pri zelo kontaminirani krmi. Dodatek istega mikotoksinskega vezalca je v raziskavi Dänicke in sod. (2003) še dodatno poslabšal priraste in konverzijo krme pri piščancih, ki so zauživali krmo okuženo z DON-om.

Številni avtorji trdijo, da T-2 toksin in DON stimulirata nastanek prostih radikalov in s tem lipidno peroksidacijo (Karppanen in sod., 1989; Rizzo in sod., 1994; Leal in sod., 1999; Vila in

sod., 2002), vendar pa si, kot ugotavlja Surai (2002), ugotovitve raziskav mnogokrat nasprotujejo. V raziskavi Hoehler in Marquardt (1996) ugotavljata, da dodatek T-2 toksina v krmo piščancev (v koncentracijah 4 mg/kg in 5 mg/kg), tako kot v naši raziskavi, ni stimuliral lipidne peroksidacije in ni povečal produkcije MDA. Ugotovila pa sta, da je dodatek toksina v krmo znižal koncentracije vitamina E v jetrih, kar kaže, da bi T-2 toksin vendarle lahko imel prooksidativne učinke, ki pa se niso pokazali z merjenjem koncentracij MDA. Nasprotno pa sta Dvorska in Surai (2001) ugotovila, da je dodatek T-2 toksina v krmo prepelic stimuliral lipidno peroksidacijo in povzročil povečano akumulacijo MDA v jetrih. Koncentracija MDA v krvni plazmi se v našem poskusu z naraščajočo koncentracijo T-2 toksina ni statistično značilno spremenila glede na poskusno skupino. Tudi v drugem našem poskusu dodatek T-2 toksina in DON-a nista statistično značilno povečala koncentracije MDA v krvni plazmi. Naši rezultati ne potrjujejo nekaterih že objavljenih raziskav. Raziskava Mezes in sod. (1999) je pokazala, da dodajanje T-2 toksina poveča koncentracijo MDA in sicer različno glede na živalsko vrsto in tkiva.

Preglednica 4. Delež DNA v repu kometa, Repni moment po Olivu (OTM), koncentracija malondialdehida (MDA) v krvni plazmi, skupna antioksidativna kapaciteta (TAS) in glutation peroksidaza (GPx) pri 1) piščancih, krmljenih z različnimi koncentracijami T-2 toksina in mikotoksinskega vezalca ter 2) pri piščancih krmljenih z dodatkom T-2 toksina, DON-a in nukleotidov

Table 4. % DNA in the tail of the comet, Olive tail moment (OTM), concentration of malondialdehyde (MDA) in blood plasma, total antoxidative status (TAS) and glutathione peroxidase (GPx) of 1) chickens exposed to different concentrations of T-2 toxin and mycotoxin binder and 2) chickens fed T-2 toxin, DON and nucleotides

	% DNA v repu	OTM*	MDA v plazmi, nmol/ml	TAS, mmol/l	GPx, U/l
1) Piščanci krmljeni z dodatkom T-2 toksina in mikotoksinskega vezalca					
Kont	$14,33^{ab}$	$3,17^{ac}$	0,49	$0,87^{ab}$	10 162
T 0,5	$10,90^{b}$	$2,56^{a}$	0,44	$1,01^{a}$	10 062
T 1,5	$16,75^{a}$	$3,86^{ac}$	0,42	$0,75^{b}$	9 896
T 4,5	$18,64^{ac}$	$4,98^{c}$	0,41	$0,94^{ab}$	8 678
T 13,5	$22,97^{c}$	$7,86^{b}$	0,40	$1,07^{ab}$	9 375
T 13,5+	$17,57^{a}$	$4,32^{ac}$	0,50	$0,99^{ab}$	10 202
p	< 0,0001	< 0,0001	0,3146	0,0256	0,7343
2) Piščanci krmljeni z dodatkom T-2 toksina, DON-a in nukleotidov					
Kont	$15,09^{a}$	$4,03^{a}$	$0,28^{ab}$	$0,64^{a}$	$18\,170^{ab}$
DON	$18,98^{b}$	$5,59^{a}$	$0,23^{a}$	$0,60^{ab}$	$20\,093^{a}$
DON+	$15,96^{ab}$	$4,27^{a}$	$0,26^{ab}$	$0,60^{ab}$	$17\,220^{ab}$
T-2	$25,22^{c}$	$9,08^{b}$	$0,32^{b}$	$0,53^{b}$	$16\,364^{ab}$
T-2+	$19,70^{b}$	$6,17^{a}$	$0,28^{ab}$	$0,54^{b}$	$15\,172^{b}$
p	< 0,0001	< 0,0001	0,0369	0,0063	0,0177

* OTM = Olive tail Moment (Olive, 1992)

[a, b, c] LS- vrednosti, ki so označene z različnimi črkami, se znotraj poskusa statistično značilno razlikujejo; $p < 0,05$.

Med živalskimi vrstami, ki so jih vključili v raziskavo, so bile najbolj občutljive gosi, sledile so race in piščanci. Če pogledamo tkiva, so najobčutljivejša jetra, sledijo krvna plazma in rdeče krvne celice. Da T-2 toksin najbolj poškoduje jetra, potrjujeta tudi raziskavi Hoehler in Marquardt (1996) ter Dvorska in Surai (2001).

Do sedaj genotoksičnost T-2 toksina in DON-a še ni bila podrobno preučena in ne vemo natančno, s katerimi mehanizmi trihoteceni poškodujejo DNA. Lahko povzročijo direktne prelome DNA ali pa delujejo preko različnih epigenetskih mehanizmov. V naših raziskavah smo ugotovili, da 10 in 13,5 mg/kg T-2 toksina in 10 mg/kg DON-a v krmi povzroči poškodbe DNA v levkocitih (pregl. 4), merjene s kometnim testom in predstavljene kot % DNA v repu kometa in kot izračunani parameter Repni moment po Olivu (OTM) (Olive, 1992). Rezultati naše raziskave glede poškodb DNA so podprti z raziskavo Atroshi in sod. (1997), v kateri so ugotovili povečane poškodbe DNA v jetrih miši, ki so bile krmljene z dodatkom T-2 tokisna in z raziskavo Rizzo in sod. (1998) v kateri so preučevali genotoksični vpliv T-2 toksina in DON-a na jetrne celice podgan.

Zmanjšana skupna antioksidativna kapaciteta (TAS), v naši drugi raziskavi, v skupini, ki je bila krmljena s T-2 toksinom, namiguje na povečan oksidacijski stres povzročen s T-2 toksinom, vendar rezultat ni podprt z rezultati glutation peroksidaze (GPx). Na drugi strani DON v krmi živali ni vplival na koncentracije TAS in GPx (pregl. 4). Nekatere študije dokazujejo, da je toksičnost mikotoksinov povezana z različnimi mehanizmi v organizmu (Surai and Dvorska, 2005), posledica česar so tudi različni vplivi in produkti. Do sedaj še ni bilo natančno raziskano, ali mikotoksini stimulirajo lipidno peroksidacijo direktno, s povečano produkcijo prostih radikalov, ali pa je povečana občutljivost tkiv na lipidno peroksidacijo povezana z antioksidativnim sistemom v organizmu.

Lipidno peroksidacijo, ki jo povzročajo toksini v krmi, lahko ublažimo z dodajanjem različnih prehranskih dodatkov. Imunski sistem piščancev, ki so izpostavljeni fuzarijskim toksinom, je pogosto oslabljen, kar pomeni, da so lahko bolj dovzetni za bakterijske in virusne infekcije (Dänicke, 2001). Naši raziskavi sta pokazali, da oba dodatka preprečita oz. odpravita poškodbe DNA imunskih celic. V naših poskusih dodatek mikotoksinskega vezalca krmi, ki je vsebovala 13,5 mg T-2 toksina/kg ter nukleotidov krmi, ki je vsebovala 10 mg T-2 toksina/kg ali DON-a nista imela vpliva na proizvodne lastnosti (pregl. 3 in 4). Tudi Dänicke in sod. (2003) so v raziskavi ugotovili, da dodatek vezalca v krmo piščancev, ki je vsebovala do 14 mg deoksinivalenola (DON), ni pokazal pričakovanih pozitivnih učinkov. Proizvodne lastnosti so se namreč, kljub uporabi mikotoksinskega vezalca, bistveno poslabšale. Tudi Karlovsky (1999) trdi, da pri omenjenem mikotoksinskem vezalcu ni odkril encimatske aktivnosti, ki naj bi bila ključna za detoksifikacijo toksinov iz skupine trihotecenov. V nasprotju z naštetimi raziskavami so naši rezultati pokazali, da dodatek mikotoksinskega vezalca krmi, ki je vsebovala 13,5 mg T-2 toksina/kg, zmanjša poškodbe DNA, prav tako smo ugotovili tudi pozitiven učinek dodatka nukleotidov, ki so zmanjšali poškodbe DNA pri živalih, ki so zauživale krmo z 10 mg/kg T-2 toksina oziroma DON-a. Iz rezultatov naših raziskav lahko zaključimo, da z dodajanjem mikotoksinskega vezalca v krmo lahko vsaj delno preprečimo absorpcijo mikotoksinov iz črevesja in tako zmanjšamo njihov negativni vpliv na imunski sistem, medtem ko z dodajanjem nukleotidov vplivamo na mehanizme popravljanja poškodb DNA imunskih celic, ki so jih povzročili toksini.

SKLEPI

T-2 toksin v krmi, pri koncentracijah nad 4,5 g/kg, značilno zmanjša dnevne priraste piščancev, medtem ko DON pri koncentraciji 10 mg/kg ni vplival na proizvodne rezultate. Mikotoksinski vezalec ni izboljšal proizvodnih lastnosti piščancev krmljenih s 13,5 mg T-2

toksina na kilogram krme. Prav tako dodatek nukleotidov ni ugodno vplival na priraste in konverzijo krme pri piščancih krmljenih z 10 mg/kg DON-a ali T-2 toksina. Visoke dodane koncentracije T-2 toksina in DON-a krmi povzročijo poškodbe DNA v levkocitih merjene s kometnim testom. Oba uporabljena dodatka lahko zmanjšata oz. odpravita poškodbe DNA imunskih celic. Z dodajanjem mikotoksinskega vezalca v krmo lahko vsaj delno preprečimo absorpcijo mikotoksinov iz črevesja in tako zmanjšamo negativni vpliv toksinov, medtem ko z dodajanjem nukleotidov vplivamo na mehanizme popravljanja poškodb DNA imunskih celic, ki so jih povzročili toksini. Oba dodatka bi bila lahko primerna za izboljšanje delovanja imunskega sistema, v primeru prisotnosti fuzarijskih mikotoksinov v krmi, vendar bodo za potrditev potrebne še nadaljnje raziskave.

VIRI

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SPREMEMBE SESTAVE KRMNIH OBROKOV ZA GOVEJE PITANCE: PRIMER UPORABE NORMATIVNIH IN POZITIVNIH MATEMATIČNIH METOD

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IZVLEČEK

Namen prispevka je prikazati možnost kombiniranja različnih matematičnih metod za analiziranje sestave krmnih obrokov v danih okoliščinah. Z matematičnimi modeli, ki temeljijo na omejeni optimizaciji, smo proučevali cenovno-stroškovna razmerja v obdobju 1998 do 2008 in iskali morebitne spremembe v sestavi racionalnih krmnih obrokov za goveje pitance. Za analizo smo uporabili normativne in pozitivne matematične metode. S pomočjo klasičnega linearnega programa, nadgrajenega s tehtanim ciljnim programom, smo izvedli normativno analizo. Da bi ugotovili, kako bi se v danih razmerah odločal povprečen rejec, pa smo simulacijo izvedli tudi s pozitivnim matematičnim programiranjem. Dobljeni rezultati kažejo, da se je sestava racionalnega krmnega obroka v zadnjih desetih letih nagnila v prid koruzne silaže, izrazito pa se je zmanjšala količina travne silaže v obroku. Zaradi naravnih danosti v Sloveniji take spremembe niso izvedljive, zato bomo morali več pozornosti posvetiti zniževanju stroškov pridelave travne silaže.

Ključne besede: govedo / biki / pitanci / prehrana živali / krmni obroki / sestava / matematične metode / linearno programiranje / tehtano ciljno programiranje

CHANGES OF BEEF RATION COMPOSITION: AN EXAMPLE OF UTILIZING NORMATIVE AND POSITIVE MATHEMATICAL METHODS

ABSTRACT

The aim of this paper is to present possibility to combine different mathematical methods for analysis of ration composition changes in actual economic environment. On the basis of mathematical programming models, based on constraint optimization, the influence of price-cost ratios on the trends of efficient beef ration formulation in the period 1998 to 2008 has been analysed. For investigation positive and normative mathematical methods have been utilized. The normative part of methods applies a common linear programming approach supported by penalty function. To find out the "reaction" of rational farmer within given circumstances, simulation was upgraded with positive mathematical programming approach. Obtained results illustrate change in ration composition by increased maize silage quantities and significantly lower amounts of grass silage during last decade. Due to Slovene natural conditions it is obvious that such a dramatic shift is impossible, therefore more attention should be paid to reduction of grass silage production costs.

Key words: cattle / beef fattening / animal nutrition / ration / composition / mathematical methods / linear programming / weighted goal programming

UVOD

Spremembe na političnem in ekonomskem področju so v zadnjem času prispevale k izrazito poslabšani stabilnosti ekonomike pitanja govedi. K temu je botrovalo več t.i. 'notranjih', kot tudi 'zunanjih' dejavnikov. V prvi vrsti lahko 'krivca' iščemo v reformi skupne kmetijke politike (SKP). Korenitejši preobrat je nakazala že MacSharrijeva reforma v letu 1992, ki odpravlja dobršen del tržno izkrivljajočih ukrepov (intervencijske cene) in daje poudarek neposrednim plačilom. Agenda 2000 z nadaljnjim zniževanjem cen in dvigom neposrednih plačil v duhu predhodne reforme je ta zasuk še stopnjevala. Za obravnavan sektor je prinesla z dohodkovnega vidika zanimive klavne premije in z njimi omilila vse slabšo ekonomsko situacijo, ki jo prinaša liberalizacija trga z govejim mesom. Zadnja, t.i. Fischlerjeva reforma, v Sloveniji vpeljana v letu 2007, pa je uvedla do tedaj še ne poznan pojem proizvodne nevezanosti plačil. Kljub vsemu je zaradi občutljivosti sektorja del plačil ostal proizvodno vezan (60 % posebne premije, premija za ekstenzivno rejo ženskih živali). Takšna politika naj bi s poudarjeno liberalizacijo kmetijskih trgov vodila do večje tržne orientiranosti kmetijstva. Slednja se vsaj zaenkrat odraža v bistveno poslabšani ekonomski situaciji pitanja govedi, saj odkupne cene ne sledijo trendu rasti stroškov. Rešitve najbrž bolj kot na prihodkovni lahko iščemo na stroškovni strani pitanja, saj so tu možnosti vplivanja rejcev številnejše.

V opazovani panogi imamo dve ključni stroškovni postavki, nakup teleta in nakup ali pridelava krme. Dokaj pogost primer v Sloveniji je, da rejci kupujejo teleta. Nakupna cena slednjih je po podatkih Kmetijskega inštituta Slovenije (KIS) v zadnjih štirih letih zopet v izrazitem porastu. Prav tako pa so se stroški krmnega obroka v zadnjih letih izrazito povečali (KIS, 2008). Na podlagi rezultatov modelnih kalkulacij KIS smo ocenili, da se stroški krmnega obroka pri pitanju govedi gibljejo na ravni okrog 55 % celotnih stroškov oziroma nekaj manj kot 70 % spremenljivih stroškov reje. Deloma so povišani stroški krme posledica večjega povpraševanja po žitih, ki ji pridelava ne sledi, bodisi zaradi suše in naravnih ujm, ki so v zadnjih letih prizadele strateška območja za pridelavo žit. Po žetvi 2008 pa se lahko opaženi trendi zopet obrnejo, saj se je situacija tako na kmetijskih kot tudi na ostalih trgih spremenila zaradi dobre letine žit in splošne zaostritve gospodarskih razmer zaradi porajajoče recesije nekaterih ključnih nacionalnih gospodarstev v svetovnem merilu.

Naša hipoteza je, da se z zviševanjem cen krme, kot posledice dodatnega povpraševanja (bio-energija, bio-masa), manjše proizvodnje in liberalizacije trgov, spreminja tudi (racionalna) sestava krmnega obroka. Pričakujemo, da so visoke cene žit botrovale višjim oportunitetnim stroškom predvsem energijske krme, kar naj bi imelo za posledico spremembo v sestavi krmnega obroka. Poleg tega vse višji vhodni stroški (npr. mineralna gnojila, gorivo) močno zvišujejo lastno ceno doma pridelane krme, kar v ospredje postavlja ekonomijo obsega (pri večjem obsegu proizvodnje se stroški na enoto zmanjšujejo). Ker konzervirana voluminozna krma pri večini rejcev v Sloveniji predstavlja poglaviten vir hranljivih snovi v krmnem obroku, se zastavlja vprašanje, ali bo izrazitemu porastu cen v preteklih letih sledil tudi preobrat v tehnologiji pitanja. Pričakujemo, da bodo rejci govejih pitancev za doseganje ekonomsko privlačnega rezultata, poleg upoštevanja ekonomije obsega, prisiljeni poiskati tudi nove tehnološke rešitve.

V tem prispevku bomo analizirali, kako naj bi se v preteklem desetletnem obdobju (1998 – 2008) sestava krmnega obroka spreminjala glede na spremembe stroškovno-cenovnih razmerij kupljene in doma pridelane krme. Analize bomo opravili s pomočjo metod matematičnega programiranja, ki temeljijo na principu omejene optimizacije. Tovrstne metode so v zadnjem času postale pomembno orodje za najrazličnejše analize v kmetijstvu in ekonomiki (Buysse in sod., 2007). Gre namreč za pristop, ki zelo dobro združi t.i. neoklasično produkcijsko teorijo z modeliranjem. Omejene vire želimo na čim boljši in čim bolj učinkovit način uporabiti in na ta način optimirati dohodek kmetijskega gospodarstva. Temeljna zamisel je, da bomo z minimiranjem stroškov krme vsako leto dobili nekoliko spremenjen krmni obrok, seveda v

odvisnosti od gibanja tržnih cen ter lastnih cen uporabljene krme. Iz sprememb v sestavi krmnih obrokov bomo nato poizkušali potegnili zaključke, kako vse dražja krma vpliva na prilagajanje tehnologije pitanja. Za analizo bomo uporabili tri vrste matematičnih modelov. Z vidika ekonomske teorije jih lahko uvrstimo v skupino normativnih in pozitivnih modelov.

Normativne analize smo se lotili s pomočjo klasičnega linearnega programa (LP). Gre za zelo pogost pristop, ki se na področju agrarne ekonomike uporablja že več kot 50 let. Njegovo uporabo zasledimo pri reševanju najrazličnejših prehranskih problemov, tako na področju humane prehrane, kot pri načrtovanju in vodenju prehrane vseh vrst domačih živali (Darmon in sod., 2002). Če se osredotočimo na področje prehrane domačih živali, lahko ugotovimo, da je najpogosteje LP uporabljen za iskanje najcenejšega obroka. Prvi je ta pristop uporabil Waugh (1951). Kljub temu, da je modeliranje z linearnim programom pogosto ostro kritizirano zaradi svoje normativne narave, pa Jones (1982) podarja, da so modeli tega tipa izrazito dobrodošli v primeru, ko gre za analiziranje odločitev v razmerah, ki so izven obsega preteklih izkušenj in zato razmere ne morejo biti modelirane z bolj pozitivnimi metodami, kot so denimo ekonometrični modeli. Zato bomo v naši analizi uporabili metodo LP, da bi ugotovili, ali bi kmetje v danih ekonomskih razmerah lahko posegali tudi po drugi krmi na trgu, da bi si pocenili stroške pitanja in si na ta način povečali konkurenčnost pitanja.

Drugo, še vedno normativno, analizo smo izvedli s pomočjo orodja, ki temelji na metodi tehtanega ciljnega programiranja (WGP). Številni strokovnjaki (Rehman in Romero 1984, 1987; Lara 1993) namreč opozarjajo na pomanjkljivosti klasičnega LP za načrtovanje krmnih obrokov. Izpostavljajo predvsem matematično togost z vidika namenske funkcije (naenkrat upoštevamo le en cilj), kot tudi z vidika 'fiksnih' omejitev. Metodo WGP sta prva uporabila Charnes in Cooper (1961, cit po Rehman in Romero, 1984). Gre za posebno obliko matematičnega programiranja, ki temelji na LP oziroma je posebna oblika le-tega (Zadnik Stirn, 2001). V primerjavi s klasičnim LP, pri katerem lahko naenkrat optimiramo le en cilj, ostale zahteve pa zajamemo v omejitvah, lahko s ciljnim programiranjem iščemo rešitev, ki zadosti večjemu številu zastavljenih ciljev. Prednost te metode je tudi, da dovoljuje odstopanje od zastavljenih ciljev (omejitev), ki pa naj bi bilo čim manjše. Vickner in Hoag (1998) ugotavljata, da je v orodjih za podporo pri odločanju (DSS) uporaba WGP zaradi omenjenih prednosti pogostejša od LP.

Za simuliranje odzivov na zunanje (eksogene) spremembe, se v literaturi najpogosteje uporabljajo metode pozitivnega matematičnega programiranja (PMP). Metoda je bila razvita z namenom, da zaobide normativne značilnosti klasičnih optimizacijskih modelov, ki temeljijo na metodah matematičnega programiranja (Buysse in sod., 2007). V večji meri se PMP uporablja v sektorskih modelih (Howitt, 2005). V primerjavi z normativnim matematičnim programiranjem (NMP), se pri PMP modelih namenska funkcija prilagodi tako, da model skoraj povsem natančno ponovi referenčno situacijo. To pomeni, da lahko uporabimo informacijo o dejanskih (opazovanih) odločitvah rejca in na tej podlagi simuliramo, kako bi se le-ta odločal v spremenjenih okoliščinah glede na svoje želje in omejitve.

Glavna ideja PMP je torej kalibriranje modela, kjer s pomočjo kalibracijskih omejitev pridemo do dualnih spremenljivk (senčnih cen kalibracijskih omejitev), ki jih nadalje vključimo v nelinearno ciljno funkcijo (Heckelei, 2002). Ta dva postopka sta bistvena za odpravo pomanjkljivosti NMP t.i. 'nezveznega obnašanja' in problema ponovitve referenčne situacije. Seveda takšen model ne more biti uporabljen za iskanje boljše – optimalnejše – rešitve za kmeta, saj PMP predpostavlja, da je njegova 'referenčna' situacija optimalna (Buysse in sod., 2007). Ta pristop torej omogoča, da s spremembo cenovno-stroškovnih koeficientov namenske funkcije simuliramo, kako bi se kmet v danih ekonomskih pogojih odzival. Seveda je ključna predpostavka, da se njegove preference in občutljivost na dražljaje iz okolja v opazovanem obdobju ne spreminjajo.

MATERIAL IN METODE

Linearni model

Analizo vpliva cen krme na sestavljanje krmnih obrokov smo z vidika ekonomske teorije izvedli s pomočjo normativnih in pozitivnih metod. Za potrebe normativne analize smo uporabili že razvito orodje (DSS) za sestavljanje krmnih obrokov bikov pitancev, ki je podrobneje opisano v prispevku Žgajnar in Kavčič (2008). Orodje je zasnovano na t.i. dvostopenjski optimizaciji, ki se izvede s pomočjo dveh pod-modelov. Ta temeljita na metodah matematičnega programiranja in sicer omejene optimizacije. Prvi pod-model je klasičen linearni model in je primer modela, ki temelji na minimiranju stroškov krmnega obroka. Orodje ga vključuje, da lahko čim bolje oceni 'raven' pričakovanih stroškov krme, ki se v drugem pod-modelu vključujejo kot eden izmed ciljev. Drugi pod-model pa temelji na pristopu WGP, ki z vidika prehrane pripelje do – po hranljivih snoveh – bolj uravnoteženega krmnega obroka.

Za namen te analize smo orodje za sestavljanje krmnih obrokov ustrezno prilagodili. Da bi čim popolneje zajeli celotno stroškovno plat, smo namesto spremenljivih stroškov uporabili lastne cene doma pridelane krme in tržne cene za kupljeno krmo. Dodatno smo izvedli tudi post-optimalno analizo pri pod-modelu LP, s pomočjo katere smo prišli do senčnih cen (angl. shadow price) posameznih omejitev. Osredotočili smo se predvsem na potrebe po energiji, beljakovinah in surovi vlaknini. S pomočjo analize občutljivosti smo izračunali meje lastnih cen travne in koruzne silaže ter tržnih cen sojinih tropin in koruznega zrnja, znotraj katerih bi ostala dobljena rešitev nespremenjena.

Simulacijski PMP model

Posebnost kmetijske pridelave je, da je z vidika prilagodljivosti na zunanje dražljaje na kratek rok relativno toga. Z drugimi besedami to pomeni, da od rejcev ne moremo pričakovati, da bodo krmni obrok med leti zaradi spremenjenih cenovnih razmerij močno spreminjali, pač pa ga bodo prilagajali le v manjši meri. Torej gre za neko 'pričakovano' obnašanje rejcev, ki temelji na njihovih osebnih lastnostih (preferencah) in omejitvah, ki jih denimo klasičen LP model ne zajame. Zaradi tega smo v našo analizo vključili tudi preprost simulacijski model, ki je umerjen (skalibriran) po pristopu standardne PMP metode.

Slednjo je uvedel Howitt (1995) in pri simuliranju uporablja tri korake. V prvem koraku je uporabljen klasičen LP model, s pomočjo katerega dobimo senčne cene klasičnih omejitev (λ_1) in senčne cene dodatnih kalibracijskih omejitev (λ_2). V drugem koraku uporabimo senčne cene kalibracijskih omejitev (λ_2) in s pomočjo teorije povprečnih stroškov izpeljemo parametre (α in β) kalibracijske stroškovne funkcije. V zadnjem (tretjem) koraku s pomočjo referenčnih podatkov in izpeljanih stroškovnih parametrov (α in β) definiramo kvadratno stroškovno-ciljno funkcijo. Tako pripravljen model omogoča avtomatsko kalibracijo modela na referenčno situacijo (Howitt, 2005).

Prehranski PMP model, ki smo ga po Howittu (1995) razvili za simuliranje odziva kmetov pri sestavljanju krmnih obrokov na eksogene cenovne spremembe, v matematični obliki zapišemo, kot je prikazano v enačbah [1] do [9].

Korak 1:

$MaxZ = -c_i x_i$ tako, da je [1]

$A_{ij} x_i \le b_j \quad [\lambda_1]$ [2]

$x_i \le x_i^0 (1+\varepsilon) \quad [\lambda_2]$ [3]

$x_i \ge 0$ [4]

Korak 2:

$$\alpha_i = c_i \qquad [5]$$

$$\beta_i = \frac{2\lambda_{2i}}{x_i^o} \qquad [6]$$

Korak 3:

$$MaxZ = -(\alpha + 0{,}5\beta x_i)x_i \text{ tako, da je} \qquad [7]$$

$$A_{ij}x_i \le b_j \qquad [\lambda_1] \qquad [8]$$

$$x_i \ge 0 \qquad [9]$$

Namenska funkcija, definirana z enačbo [1], predstavlja vsoto zmnožkov tržnih cen ($-c_i$) ter polnih lastnih cen ($-c_i$) *i*-te krme s količino izbrane *i*-te krme v sestavljenem krmnem obroku. Ker smo pri cenah upoštevali negativen predznak, je posledično namenska funkcija predmet maksimiranja. Druga enačba predstavlja prehranske normative, katerim mora biti zadoščeno, da model najde rešitev. S pomočjo dualnega programa lahko dobimo senčne cene (λ_1) posameznih omejujočih omejitev. V primerjavi s klasičnim linearnim programom smo naš primarni LP model razširili s t.i. kalibracijskimi omejitvami [3]. Z njimi model 'prisilimo', da raven izbrane *i*-te krme (x_i) ne preseže referenčne količine *i*-te krme, kateri je prišteta zelo majhna vrednost, t.i. perturbacija (ε). Slednja je vpeljana v kalibracijsko omejitev z namenom, da preprečimo linearno odvisnost med klasičnimi omejitvami (prehranskimi) in kalibracijskimi omejitvami (Heckelei in Britz, 2000). Z dodanimi kalibracijskimi omejitvami pridemo do rešitve samo v primeru, da je referenčni obrok skladen z omejitvami modela. Na prvi pogled gre za povsem trivialno trditev, vendar se le-ta lahko izkaže kot problematična, če z modelom analiziramo sestavo podobnih krmnih obrokov, katerih sestavine imajo zaradi najrazličnejših vzrokov povsem različne hranilne vrednosti.

S pomočjo dualnega programa dobimo senčne cene kalibracijskih omejitev. V drugem koraku (5 in 6) na podlagi senčnih, lastnih in tržnih cen izračunamo parametre stroškovne funkcije. S pomočjo teorije povprečnih stroškov izpeljemo α, ki predstavlja presečišče stroškovne funkcije in parameter β, ki predstavlja naklon stroškovne funkcije.

V zadnji fazi kalibriranja uporabimo izračunane parametre stroškovne funkcije. Namenska funkcija [7] se zaradi kvadriranja (x_i^2) spremeni v nelinearno, pri kateri zopet iščemo maksimum. Tako prilagojena in 'uravnotežena' namenska funkcija, ob upoštevanju prehranskih omejitev, nam brez kalibracijskih omejitev vrne sestavo referenčnega krmnega obroka. Na tako pripravljenem modelu lahko nato študiramo npr. vpliv sprememb cen in stroškov preteklega desetletnega obdobja. V našem primeru smo kot referenčni krmni obrok izbrali nekoliko prilagojen krmni obrok, predpostavljen v modelnih kalkulacijah (KIS, 2008).

Prehranske potrebe bikov pitancev

Seveda gre pri sestavljanju krmnih obrokov za številne dejavnike, ki vodijo kmeta pri njegovem odločanju. Poleg izbrane pasme, velikosti kmetijskega gospodarstva ter razmerja med ornimi in travnimi površinami, ki določajo potrebe po krmi ter razmerje med doma pridelano in kupljeno krmo, je pomembna tudi tehnologija pitanja. Za analizo smo izbrali tehnološke predpostavke analitične modelne kalkulacije (KIS, 2008). Predpostavili smo, da se pitanje začne pri telesni masi 120 kg in se konča pri telesni masi 550 kg. Povprečen dnevni prirast preko celotnega obdobja znaša 0,9 kg/dan, kar pomeni, da pitanje traja 478 krmnih dni.

Ker se za potrebe modelnih kalkulacij uporablja starejši sistem škrobnih enot, smo prehranske potrebe pitancev ocenili s pomočjo simulacijskega modela za ocenjevanje prehranskih potreb

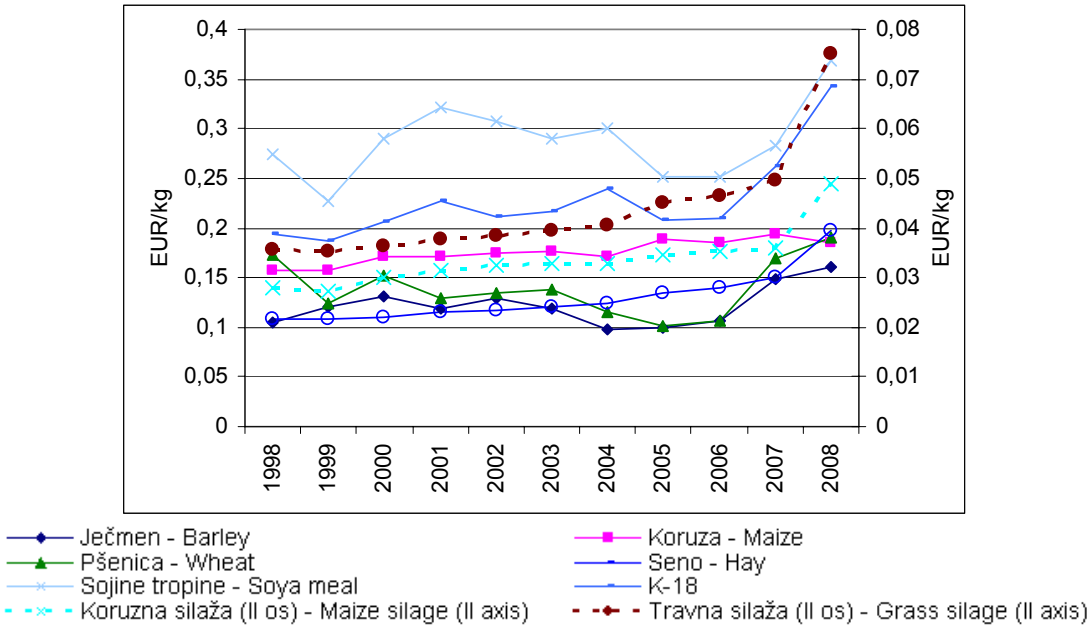
prežvekovalcev, ki temelji na presnovni energiji. Simulacijski model je podrobneje opisan v Žgajnar in sod. (2007).

Kupljena in doma pridelana krma

Za analizo smo izbrali najpogostejši način pitanja v Sloveniji. Predpostavili smo, da kmetijsko gospodarstvo večji del krme pridela na lastnih zemljiščih, del močne krme pa dokupi na trgu po tržnih cenah. Ker voluminozne krme večinoma ni tržna dobrina, smo na podlagi izračunov modelnih kalkulacij ocenili skupne stroške pridelave posamezne krme in jih nadalje ovrednotili s polno lastno ceno (brez upoštevanja morebitnih subvencij). Za razliko od metode pokritja, kjer so zajeti zgolj spremenljivi stroški, modelne kalkulacije vključujejo vse stroške, ki so povezani s pridelavo, kamor prištevamo tudi stroške dela (Rednak, 1998). Ob tem je potrebno podariti, da smo upoštevali zgolj stroške, povezane s pridelavo glavnega pridelka oziroma pridelka, ki ga lahko vključimo v krmni obrok.

Pri vrednotenju krme po polni lastni ceni ima ekonomija obsega ključno vlogo. Zato je potrebno izpostaviti, da kalkulacije temeljijo na predpostavki, da je velikost parcel 1 ha in so od kmetijskega gospodarstva oddaljene 1 km. Serijo osnovnih podatkov med leti 1998 in 2008 smo pridobili na spletni strani Kmetijskega inštituta, kjer imajo objavljene t.i. zbirnike podatkov na letni ravni (KIS, 2008).

Prva dva modela (LP in WGP) lahko pri sestavljanju krmnega obroka izbirata med osmimi vrstami krme (slika 1). Na razpolago imata pet vrst močnih krmil (ječmen, koruza, pšenica, dopolnilna krmna mešanica K-18 in sojine tropine), ter tri vrste voluminozne krme (seno, koruzna silaža in travna silaža). Predpostavili smo, da rejci vsa močna krmila dokupijo po tržnih cenah. Na lastnih zemljiščih pa pridelajo seno, travno in koruzno silažo. Slednje lahko ovrednotimo po njihovi polni lastni ceni. Kot je razvidno s slike 1, se je v opazovanem obdobju vsa krma podražila.



Slika 1. Gibanje tržnih cen močne krme ter polnih lastnih cen doma pridelane voluminozne krme v obdobju 1998–2008.

Figure 1. Changing market prices and total unit costs for feed and voluminous forage in the period 1998–2008.

Izračunane polne lastne cene doma pridelane voluminozne krme so se vse od leta 1998 nenehno zviševale. S podrobnejšo analizo smo ugotovili, da je zviševanje cen voluminozne krme posledica predvsem vse dražjih strojnih storitev in vse višjih postavk domačega dela ter kapitala. Poleg tega so se v opazovanem obdobju tudi mineralna gnojila nenehno dražila, kar je bilo še posebej izrazito v zadnjih dveh letih. V letu 2008 so se denimo cene mineralnih gnojil zvišale skoraj za trikrat. Slednje je tudi ključni razlog, da so se lastne cene pridelkov v zadnjem letu tako povečale. Slika bi bila nekoliko drugačna, če bi pretežen del rastlinskih hranil rejci zagotovili z gnojem domačih živali. S slike 1 je razvidno, da se je cena travne silaže v primerjavi s koruzno silažo relativno hitreje zviševala. Izrazit razkorak se kaže od leta 2002 dalje. Na prvi pogled nelogično dejstvo je moč pojasniti s količino pridelka na enako površino zemljišča. Pridelek travne silaže je bistveno manjši v primerjavi s sicer že tako ali tako cenejšo koruzno silažo, zato so stroški pridelave travne silaže na enoto pridelka večji.

Nihanja so opazna tudi pri kupljeni močni krmi. Dvig cen je nedvomno posledica kompleksnih pojavov in vplivov, ki pa jih ni mogoče enoznačno opredeliti. S slike 1 je razvidno, da so energijska krmila (koruza, pšenica in ječmen) v primerjavi s pretežno beljakovinsko krmo (sojine tropine) in sestavljenim močnim krmilom K-18 bistveno cenejša. Koruzno zrnje je v vsem opazovanem obdobju dražje od pšenice in ječmena, ki se izraziteje podražita šele v zadnjih treh letih.

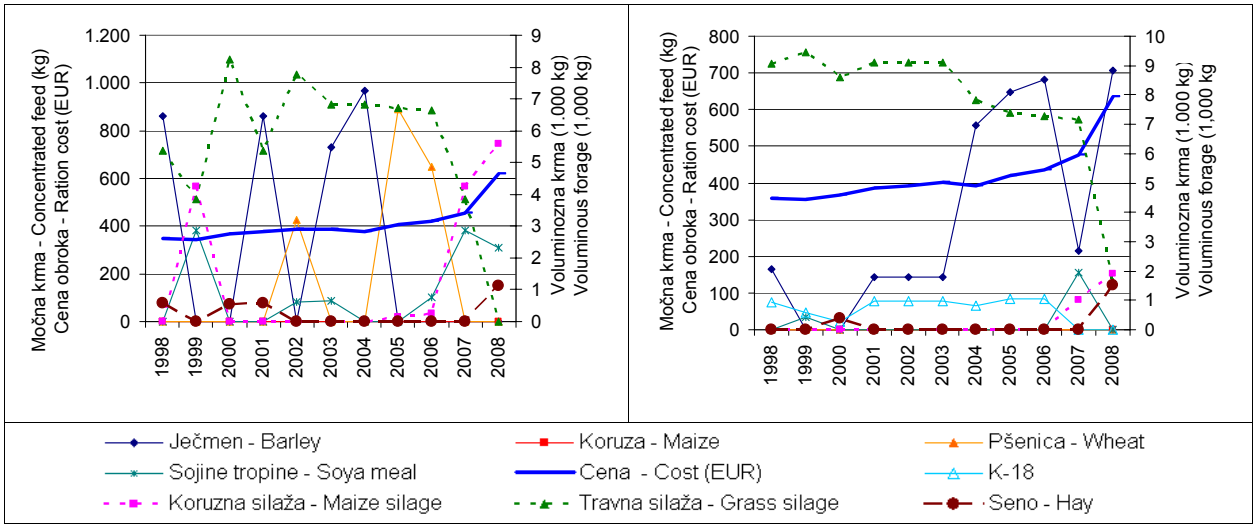
Za pokrivanje potreb po rudninskih snoveh so v nabor krmil vključene tudi štiri rudninsko vitaminske mešanice. Sprememb njihovih cen v danem obdobju ne prikazujemo, saj zaradi manjše količinske zastopanosti v obroku ne vplivajo na našo analizo.

Pri optimiranju sestave krmnega obroka sta, poleg ekonomskega vidika, ključnega pomena hranilna vrednost in kakovost krme. Obe sta odvisni od številnih dejavnikov kot so kakovost tal, klimatski dejavniki, količine padavin, tehnologije pridelave in tehnologije spravila. Iz tega sledi, da lahko kakovost krme med leti močno niha, kar lahko povzroči, da obroki s povsem enako sestavo ne pokrijejo vedno vseh potreb živali po hranljivih snoveh. V naši analizi smo ta vidik zanemarili; v izračunu smo upoštevali nekoliko nadpovprečno hranilno vrednost krme, ki je bila enaka v celotnem obdobju opazovanja.

REZULTATI IN RAZPRAVA

Rezultate modelov prikazujemo v enakem vrstnem redu, kot so opisani uporabljeni pristopi. Najprej pokažemo, kako bi se sestava obroka spreminjala, če bi bil rejec povsem prilagodljiv in bi bil njegov edini cilj minimiranje stroškov (rezultati LP). Sledijo krmni obroki, ki so sestavljeni s pomočjo tehtanega ciljnega programiranja. Nadaljujemo s predstavitvijo rezultatov post-optimalne analize in njihovega pomena. V zadnjem delu se osredotočimo na simulirane krmne obroke s pomočjo PMP modela. Vsi grafikoni, ki prikazujejo skupne stroške in strukturo krmnih obrokov, se nanašajo na celotno obdobje pitanja.

V prvi model smo vključili 12 vrst krme, iz katerih smo sestavili najcenejši možni krmni obrok. S slike 2 je razvidno, da se sestava tega krmnega obroka med leti močno spreminja. Značilnost LP je namreč prekinjen (nezvezen) odziv na spremenjene zunanje razmere – v našem primeru tržne cene oziroma izračunane polne lastne cene. Posledično se dobljena rešitev izrazito spreminja med leti in kar je bolj problematično, iz dobljenih rezultatov se ne da izluščiti neke splošne zakonitosti, kaj se je v opazovanem obdobju dogajalo s sestavo krmnega obroka in napovedati, kakšna bodo gibanja v prihodnosti. To dejstvo je še zlasti izrazito pri vključevanju energijskih močnih krmil v obrok.



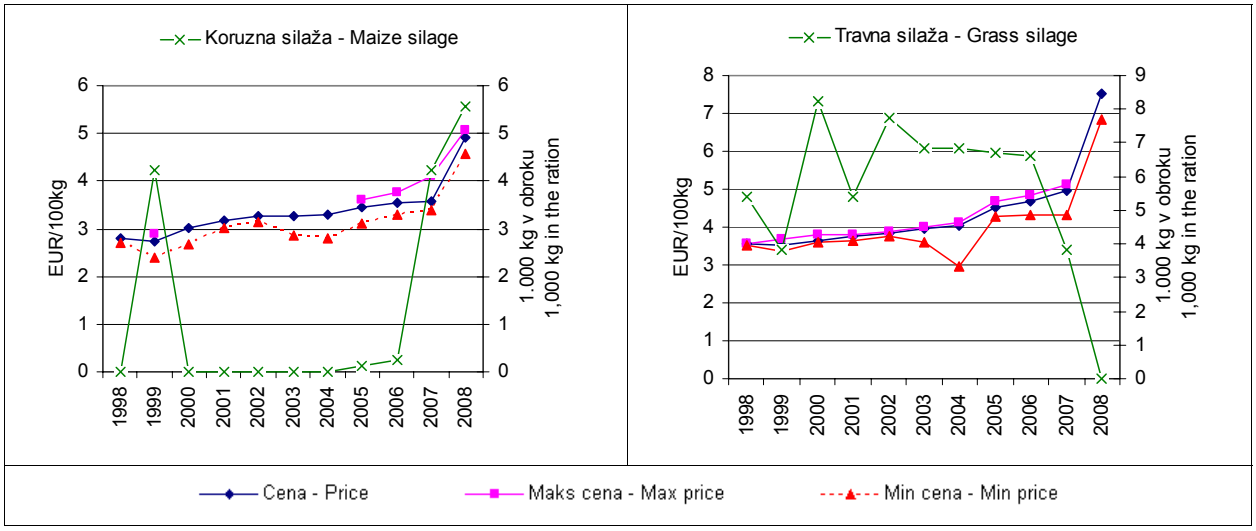
Slika 2. Krmni obroki v obdobju 1998–2008, sestavljeni s pomočjo linearnega in tehtanega ciljnega programa.

Figure 2. Rations for the period 1998–2008, calculated with linear and weighted goal program.

S slike 2 je razvidno, da manjkajoče potrebe po energiji z izjemo leta 1999 in zadnjih dveh let, kjer v krmni obrok vstopa koruzna silaža, pokrijemo s pšenico in ječmenom, ki se v krmnem obroku linearnega modela pojavljata kot alternativi. Predvsem v zadnjem obdobju se zaradi vse dražje doma pridelane travne silaže količina sojinih tropin, kot vira beljakovin, v obroku povečuje. Zviševanje cen sojinih tropin je vse od leta 2005 dalje na enoto beljakovin manjše kot pri travni silaži. Drago koruzno zrnje ni vključeno v rešitev. Svoj delež k temu doprinese tudi povečana količina koruzne silaže v krmnem obroku. Kljub rahlemu povečanju količine koruzne silaže v obrokih, postaja zagotavljanje ustrezne strukture obroka (strukturna vlaknina iz voluminozne krme) ključen problem. Na to je pokazala tudi dodatna analiza senčnih cen, ki so pri omejitvi zagotavljanja najmanjšega deleža strukturne vlaknine v obroku najvišje. Nedvomno je to posledica dragega in kakovostnega sena. Izračunane senčne cene bi bile tako bistveno drugačne, če bi imeli v naboru voluminozne krme cenejše seno slabše hranilne vrednosti.

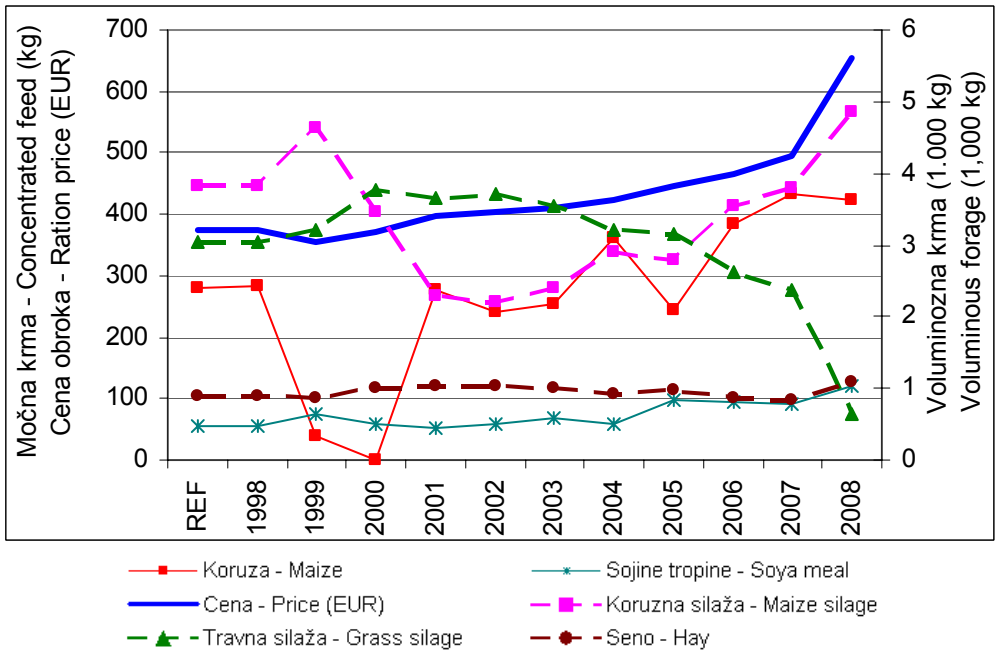
Do nekoliko drugačnih zaključkov pridemo pri rešitvi WGP, ki po definiciji išče s prehranskega vidika bolj uravnotežen krmni obrok. Ker ima sama cena nekoliko manjši vpliv, je pričakovano, da je obrok v primerjavi z obrokom linearnega modela nekoliko dražji. Razvidno je, da se med leti sestava obroka spreminja predvsem na račun zmanjševanja količine travne silaže in povečevanja količine ječmena v obroku. To je tudi edini obrok, ki vključuje relativno drago krmno mešanico K-18, kar je nedvomno posledica manjšega pomena stroška krmnega obroka pri ciljnem programiranju. Zanimivo je, da koruzna silaža z izjemo zadnjih dveh let ni zastopana v nobeni rešitvi WGP.

V vsem opazovanem obdobju je pri rezultatih obeh metod opazen izrazit trend podražitve krmnega obroka. S pomočjo dodatnih izračunov smo ugotovili, da se pri linearnem programu med leti stroški vsebovane močne krme praktično ne zvišujejo. V desetletnem obdobju vseskozi ostajajo na ravni 100 EUR, z izjemo zadnjih dveh let, ko se zvišajo za slabih 20 EUR na pitanca. To pomeni, da dvig cen vodi do vse večjega deleža voluminozne krme v skupnem strošku krmnega obroka. Precej drugačne zaključke lahko potegnemo iz rezultatov WGP. Do leta 2003 so namreč stroški močnih krmil predstavljali okrog 10 %, po tem letu pa zelo hitro narastejo na nekaj manj kot 20 % celotnih stroškov krmnega obroka.



Slika 3. Stabilnost dobljenih rešitev linearnega programa:primer koruzne in travne silaže.
Figure 3. Stability of obtained linear program solutions: maize and grass silage.

Na sliki 3 prikazujemo izračunane meje, znotraj katerih se lahko gibljejo lastne cene koruzne in travne silaže, ne da bi ob tem prišlo do sprememb v sestavi krmnega obroka. Prikazujemo zgolj rezultate za obe silaži, ki sta se izkazali za ključni pri sestavi krmnega obroka. S slike 3 je razvidno, da se cena travne silaže praktično ves čas giblje na zgornji meji. V danih razmerah to pomeni, da bi se količina v obroku zmanjšala takoj, ko bi cena narasla. Nasprotno lahko ugotovimo pri koruzni silaži, kjer je cena, z izjemo leta 1999 in zadnjih štirih let, ves čas nad najvišjo dovoljeno ceno in zato koruzna silaža ni vključena v rešitev. Kljub izrazitemu dvigu lastne cene koruzne silaže v zadnjih dveh letih pa bi le-ta lahko še narasla, ne da bi to vplivalo na dobljeno rešitev. Glede na aktualna cenovna razmerja je bilo koruzno zrnje v primerjavi z ostalo krmo, dostopno na trgu, predrago, zato ni bilo zajeto v rešitvi.



Slika 4. Simuliranje sprememb v sestavi krmnega obroka s pomočjo pozitivne analize.
Figure 4. Simulation of ration structure changes with positive analysis.

S pomočjo tretjega modela (PMP), ki je bil razvit za potrebe te analize, smo simulirali, kako bi se rejec glede na svoje preference odzival na 'zunanje' spremembe. Kot referenčno situacijo smo izbrali cenovno – stroškovna razmerja iz leta 1998. Predpostavili smo, da je t.i. referenčni krmni obrok (REF) nekoliko prilagojen krmni obrok iz modelnih kalkulacij. Na sliki 4 prikazujemo, kako bi sprememba cen po posameznih letih vplivala na sestavo krmnega obroka 'racionalnega' rejca. Referenčni obrok za celotno obdobje pitanja naj bi tako vključeval nekaj manj kot štiri tone koruzne silaže, dobre tri tone travne silaže, nekaj manj kot 900 kg sena, 280 kg koruznega zrnja in dobrih petdeset kilogramov sojinih.

S slike 4 je razvidno, da vse dražja travna silaža počasi izgublja pomen v krmnem obroku. Hkrati se delež koruzne silaže v obroku povečuje vse od leta 2002 naprej. V tem letu se je namreč travna silaža v primerjavi s koruzno silažo začela močneje dražiti. Simulacija je pokazala tudi, da je z izjemo leta 2000 koruzno zrnje vključeno v krmni obrok, kar je glede na rezultate prejšnjih modelov dokaj neracionalno. Na tem preprostem primeru se izkaže značilnost (pomanjkljivost) PMP metode, saj lahko kalibriramo zgolj tiste aktivnosti (v našem modelu vrste krme), ki so vključene že v referenčni (izhodiščni) rešitvi, ne pa tudi tistih, ki so prav tako na razpolago in jih kmet v referenčni situaciji zaradi takšnih ali drugačnih razlogov ni uporabil. Z drugimi besedami to pomeni, da je lahko pri klasičnem PMP pristopu v rešitev zajeta zgolj tista krma, ki jo je vključevala referenčna (REF) situacija.

Pri kalibriranju modela je bila senčna cena kalibracijske omejitve koruznega zrnja enaka nič. Posledično je tudi parameter β enak nič, kar pomeni, da je 'odzivanje' rejca pri vključevanju koruznega zrnja v obrok ostalo linearno. Heckelei (2002) takšne aktivnosti imenuje mejne aktivnosti (marginal activities). Značilnost slednjih je, da so omejene s strani 'klasičnih' omejitev in ne zgolj s strani dodatnih kalibracijskih omejitev, kar je značilnost bolj zaželenih aktivnosti. Heckelei (2002) opozarja na t.i. fenomen substitucije, kar se v našem primeru zaradi podražitve bolj zaželenih aktivnosti odraža v večjem vključevanju koruznega zrnja v obroku.

SKLEPI

Na podlagi dobljenih rezultatov lahko zaključimo, da se je strošek krmnega obroka v zadnjih desetih letih tudi pri optimizaciji nenehno povečeval. S pomočjo normativnih modelov smo ugotovili, da bi teoretično bolj prilagodljivi rejci s pogostejšim optimiranjem sicer lahko zniževali stroške krmnega obroka, ki pa bi v zadnjih desetih letih še vedno kazali izrazit trend rasti. Bistven problem pri normativni analizi, zlasti LP, se je izkazal zaradi značilnega prekinjenega (nezveznega) odziva na spremenjene tržne cene oziroma izračunane polne lastne cene. Posledično se dobljena rešitev močno spreminja med leti in, kar je še bolj problematično, iz dobljenih rezultatov se ne da izluščiti nekega splošnega trenda, v katero smer se je spreminjala sestava krmnega obroka v obravnavanem obdobju. Prav tako nam takšen normativen matematičen model ne omogoča, da bi izračunali dejansko - referenčno stanje. To se izkaže kot problem v primeru, če na določenem kmetijskem gospodarstvu rejec krmi obrok, ki z ekonomskega vidika ni racionalen, je pa zaradi določenih okoliščin edino možen (npr. razmerje med njivskimi in travnatimi površinami). V takšnem primeru ne moremo opazovati, kako sprememba cen krme in krmil vpliva na sestavo krmnega obroka, saj z modelom kljub dodatnim omejitvam ne moremo simulirati realnega stanja.

S pomočjo PMP modela smo simulirali, kako bi se povprečen rejec, glede na svoje preference, odzival na zunanje spremembe. Dobljeni rezultati kažejo, da se je sestava krmnega obroka v zadnjih desetih letih nagnila v prid koruzne silaže na račun travne silaže. Vsekakor dobljenih rezultatov ne gre posploševati, saj je struktura lastnih cen v veliki meri odvisna od stalnih stroškov, ki pa se med posameznimi kmetijskimi gospodarstvi močno razlikujejo.

Posledično bi bila lahko situacija na posameznem kmetijskem gospodarstvu precej drugačna, v največji meri pa odvisna od ekonomske učinkovitosti gospodarstva.

V praksi seveda nismo in tudi ne bomo zasledili tako izrazitih trendov, zlasti ne tako izrazitega zmanjšanja količine travne silaže v obroku. Realnejše rezultate bi dobili s t.i. kmetijskim modelom, ki bi kot omejitev vključeval tudi površine obdelovalnih zemljišč, hkrati pa bi zajel tudi druge omejitve, ki so prav tako pomembne in posredno vplivajo na vodenje kmetijskega gospodarstva. Značilnost oziroma predpostavka modelov, ki smo jih uporabili pri analizi, je, da ne upoštevajo razmerja zemljišč (travniki : njive), kot tudi ne, da le-ta ne smejo ostati neizkoriščena in ostalih omejitev, ki jih SKP nalaga kmetom. Dobljene rezultate zato lahko tolmačimo tudi na nekoliko drugačen način. Dobljeni trendi kažejo na nujnost iskanja možnosti cenejše pridelave krme, bodisi z izboljševanjem tehnologije oziroma kjer je možno, z ekonomijo obsega. Iz dobljenih rezultatov bi tako lahko zaključili, da bo predvsem pri spravilu travne silaže potrebno posvetiti več pozornosti zniževanju stroškov.

SUMMARY

Due to changing political and economic environment, beef fattening has recently become one of the most sensible sectors within EU agriculture. Its poor economic position is mainly caused by the change of the common agricultural policy (CAP). Strongly protectionist oriented policy from the past has changed fundamentally in the last few years. Starting with MacSharry reform in 1992 that was continued with Agenda 2000 and the reform in 2003, implemented in Slovenia in 2007, CAP is abandoning previous market-price policy instruments in favour of new decoupling concept. It is expected that such approach should yield more market oriented farmers. But in the beef sector this is so far more reflecting in worsening economic position. Production costs are much higher than price achieved at the global market. The main cost in fattening, beside calve purchase, goes to ration costs. In Slovene circumstances it presents up to 55% of total cost or around 70% of variable cost. This is especially important, since feed and fodder price trend is positive and in last few years more and more steep. This is due to additional demand for grains caused by bio-energy production, decreased grains production (draughts, floods etc.) in strategic regions in the world as well as energy price rise (fertilizers and gasoline).

In this study we have analysed how optimal beef rations have been changing in the last decade due to changes in feed price and estimated fodder total costs. Model calculations, prepared by Slovene Agricultural Institute (KIS, 2008) have been used for estimation of total costs for home produced forage.

Three mathematical programming methods based on constraint optimization have been applied. They tend to solve basic economic problem – making the best use of limited resources, which is the basic concept of neoclassical economic theory (Buysse *et al*, 2007). Constrained optimisation models could be split into normative and positive ones.

The normative analyses have been done with already developed tool - decision support system (DSS) for beef ration formulation (Žgajnar and Kavčič, 2008). According to the purpose of this study it has been slightly adapted in the sense of total cost approach and fattening periods, which have been jointed into one period. Both sub-models could formulate rations from five purchased feed and three home produced voluminous forages. With the common LP approach (the first sub-model) the least cost ration has been formulated as well as post optimal analyses have been performed to estimate the stability of obtained solutions. The second sub-model, based on weighted goal programming (WGP), served for calculation, how farmer that pays more attention to the quality and not mainly on economics would have formulated the ration. To estimate how an average farmer (we took the presumptions of the model calculation (KIS, 2008)) would have react on external – feed and fodder price changes, an model based on positive

mathematical programming (PMP) approach has been developed. Calibration process is done by the most widely applied approach, proposed by Howitt (1995).

All three models have shown that ration costs have significantly increased in the last decade. With optimization process it was possible to reduce costs, more efficiently in the case that ration costs was, besides satisfying nutrition constraints, the most important objective of the farmer (LP). On the basis of calibrated model (PMP), we have simulated how an average farmer would have change the manner of ration formulation. The main drawback of this method is, that model can 'play' only with the feeds that are included in the reference ration (REF). It comes from the analysis that the main problem is expensive grass silage that has explicit negative trend in ration structure and vice versa holds for maize silage. Of course this is only applicable when there are no constraints on tillage area, policy rules etc., what is assumed in our study. Obtained results could be therefore interpreted in the way that for improving economic position of the fattening, home produced fodder costs have to be reduced, either with improved cost – efficient technology or economics of scale.

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FARM INCOME ANALYSIS OF AGRICULTURAL HOLDINGS IN MACEDONIA USING FADN METHODOLOGY

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ABSTRACT

No consistent farm income data on micro level is available in Macedonia. The FADN methodology, being the only standardised and harmonised farm accountancy system in EU, was applied and tested on a sample of agricultural holdings. In addition, data on quantity of inputs and applied technology, as well as occurrence of non-agricultural income were recorded. The structure and farm income of Macedonian farms in 2002–04 was presented upon a sample of farms belonging to different economic sizes and farm types. The results were analysed and interpreted in EU context, by comparison with a panel of EU member countries. The size of Macedonian farms in economic terms (5.9 ESU) was five times smaller than the EU-25 average (32.7 ESU). The gross farm income of the Macedonian sample was 5,500 EUR/farm, representing about 15% of what an average EU farm generated at that level. The family farm income reached 4,100 EUR, four times lower than the EU-25 average. Due to differences in income will the EU-accession pose major challenges to Macedonian farmers, but also expectedly bring improvement of the income situation of commercial-orientated farms. Very little support was available to Macedonian farms until 2004, thus the farm income includes no subsidies, in contrast to the EU farms. The benefits of farm income data on micro level will contribute to the creation and evaluation of the agricultural policy, as well as the measurement of the recently launched national policy support schemes and the imminent pre-accession funds.

Key words: agriculture / agricultural holdings / farm income / FADN methodology / Macedonia

ANALIZA DOHODKA KMETIJSKIH GOSPODARSTEV V MAKEDONIJI Z UPORABO FADN METODOLOGIJE

IZVLEČEK

Makedonija ne razpolaga s konsistentnimi podatki o dohodku kmetijskih gospodarstev. S ciljem zapolnitve te vrzeli je bila na vzorcu kmetijskih gospodarstev (KG) uporabljena FADN metodologija, standardiziran in hamoniziran sistem kmetijskega računovodstva EU. Metoda je bila nadgrajena z dodatnimi podatki o proizvodnih vložkih, apliciranih tehnologija in prisotnosti nekmetijskih virov dohodka. Struktura in kmetijski dohodki v Makedoniji v letih 2002–04 so predstavljeni s pomočjo vzorca, ki je vključeval različne ekonomske velikosti in tipe kmetijskih gospodarstev. Rezultati so predstavljeni v primerjavi s podatki držav članic EU. Ekonomska velikost kmetijskega gospodarstev je z 5,9 ESU pet krat manjša kot velja za povprečje EU-25 (32,7 ESU). Bruto dodana vrednost iz kmetijstva je 5.500 EUR/KG, kar predstavlja okoli 15 % EU povprečne vrednosti. Dohodek kmečke družine je dosegel 4.100 EUR v povprečku, štiri krat manj kot povprečje EU-25. Analiza kaže, da bo zaradi razlik v dohodkih, pristop k EU lahko prinesel tudi izboljšanje ekonomskega položaja na tržno usmerjenih KG. Za razliko od EU, vse

do leta 2004, makedonsko kmetijstvo ni bilo deležno neposrednih podpor. Vzpostavitev podatkovnega sistema o kmetijskih dohodkih na mikro ravni lahko pomembno izboljša oblikovanje in presojo ukrepov kmetijske politike v predpristopnem obdboju.
Ključne besede: kmetijstvo / kmetijska gospodarstva / dohodki / metodologija FADN / Makedonija

INTRODUCTION

The agricultural sector in Macedonia participates with 11% in the GDP (Ministry for Agriculture, Forestry and Water Management, 2006) and is traditionally one of the most important in the economy. Coupled with the processing industry, the share of the agri-food sector in the GDP increases to about 17%.

The sector is characterized by a large number of small and heterogenic holdings. Preliminary data from the 2007 Agricultural Census indicate 192,378 agricultural holdings, which cultivate 264 338 ha. According to this source, the average Macedonian farm utilises agricultural area of as low as 1.37 ha (87.5% of all holdings cultivate less than 10 ha of utilised agricultural area). More than 80% of the land is owned or rented by family farms (State Statistical Office, 2008). The major crops are cereals, early vegetables, grapes and fodder crops; as for the livestock production, dairy farming and sheep breeding are the most significant (State Statistical Office, 2008).

Macedonia has been an EU candidate country since 2005. The EU approximation process will have an impact on the Macedonian agriculture; prices and trade flows will change, and that will inevitably have an effect on the farm income.

Farm data available on micro level is scarce. Official statistical sources in Macedonia provide insufficient quantity and quality of farm level data. The farmers in Macedonia are not obliged to keep farm books or conduct farm accounting; hence, they do not have accurate farm income calculations. Farm records can provide valuable information which can indicate the profitability, support the decision-making process and facilitate the farm business planning. It is generally assumed that the introduction of accounting will improve the farm management and produce better farm performance (Luening, 1989).

Furthermore, policymakers and other stakeholders involved in agriculture will get greater efficiency and effectiveness in their decisions when they base the farm income analysis on accounting-derived information of the farms (Argiles, 2001). The policies operated by the EU, particularly the CAP, require reliable statistical information on the farmers' economic situation. A central requirement of a policy as complex as the CAP is data on the incomes of farmers, which can be used to assist the policy design and at the same time be a part of the monitoring of its performance (Hill, 1991). Thus, a functional farm accountancy data system can be useful for the decision-makers in creating adequate agricultural policy, but also in validation of the results from the appropriate measures and the integration effects. In addition, it can support the advisory and extension segment, as well as the research and academic community.

The objective of this paper is to apply and test the EU-FADN methodology on a sample of agricultural holdings in Macedonia. Additionally, the structure and income of the Macedonian farms are compared to the EU countries by using the harmonised methodology. This is the first attempt to fill in the gap of lacking farm data on micro level in Macedonia and functionally interpret them in EU context. With the EU pre-accession in mind, the paper stresses the availability of farm data as an important tool for the national policy support schemes and the pre-accession funds.

The paper starts with an overview of the applied FADN methodology, followed by explanation of the additional methods used. Further, the sample is described along with the classification of the farms. Then we provide presentation of the Macedonian results of the farm

income indicators. These results are compared with farm cost and income data from EU countries. The main conclusions are drawn in the end.

MATERIAL AND METHODS

Farm Accountancy Data Network (FADN)

The Farm Accountancy Data Network was established in 1965 as an information tool for collecting accountancy data from agricultural holdings in the European Union (EEC/79/65). FADN was primarily set up to support the creation and assessment of the Common Agricultural Policy (CAP). The system is a unique instrument for evaluating the income of agricultural holdings providing information about the economic conditions on European farms.

FADN is the only harmonised and standardised source of data obtained from a sample of individual farms across all member states. Given the common methodology, the network provides comparable data on a European level. The field of observation of FADN are commercial farms. A minimum European Size Unit is established in each member state to define the commercial farm threshold. The sample is stratified by region, farm size and type of farming.

The farm income indicators in FADN are derived from the income statement; the subtraction of the total intermediate consumption (SE275), production and input subsidies (SE605) and taxes (SE390) from the total output (SE131), provide the gross farm income indicator (SE410). The depreciation costs are further subtracted in order to reach the farm net value added indicator (SE415). Finally, the family farm income (SE420) is produced by deducting the external factors – wages, rent and interest paid (SE365) from the farm net value added.

Methods used in addition to FADN

FADN data on EU level still provide limited analysis in some respects; one of the major issues is that the FADN data completely lack information in terms of quantity of inputs used by the farm for each farm enterprise and the applied technology (Paris and Arfini, 1999). In this respect, analytical gross margin budgets were calculated for each farm enterprise, being any coherent portion of the general input-output structure of the farm business that can be separated and analysed as a distinct entity (Eidman *et al*, 2000).

FADN does not cover the non-agricultural income of the farm household; however, having in mind the structure of Macedonian farms, the off-farm income as an important source of revenue was therefore included into the survey questionnaire.

A range of alternatives should be considered for regular calculation (Hill, 1991), one of them being the net profit margin: Family Farm Income (SE420)/Total Output (SE131).

Selection of the sample

A detailed in-depth farm survey was conducted on 50 representative farms in 4 regions throughout the country (Skopje, Bitola, Negotino and Strumica). The selection of farms was based on the National Extension Agency farms sample.[*] The regional coverage, major farm activity and minimum of 1 ha of utilised agricultural area were set as farm selection criteria. We should note that the sample is not statistically representative for all commercial farms in the country.

[*] The Farm Monitoring System (FMS) is a survey conducted by the National Extension Agency of the Republic of Macedonia. This survey includes around 450 representative farms countrywide with basic farm economics indicators.

The data derived from the survey were processed using an applicative model for farm business analysis, specifically developed for this purpose in Microsoft Excel in accordance with FADN methodology. The data from the Macedonian farms were collected for the period of 2002–2004. The year 2004 was taken as the most suitable year for the EU-member states comparison, since it was the first year that included EU-25 countries in the FADN database.

The EU countries included in the comparison panel were selected upon three criteria: new member states that joined the EU in 2004 (e.g. Slovenia); bordering countries, with similar agro-climatic conditions (e.g. Greece); and EU countries with intensive agricultural production (e.g. The Netherlands).

Classification of Macedonian farms according to FADN

According to the FADN regulative (78/463/EEC), classification of farms in the European Union is principally done according to two major criteria: economic size of the agricultural holding and type of farming.

The economic size of the farm is determined as the value of its total farm standard gross margin, expressed as a Community unit of measurement, the European Size Unit (ESU), currently estimated at 1,200 EUR. The standard gross margin (SGM) is the balance between the standard value of the output and the standard value of certain direct specific costs, calculated in average for a period of 3 to 5 years. The SGM is an economic criterion expressed in monetary terms, either per hectare of utilised agricultural area in the case of crop enterprises or per head of livestock in the case of livestock farming.

The size structure of agricultural holdings in Macedonia is very unfavourable, as it is reflected in the sample (see Table 1); almost one-half of the surveyed holdings belong to the economic class of very small farms (less than 4 ESU, i.e. SGM < 4,800 EUR). In the class of small farms, ranging from 4 to 8 ESU, belongs 37% of the surveyed farms. The economic class of medium-low farms (8–16 ESU) is represented by 14% of the holdings, while only 4% belong to the class of medium-high sized farm (> 16 ESU).

The type of farming is the other classification criterion, defined as the production system of a holding which is characterised by the relative contribution of different enterprises to the holding's total standard gross margin. The general type of farming level (TF8) is applied in this paper.

The largest share of surveyed farms in Macedonia is taken by dairy producers (27%), followed by vegetable and grape producers (each 18%), grazing livestock – sheep and mixed farms (each 12%), and finally the fruit and field crops producers (Table 2).

RESULTS

Macedonian sample

Using the farm economic size classification, the highest level of farm income (gross farm income, farm net value added and family farm income) was attained by medium-high farms. This is logical since the highest total output was also achieved by this group, and the level of total intermediate consumption followed a declining input/output coefficient. Subsequently, the profitability is the highest for this group, as the net profit margin reaches 50.6%. Lowest profitability is noted in the very small farms group, reaching 21.7% margin.

The level of non-agricultural income (pensions, social security, off-farm salary) is mostly elevated on very small farms, and declines proportionally with the economic farm size. This leads us to the assumption that smaller farms are more dependent on supplementary sources of

income and most likely practicing farming as part-time activity, while larger farms are more commercialised.

Table 1. Average value per economic size groups of Macedonian farms included in the survey 2002–04, EUR

Preglednica 1. Povprečne vrednosti makedonskih kmetijskih gospodarstev vključenih v vzorec 2002–04 razvrščenih po skupinah ekonomske velikosti, EUR

Average value per agricultural holding Povprečne vrednosti	FADN code	Very small farm Zelo majhne KG (< 4 ESU)	Small farm Majhne KG (4–< 8 ESU)	Medium-low farm Srednje-majhne KG (8–< 16 ESU)	Medium-high farm Srednje-velike KG (> 16 ESU)	Average Povprečje
Structure in sample / Struktura	%	45	37	14	4	-
Total UAA, ha Kmetijska zemljišča v uporabi	(SE025)	2.9	4.5	6.1	7.5	4.2
Total livestock units Število glav velike živine (GVŽ)	(SE080)	3	4	7	3	4
Total output crop production Prihodek od rastlinske pridelave	(SE135)	2,282	5,489	9,527	22,685	5,271
Total output livestock prod. Prihodek od živinoreje	(SE206)	2,307	4,579	10,831	3,869	4,384
Other output Ostali prihodki	(SE256)	43	72	239	407	95
Total output **Skupni prihodek**	**(SE131)**	**4,603**	10,206	20,599	27,590	9,788
Total specific costs Posebni stroški	(SE281)	1,896	3,389	6,552	6,229	3,261
Total farming overheads Splošni stroški	(SE336)	907	1,568	3,075	4,511	1,592
Total intermediate consumption Vmesna poraba	(SE275)	2,803	4,957	9,627	10,740	4,854
Gross farm income **Bruto dodana vrednost**	**(SE410)**	**1,800**	5,249	10,972	16,850	4,934
Depreciation Amortizacija	(SE360)	520	887	1,450	2,298	854
Farm net value added **Neto dodana vrednost**	**(SE415)**	**1,267**	4,349	9,492	14,541	4,065
Total external factors Stroški z zunanjimi dejavniki	(SE365)	268	506	899	585	456
Family farm income **Dohodek kmečke družine**	**(SE420)**	**999**	3,843	8,593	13,955	3,609
Net profit margin, % **Neto profitna marža**	(SE420/131)	**21.7**	37.7	41.7	50.6	36.9
Off-farm income Dohodek izven kmetijstva	OFI	1,884	1,142	465	-	1,339

When looking at the farm income by type of farm classification, the highest income generating farms are cereal farms, vegetable farms and grape farms. This result was somehow anticipated for the vegetable, grape and dairy farms, since generally vegetables and grapes are achieving high gross margins per capacity unit. The cereal farms that participated in the survey, besides from product sales, showed high output value from providing machinery services to other farmers (harvesting and baling), so their farm income was higher than expected. The dairy farming in Macedonia attained average income, which comes as no surprise since the breeds are mostly domestic or mixed; the milk yield is low and the feed input value was high.

Table 2. Average value per type of farm in Macedonia, survey results 2002–04, EUR
Preglednica 2. Povprečne vrednosti makedonskih kmetijskih gospodarstev vključenih v vzorec 2002–04 razvrščenih po tipih kmetijskih gospodarstev, EUR

Farm type (TF8) Tip kmetovanja	% in sample	Total output / Skupni prihodek (SE 131)	Gross farm income / Bruto dodana vrednost (SE410)	Farm net value added / Neto dodana vrednost (SE415)	Family farm income / Dohodek kmečke družine (SE420)
A (Field crops) Poljedelska kmetija	4	12,058	7,378	6,325	4,845
B (Horticulture) Vrtnarska kmetija	18	11,103	6,084	4,759	4,393
C (Wine) Vinogradniška kmetija	18	8,488	5,162	4,239	4,201
D (Permanent crops) Sadjarska kmetija	10	4,092	2,210	1,569	1,337
E (Milk) Mleko	26	11,860	5,107	4,416	3,760
F (Grazing, sheep) Drobnica	12	9,766	4,315	3,555	2,953
H (Mixed) Mešana kmetija	12	8,714	4,405	3,674	3,239
Average Povprečje	-	9,788	4,934	4,065	3,609

Comparison with EU

The agricultural holdings in the European Union are on average more than five times the size of the agricultural holdings in Macedonia. The average economic size of EU farms in 2004 was 32.7 ESU, while the Macedonian match was determined to be 5.9 ESU.

In terms of engaged labour, the Macedonian farm averagely employs two annual working units (AWU), which is even higher than the EU-25 average of 1.7 AWU (ranging from 1.2 AWU in Greece to 2.4 AWU in The Netherlands). We have to consider that many operations on Macedonian farms, such as sorting and grading of vegetables or even milking of cows, are performed manually and are very labour consuming. The labour productivity and technological level are lower on Macedonian farms, as compared to the EU.

In most of CEEC countries that joined the EU in 2004, for instance Slovenia, the production potential of family farms in the pre-accession period was low, in particular due to the limited land and capital resources (Erjavec *et al*, 2003). In addition, subsistence farming was largely practiced, which is to a large extent corresponding to the Macedonian situation.

The utilised agricultural area (UAA) per agricultural holding shows high variability among EU member countries, ranging from 6.3 ha in Greece, up to 93.3 ha in Sweden, with EU average of 34.3 ha in 2004. The average derived from the Macedonian sample farms is 4.2 ha UAA/farm, which is higher than the official statistical mean of 1.37 ha per farm (State Statistical Office, 2007), meaning that the farms included in the sample were slightly larger than the average.

The livestock units per agricultural holding in the EU in average reach 29.0. The Macedonian average equals 3.8 LU/holding, which is logical when compared to the statistical information that 86.4% of the farms have 1–5 heads of cattle (Brandt, 2006). Macedonian farms are lagging behind the EU average wheat yields; according to the research in 2004 the Macedonian average is 3.8 t/ha (the official statistics provides a figure of 3.5 t/ha for the same period), as compared to the average of 6.7 t/ha in EU-25. In respect to cow milk, the research results demonstrated a mean of 4 557 l/head (according to the official statistics, this average is much lower - 2 362 l/head), compared to the EU average of 6 908 l/head.

Table 3. Structure of agricultural holdings and major indicators in 2004
Preglednica 3. Struktura kmetijskih gospodarstev in ključni indikatorji v letu 2004

	Economic size Ekonomska velikost (ESU)	Annual working units Število polnovrednih delovnih moči	Utilised agricultural area – UAA Kmetijska zemljišča v uporabi (ha)	Rented UAA Kmetijska zemljišča v najemu (ha)	Livestock units Število glav velike živine (LU)	Wheat yield Pšenica (kg/ha)	Cow milk yield Mlečnost (l/head)
FADN code	(SE005)	(SE010)	(SE025)	(SE030)	(SE080)	(SE110)	(SE125)
Greece	9.4	1.2	6.3	2.5	4.1	3 158	4 521
France	75.9	1.9	73.7	61.1	60.6	7 655	6 899
Hungary	17.1	1.9	49.4	33.0	21.2	5 278	6 747
Italy	25.4	1.4	16.8	6.3	14.2	5 657	6 817
Netherlands	127.2	2.4	31.2	12.6	99.6	8 607	8 283
Poland	9.4	1.8	15.7	4.0	12.7	5 463	4 432
Sweden	55.7	1.4	93.3	45.7	54.0	5 952	8 829
Slovenia	7.3	2.0	12.7	4.3	13.5	4 648	5 576
EU-25	**32.7**	**1.7**	**34.3**	**18.0**	**29.0**	**6 676**	**6 908**
Macedonian sample Makedonski vzorec	5.9	2.0	4.2	1.6	3.8	3 791	4 557

Source: Survey and own calculations based on the FADN public database

It is interesting to compare the specific costs of Macedonian and EU farms on two levels i.e. on agricultural holding level or 1 ha UAA level. When the total amount of average specific costs is analysed per holding, then the Macedonian results (3,214 EUR) are close to those of the Greek farms (4,008 EUR) and Slovenian farms (4,894 EUR), but at the same time are almost seven times lower than the EU average (21,558 EUR). This situation has been anticipated, since the size of agricultural holdings is the smallest in these countries.

The Macedonian farms had higher average values of specific costs per one hectare of UAA (780 Euro/ha) compared to the EU-25 average (628 Euro/ha). The major contributors to this phenomenon were the high livestock feed costs, due to the high input prices and often imported inputs.

Using a survey supported by harmonised methodology provided grounds for processing comparable data. The gross farm income at Macedonian holdings is twice as low as compared to some of the countries that joined the EU in 2004 (such as Poland and Slovenia) and compared to the EU countries that apply high-end technology and intensive production is more than 20 times lower (for instance, The Netherlands). An issue relevant to the gross farm income is the production or input support evident through the subsidy levels. No subsidies were provided to the farmers in Macedonia in 2004; quite the opposite, the EU farmers received various types of support, which had an impact on the income levels.

It is evident that at the Macedonian holdings the difference between the various income indicators is very small, unlike the EU countries. This is explained by the lower depreciation costs (little and often depreciated machinery) and avoidance or exemption of land taxes payment. Moreover, many of the farmers would use unemployment social and health benefits. Therefore the margin between the gross farm income (SE410) and the farm net value added (SE415) derived from the Macedonian sample results is inconsiderable.

The edge between the farm net value added (SE415) and the family farm income (SE420) at the sample farms is again rather small – around 10%, and is basically caused by the low level of costs for external factors (rent paid, wages paid and interest paid). In contrast, the EU average farm net value added is almost double than the family farm income.

Table 4. Comparison of the specific costs per holding in 2004, EUR
Preglednica 4. Primerjava specifičnih stroškov po kmetijskem gospodarstvu v 2004, EUR

	Total specific costs Posebni strošk (SE 281)	Seed and seedlings Stroški s semenom in sadikami (SE 285)	Plant nutrition Stroški z gnojili (SE 295)	Plant protection Stroški z varstvom rastlin (SE 300)	Other crop specific costs Ostali pos. stroški z rast. pridelavo (SE 305)	Livestock feed Stroški s krmo (SE 310-SE 325)	Other livest. spec. costs Ostali pos.stroški z živinorejo (SE 330)
Average per holding / Povprečje na kmetijsko gospodarstvo							
Greece	4,008	547	935	678	219	1,324	104
France	38,096	4,988	7,457	6,837	1,052	8,422	2,506
Hungary	22,836	2,689	2,733	2,888	857	4,311	1,222
Italy	17,157	2,352	1,679	1,607	1,955	6,239	636
Netherlands	95,751	21,142	4,264	5,332	12,720	13,617	9,873
Poland	8,079	819	1,329	611	336	1,008	266
Sweden	55,279	3,913	6,992	2,714	2,622	24,649	4,950
Slovenia	4,894	421	517	286	415	1,674	639
EU-25	**21,558**	**2,627**	**2,885**	**2,285**	**1,312**	**5,542**	**1,771**
Macedonian sample Makedonski vzorec	3,214	288	389	317	355	1,608	257
Average per 1 ha UAA / Povprečje na ha UAA							
Greece	632	86	147	107	35	209	16
France	517	68	101	93	14	114	34
Hungary	462	54	55	58	17	87	25
Italy	1,024	140	100	96	117	372	38
Netherlands	3,073	678	137	171	408	437	317
Poland	514	52	85	39	21	64	17
Sweden	592	42	75	29	28	264	53
Slovenia	387	33	41	23	33	132	51
EU-25	**628**	**77**	**84**	**67**	**38**	**161**	**52**
Macedonian sample Makedonski vzorec	780	70	94	77	86	390	62

Source: Survey and own calculations based on the FADN public database

This situation can be explained to certain extent: land rent is rarely paid or is quite insignificant in Macedonia. Family labour is dominant and occasionally seasonal labour is hired. The Macedonian farmers rarely engage external labour on permanent basis (except in sheep production, where a shepherd is regularly hired). Some of the potential expenses are actually omitted; for instance, hired or contracted labour, and even permanent labour, is paid directly or in kind, meaning that payment of social benefits and health insurance is avoided. Also, investments in agriculture are very low and therefore the farmers are rarely users of borrowed capital (only 2% of the surveyed farmers used commercial bank loans). The main reason behind this is the lack of available sources of financing in agriculture, high collateral demands and unfavourable interest rates.

When farm family income indicators are compared on farm level, the Macedonian holdings achieve the lowest average value of 4,113 EUR, the EU-25 average being 18,097 EUR/holding. Linking the farm income to the utilised agricultural area, Macedonia is among the countries with highest farm income per 1 ha. It has to be stated that the results from the Macedonian sample are higher than the actual situation, since the farms included in the sample were in average larger and business-oriented.

All this leads us to an argument that perhaps the most realistic indicator for comparison, at this stage of Macedonian agriculture, is the gross farm income, given that the total output value and the intermediate consumption are fairly accurate (also noted by Keszthelyi, 2005).

Table 5. Farm income comparison in 2004, EUR
Preglednica 5. Primerjava dohodkov za leto 2004, EUR

	Total output Skupni prihodek (SE 131)	Gross farm income Bruto dodana vrednost (SE410)	Farm net value added Neto dodana vrednost (SE415)	Family farm income Dohodek kmečke družine (SE420)
Average per agricultural holding / Povprečje na kmetijsko gospodarstvo				
Greece	16,982	14,478	12,171	10,380
France	122,742	71,697	49,221	27,579
Hungary	55,792	24,966	18,111	6,607
Italy	55,281	37,174	30,676	24,555
Netherlands	278,710	123,037	88,687	29,793
Poland	19,027	9,656	6,850	5,872
Sweden	119,831	55,647	28,491	6,529
Slovenia	15,537	10,900	6,472	4,895
EU-25	**61,935**	**36,615**	**28,086**	**18,097**
Macedonian sample Makedonski vzorec	10,371	5,474	4,575	4,113
Average per 1 ha UAA / Povprečje na ha KZU				
Greece	2,679	2,284	1,920	1,637
France	1,666	973	668	374
Hungary	1,129	505	366	134
Italy	3,300	2,219	1,831	1,466
Netherlands	8,944	3,949	2,846	956
Poland	1,210	614	436	374
Sweden	1,284	596	305	70
Slovenia	1,228	862	512	387
EU-25	**1,804**	**1,067**	**818**	**527**
Macedonian sample Makedonski vzorec	2,517	1,328	1,110	998

Source: Survey (applicative model) and own calculations based on FADN public database

DISCUSSION AND CONCLUSIONS

Having a functional farm accountancy data system in Macedonia, in compliance with the EU-FADN, will be useful both on micro level (farm management purposes) and macro level (agricultural policy makers, extension and science).

The research revealed that the Macedonian farms, in physical and economic terms, are far smaller than the EU average. With high level of production factors, especially labour, the farms reach a low level of economic output. Most of the farms (82%), according to the FADN methodology and economic size classification, fall into the group of very small and small farms.

The average engagement of annual working units in Macedonia was 2.0 AWU at 4.2 ha UAA/farm, while the European parallel was 1.7 AWU at average 34.3 ha UAA/farm. This indicates that the Macedonian farms have major issues to confront, mainly with regard to the possibilities to improve the farm efficiency and labour productivity.

Still, the general conclusion is that the structure is comparable to at least a portion of the EU countries. The size of Macedonian farms in economic terms (5.9 ESU) was five times smaller

than the EU-25 average (32.7 ESU), but still relatively close to farm's size in Slovenia, Greece or Poland (ranging from 7.3–9.4 ESU). It is mostly probable that the structure and size does not demonstrate high competitiveness of the sample farms, but still taking into consideration that no subsidies were available in the research period, certain development potentials are displayed.

The inclusion of the non-agricultural income in the survey proved to be a useful indicator of the farmers activity. The Macedonian farms off-farm income demonstrated consistent decline as the economic size of the farm increased. The smaller the farm in the sample, the higher the off-farm income i.e. very small farms realised an additional income of approximately 1,900 EUR; small farms - 1,140 EUR; medium-low farms - 470 EUR and medium high farms generated no extra income.

When farm family income indicators are compared at farm level, the Macedonian holdings achieve the lowest average value when weighted against the EU countries comparison panel. One of the major conclusions with regard to the farm income comparison is the observation that the gross farm income is possibly the most appropriate level to consider. In this context, the gross farm income of the Macedonian sample was around 5,500 EUR/farm, representing about 15% of what an average EU farm generated at that level. The differences between the various income levels indicate methodological weaknesses. The family farm income participates with 50% in the gross farm income in the EU, with high variability from 12 to 73 percentages; the same ratio calculated the Macedonian sample is 75%. When comparing the Macedonian results to those from the EU farms, the total output and intermediate consumption (specific and overhead costs) can be regarded as consistent, and accordingly the gross farm income indicator as considerably accurate.

The Macedonian farmers will face major challenges in the EU pre-accession period. The expected effect of EU-integration process is that the structure of the holdings will gradually change towards larger, primarily commercial and competitive farms; subsequently the income of farms will assumingly improve and move closer to EU levels, at least to those of the countries that joined in the last two enlargement cycles. The subsistence farmers will not gain a lot from the accession, especially if the regional and rural development policy does not increase employment opportunities (Erjavec and Dimitrievski, 2008).

The country's strategic policy aims at strengthening the competitive ability of Macedonian agriculture by increasing the sector efficiency. The investments in agricultural holdings targeting farm modernisation, reconstruction and renewal of the assets, supported by the national agricultural policy and the imminent pre-accession funds, will increase the competitiveness of Macedonian farms and ultimately improve the farm income.

The FADN methodology prospectively could be complemented with more analytical approach when gathering farm enterprises cost data by including input specific quantitative data. The presence of this information (quantity of inputs and applied technology) could be used to build 'technical matrixes' in the standard-type models for the ex-ante analysis of the effects of certain agricultural policies and to tackle with a greater degree of accuracy the problems linked to the technical efficiency and the analysis of the production processes (Paris and Arfini, 1999).

The technical and economic parameters resulting from an accountancy information system are a valuable source for further scientific and applicative research. Once a functional farm accountancy system has been established, one of the directions in which the analysis may focus is by applying the Positive Mathematical Programming (PMP). PMP methodology is suitable for policy analysis using the FADN data. This methodology features exploitation of positive information which reflect the farmer behaviour and estimate the level of gross margin for the whole farm. In just one PMP model it is possible to include all the farms having homogeneous character improving its ability as tool for policy analysis (Paris and Arfini, 1999).

The farm income analysis of Macedonian farms proved to provide comparable data using the harmonised EU-FADN methodology. The methodology was tested so it can further be applied

on a representative sample. The interpretation and analysis of farm level data supply sufficient information on the farm income for decision makers, thus enabling them to make informed decisions.

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INFLUENCE OF RAW MATTER ORIGIN AND PRODUCTION PERIOD ON FATTY-ACID COMPOSITION OF DRY-CURED HAMS

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ABSTRACT

We have here investigated the fatty acid composition in the muscle (Mm. *biceps femoris, semitendinosus and semimembranosus*) of Vipava and Vipava-style hams made from fresh pork legs that originate from Slovenia, and from Germany and Italy, respectively. Dry-cured hams are produced under technology protected according to recognized geographical indications for Vipava ham, a salt-cured ham that is air-dried rather than smoked. The fatty acid compositions of samples were determined by gas-liquid chromatography following *in-situ* transesterification. On average, hams contained 6.89% of intramuscular and intermuscular fat, with the fatty-acid composition as 50.0% monounsaturated, 11.8% polyunsaturated (PUFA) and 38.0% saturated fatty acids. The origin of the raw matter has significant influence the PUFAs in hams, which were 2.0% lower in products from pigs of Slovenian (own) rearing. The important indicators of lipid nutritive value, as P/S ratio (0.31), content of *n*-3 (0.68%) and *n*-6 (9.02%) PUFAs, and ratio of *n*-6/*n*-3 PUFAs (14.1), are similar to those cited in the literature for other types of dry-cured hams from south European regions produced from pigs reared under intensive systems.

Keywords: meat products / dry-cured ham / Vipava ham / pork legs / origin / fat / composition / fatty acids / Slovenia

VPLIV POREKLA STEGEN IN STOPNJE ZRELOSTI NA MAŠČOBNOKISLINSKO SESTAVO LIPIDOV (VIPAVSKEGA) PRŠUTA

IZVLEČEK

V članku je predstavljena maščobnokislinska sestava intra- in intermuskularne maščobe mišic (Mm. *biceps femoris, semitendinosus* in *semimembranosus)* pršutov, zaščitenih z geografsko označbo 'Vipavski pršut' in proizvedenih iz slovenske surovine, in pršutov, proizvedenih po isti tehnologiji (v tipu Vipavskega pršuta), vendar sveža stegna izvirajo iz Nemčije ali Italije. Proizvedeni so bili po tradicionalni tehnologiji, t.j. na način, ko se suho soljena prašičja stegna sušijo na zraku brez predhodnega prekajevanja. Maščobno kislinski profil intramuskularne maščobe smo določili s plinsko-tekočinsko kromatografijo po metodi *in situ* transesterifikacije. V povprečju pršuti vsebujejo 6,89 % intra- in intermuskularne maščobe, glede maščobnokislinske sestave pa 50 % pripada enkrat nenasičenim, 11,8 % večkrat nenasičenim (VNMK) in 38 % nasičenim maščobnim kislinam. Poreklo surovine značilno vpliva na delež VNMK v pršutih, ki je dva odstotka (od skupnih maščobnih kislin) manjši v pršutih izdelanih iz slovenske kot nemške in italijanske surovine. Pomembni pokazatelji prehranske vrednosti

lipidov, kot so razmerje P/S (0,31), vsebnost *n*-3 (0,68 %), vsebnost *n*-6 (9,02 %) VNMK in razmerje *n*-6/*n*-3 (14,1), so podobni podatkom, ki jih navaja literatura za druge vrste pršutov iz južnih držav evropske regije, proizvedenih iz intenzivno vzrejenih prašičev.

Ključne besede: mesni izdelki / pršut / Vipavski pršut / stegna / poreklo / maščobe / sestava / maščobne kisline / Slovenija

INTRODUCTION

Dry-cured hams are appreciated by consumers because of their sensory traits and their image as traditional products. At present, traditional technologies are used to produce high quality dry-meat products with attractive sensory qualities, such as colour, aroma and texture, and high nutritional value due to a very high proteins content. The drying and long ripening periods are processes that can form not only typical sensory traits and microbiological stability, but also change the composition and nutritional value of proteins and lipids.

Lipids in muscle and adipose tissues undergo intense degradation during processing, including lipolysis and oxidation, which generates numerous volatile compounds and consequently the typical aroma notes of dry-cured meat products (Gandemer, 1999, 2002). This degradation also has key roles for changes in the lipid composition and nutritional value of dry-cured ham. In southern European countries, the production processes include the standard steps of salting, drying and ripening. However, there are large differences in the relative times and temperature humidity cycles, mainly during drying and ripening, according to the processes used for each product in each country (Toldra and Flores, 1998). These large variations in processing conditions can affect the kinetics of lipolysis and oxidation reactions to great extents (Gandemer, 2002).

The quality of dry-cured hams is related to both the quality of the raw matter (adipose tissue and muscle) and the control of the complex biochemical reactions that take place during the ham processing. The quality of the raw matter is directly related to the rearing condition of the pigs (Lopez-Bote, 1998; Toldra and Flores, 1998). In European countries, most of the dry-cured and dry-fermented meat products are manufactured from muscle and adipose tissue of pigs reared under intensive systems. The pigs are from industrial genotypes and their crossbreeds, and they are slaughtered at 100–120 kg, at around 5–6 months of age. For dry-cured ham production, e.g. Parma and San Daniele hams, the pigs are slaughtered when they are heavier (160–180 kg) and older (9–12 months). The muscle and adipose tissue of these pigs show very similar compositional traits (Bosi *et al.*, 2000). The raw matter of dry-cured hams produced in Slovenia is mainly from European (Carso hams) or solely Slovenian pig production (Vipava hams) from middle weight pigs (120–140 kg) reared under intensive systems. Vipava ham is a Slovenian product that has protected geographical indications (IGP) (Regulations on Dry-ham of Vipava…, 2007), and it is from pigs of Slovenian production; it is processed by an original technology that uses a longer ripening. This provides a product of specific sensory quality, with a nutritional value that is most probably related to changes in the lipid fatty-acid composition.

The lipid composition of meat products is important in the context of balanced nutrition. Fresh pork leg (*m. biceps femoris*), which is also known as fresh ham, is the uncured hind leg of the hog, and shows a lipid fatty-acid composition mostly of monounsaturated fatty acids (MUFAs), such as oleic acid (18:1*c*-9), and saturated fatty acids (SFAs), such as palmitic (16:0), stearic (18:0) and myristic (14:0) acids. There are lower levels of polyunsaturated fatty acids (PUFAs), such as the *n*-6 linoleic (18:2*c*-9,12), α-linolenic (18:3*c*-9,12,15) and arachidonic (20:4*c*-5,8,11,14) PUFAs, and the long-chain *n*-3 eicosapentaenoic (EPA; 20:5*c*-5,8,11,14,17), docosapentaenoic (DPA; 22:5*c*-7,10,13,16,19) and docosahexaenoic (DHA; 22:6*c*-4,7,10,13,16,19) PUFAs (Golob *et al.,* 2006). Similar fatty-acid compositions are seen for the adipose tissue from pigs of various European areas that are used also for the production of

different protected dry-cured hams (e.g. Parma, Bayonne and Serrano), as indicated by Gandemer (2002), Fernandez *et al.* (2007) and Webb and O'Neill (2008).

The fatty-acid profile of the fresh pork leg has been changing under traditional technologies, and consequently in Carso ham some indicators of the lipid nutritional value have been improved, such as the PUFA/SFA (P/S) ratio (from 0.36 to 0.43) and the *n*-6/*n*-3 ratio (from 12.4 to 9.7) (Golob *et al.*, 2006). Spanish (Iberian, Serrano) and French (Bayonne, Corsican) dry-cured hams also show significantly lower SFA content (27.6%–37.1%) and higher MUFA content (55.9%–65.1%) (Gandemer, 2002). High amounts (10.2%) of linoleic acid have been reported for Serrano hams (Fernandez *et al.*, 2007).

However, as the components of the raw matter, the lipids in the muscle and adipose tissues can vary greatly, both quantitatively and qualitatively, according to a range of factors, including the animal species, age, sex and diet (Toldrá, 1998; Fernandez *et al.*, 2007). There is little data available on the fatty-acid composition of dry-cured hams (and none available for Vipava ham) and its nutritional value in the human diet. Likewise, the influence of the ripening duration and the origin of the raw material on fatty-acid composition has rarely been investigated. Thus, the purpose of the present study was to determine the content of intermuscular and intramuscular fat and the fatty-acid composition in the muscle (Mm. *biceps femoris, semitendinosus and semimembranosus*) of Vipava and Vipava-style hams made from fresh pork legs that originate from Slovenia, and from Germany and Italy, respectively.

MATERIALS AND METHODS

Materials

A total of 25 fresh pork legs (10 from Slovenia, 10 from Germany and five from Italy) were included in this study. The weights of the fresh legs shaped in the Vipava style were between 10 kg and 12 kg. The traditional technology that is protected according to the geographical indication as 'Vipava ham' includes the salting of the hams with sea salt, post-salting (over 11 days at temperatures of 0 °C and 5 °C), and rest ('riposo'; with or without ventilation at temperatures of 0 °C and 8 °C, with a total duration of these initial phases at a minimal 70 days. This is followed by the drying/ ripening at 12 °C to 22 °C, for a total of 12 months or 18 months.

Five experimental groups were included: V-S (Vipava hams produced from Slovenian fresh pork legs and ripened 12 months, as the standard procedure), V-L (Vipava hams produced from Slovenian fresh pork legs and ripened 18 months), Vs-GS (Vipava-style hams produced from German fresh pork legs and ripened 12 months), Vs-GL (Vipava-style hams produced from German fresh pork legs and ripened 18 months) and Vs-IS (Vipava-style hams produced from Italian fresh pork legs and ripened 12 months).

Samples for the determination of fatty acids were taken as 1-cm-thick slices of ham (intramuscular and intermuscular fat without subcutaneous fat). The slices were taken from caudal parts of each ham, and transversal on *os femoris* – the central part containing the *biceps femoris, semimembranosus* and *semitendinosus* muscles. The samples were homogenised, vacuum packed, and frozen at –20 °C until their analysis.

Intramuscular and intermuscular fat content

The intramuscular and intermuscular fat (IMF) contents were determined by the method described in AOAC Official Method 991.36. Fat (Crude) in Meat and Meat Products (AOAC, 1997). The total lipids were extracted by hot treatment with petroleum ether as the solvent.

Fatty-acid composition

The fatty-acid compositions of the samples were determined by gas-liquid chromatography (GLC), using *in-situ* transesterification (Park and Goins, 1994). The fatty acid methyl ester (FAME) contents were determined by GLC, on an Agilent Technologies 6890 gas chromatograph, with a flame ionisation detector and an Agilent Technologies HP-88 capillary column (Cat.No. 112-88A7) (100 m × 0.25 mm × 0.2 μm). The separation and detection were performed under the following conditions: temperature programme, 150 °C (held for 10 min), 2 °C/min to 180 °C (40 min), 3 °C/min to 240 °C (85 min); injector temperature, 250 °C; detector temperature 280 °C; injector: split:splitless, 1:30; volume, 1 μL; carrier gas, He 2.3 mL/min; make-up gas: N_2 45 mL/min; detector gases: H_2 40 mL/min; synthetic air (21% O_2) 450 mL/min.

The FAMEs were determined through their retention times in comparison to those of the following standard mixtures: Supelco fatty acid methyl ester mix – 37 components (Cat. No. 18919-1AMP); Supelco PUFA No.1: Animal source (Cat. No. 47015-U); Supelco Linoleic Acid Methyl Ester cis/trans Isomer Mix (Cat.No. 47791); Supelco cis-7-octadecenoic methyl ester (Cat.No. 46900-U); cis-11-octadecenoic methyl ester (Cat.No. 46904); Fluka Methyl stearidonate (Cat.No. 43959); Natural ASA CLA 10t, 12c in CLA 9c, 11t; and NuChek standards: GLC-68D, GLC-85, GLC-411 and GLC-546.

The NuChek GLC-68D and GLC-85 standards mixtures were used to determine the response factor, Rf_i, for each fatty acid. The weight portion of each fatty acid in the sample was determined using the Rf_i and the factor of transformation of fatty-acid content from FAME content. The determination of reliability and accuracy of the analytical method for the detection of fatty acids was ensured by the use of the certified reference matrix, CRM 163 (blend beef-pork fat, BCR), which was in good agreement with the certified values. The FAMEs were expressed as percentages of the total fatty-acid content.

Data analysis

The data for the fatty-acid compositions were processed by the GLM procedure (SAS, 1999). The statistical model included the main effects of fresh pork leg supplier group connected with ripening time (V-S, V-L, Vs-GS, Vs-GL, Vs-IS). The means of the experimental groups were obtained using the Duncan procedure, and were compared at the 5% probability level (SAS, 1999).

RESULTS AND DISCUSSION

Recently with human nutrition, the emphasis has shifted away from fat quantity to fat quality, as related to fatty-acid composition. SFAs have generally been labelled as the culprits for cancers and coronary heart disease, although C18:0 is considered as a neutral fatty acid. It is recommended that the total lipid intake should be 30% of the total energy intake. From 10% to 30% of the lipid energy should be from SFAs (Enser *et al.*, 1996). However, more recently, nutritionists have focused on the type of PUFA and the balance in the diet between the *n*-3 PUFAs, such as α-linolenic acid (18:3), and the *n*-6 PUFAs, such as linoleic acid (18:2). It has also been reported that the ratio of *n*-6:*n*-3 PUFAs can provide a risk factor for cancers and coronary heart disease, and especially for the formation of blood clots leading to a heart attack. The recommendation is for a ratio of less than 4.0 (Wood *et al.,* 2003). As with the P/S ratio, the meats can be manipulated to provide a more favourable *n*-6:*n*-3 ratio.

The IMF contents for the dry-cured hams produced from fresh pork legs that originated from three different countries and according to different ripening times are shown in Table 1. Across

all five groups, the IMF contents were similar, at a mean of 6.9%. These IMF contents are in agreement with findings in other studies, with levels reported of 8.8% in Parma (Fiego *et al.*, 2005), 9.3% in Iberian, 5.3% in Corsican, 3.5% in Serrano and 2.6% in Bayonne (Gandemer, 2002) hams. We also note that the chemical analyses in the present study were carried out with average samples that were obtained by mixing the *biceps femoris, semimembranosus* and *semitendinosus* muscles.

Table 1. Intramuscular and intermuscular fat (%) content (IMF) of dry-cured hams produced from fresh pork legs from three different countries and according to ripening times

Preglednica 1. Vsebnost mišične in medmišične maščobe (%) v pršutih, proizvedenih iz svežih prašičjih stegen različnega izvora in stopnje zrelosti

	Ham source (N = 5 × 10 = 50)						
	V-S	V-L	Vs-GS	Vs-GL	Vs-IS	Sign.	Overall mean
IMF ($\bar{x} \pm$ SD)	7.32 ± 2.2^a	7.27 ± 1.1^a	6.30 ± 1.06^a	6.26 ± 1.0^a	7.18 ± 0.8^a	Ns	6.89 ± 1.4

Mean values ± standard deviation for each group. N, number of observations. V-S (Vipava hams, ripened 12 months), V-L (Vipava hams, ripened 18 months), Vs-GS (Vipava-style hams, German origin, ripened 12 months), Vs-GL (Vipava-style hams, German origin, ripened 18 months) and Vs-IS (Vipava-style hams, Italian origin, ripened 12 months). Sign., statistical significance: not significant, Ns, $P > 0.05$.

The weight percentages of all of the fatty acids and some of the calculated indicators of the lipid nutritive values for the Vipava and Vipava-style hams are shown in Table 2.

The total SFA content in the hams was on average 38.0%, and the differences between all of the groups are not significant. The most prevalent SFAs were palmitic (16:0) (23.7% of total fatty acids) and stearic (18:0) (12.1%) acids. The Italian (Parma), Spanish (Serrano, Teruel) and French (Bayonne) dry-cured hams had similar contents of palmitic and stearic acids (Gandemer, 2002; Larrea *et al.*, 2007). The origins of the raw matter in our study did not affect the SFA compositions of the hams. Only two SFAs (lauric 12:0 and myristic 14:0 acids) showed low concentrations that decreased significantly with longer ripening (18 months; products V-L and Vs-GL). Both of these SFAs are known to be atherogenic (Ulbricht and Southgate, 1991).

The overall mean level of MUFAs was 50.0% of the total fatty acids, which did not differ significantly between the five groups of dry-cured hams. The most prevalent was oleic acid (18:1*n*-9; 44.6%), with no significant effects seen for the raw matter origins and ripening times of the hams. Only two MUFAs with lower proportions differed significantly ($P \leq 0.05$) between the five groups; V-L showed the lowest proportion (2.98%) of palmitoleic acid (16:1*c*-9) and the highest proportion (0.95%) of gadoleic acid (20:1*c*-9).

The PUFA content in the hams was on average 11.8% of the total fatty acids, and was significantly ($P \leq 0.05$) influenced by the raw matter origin, but not by time of ham ripening. The products of Slovenian origin (V-L and V-S) contained approximately 2% lower levels of PUFAs compared to the other groups. Of note, linoleic acid (18:2*n*-6) showed an overall high mean proportion (8.95%), which was significantly ($P \leq 0.05$) lower in products of Slovenian origin (V-S, 7.85%; V-L, 8.63%) compared to the other hams (Vs-GL, Vs-GS, Vs-IS), where it was between 9.23% and 9.86%, close to the 10.2% linoleic acid reported previously for Serrano ham (Fernandez *et al.*, 2007).

There were some long-chain *n*-3 PUFAs in the hams. The content of DPA was significantly ($P \leq 0.05$) lower in the V-L (0.13%) and V-S (0.15%) hams, compared with the samples with other origins, such as Vs-GL (0.22) and Vs-GS (0.26%). An extended ripening of hams had no significant effects on the *n*-3 PUFAs.

Table 2. Fatty acids (g/100 g fatty acids) content (means ±standard deviation) of dry-cured hams produced from fresh pork legs from three different countries and according to ripening times

Preglednica 2. Maščobnokislinska sestava (g/100 g skupnih maščobnih kislin; povprečne vrednosti ± standardni odklon) pršutov, proizvedenih iz svežih prašičjih stegen različnega izvora in stopnje zrelosti

	Ham source (N = 5 × 5 = 25)						
Fatty acids, $\bar{x} \pm SD$	V-S	V-L	Vs-GS	Vs-GL	Vs-IS	Sign.	Overall means
8:0	0.01 ±0.01^{a}	0.02^{a}	0.02^{a}	0.02^{a}	0.02^{a}	Ns	0.02
10:0	0.13 ±0.03^{a}	0.11 ±0.01^{a}	0.12 ±0.05^{a}	0.11 ±0.02^{a}	0.12 ±0.02^{a}	Ns	0.12 ±0.03
11:0	0.01 ±0.01^{a}	0.00^{a}	0.01 ±0.01^{a}	0.00 ±0.01^{a}	0.00 ±0.01^{a}	Ns	0.00 ±0.01
12:0	0.10 ±0.02^{abc}	0.08 ±0.01^{c}	0.12 ±0.02^{a}	0.10±0.04^{bc}	0.11 ±0.01^{ab}	**	0.10 ±0.02
12:1*c*-3	0.07 ±0.03^{a}	0.05 ±0.02^{b}	0.06 ±0.02^{ab}	0.06 ±0.02^{a}	0.05 ±0.01^{ab}	Ns	0.06 ±0.02
13:1*c*-3	0.04 ±0.01^{a}	0.03 ±0.01^{b}	0.04 ±0.01^{ab}	0.04 ±0.01^{a}	0.03 ±0.01^{ab}	*	0.03 ±0.01
14:0	1.43 ±0.16^{a}	1.35 ±0.19^{ab}	1.45 ±0.17^{a}	1.27 ±0.08^{b}	1.43 ±0.09^{a}	*	1.39 ±0.15
14:1*t*-5	0.02^{a}	0.02^{a}	0.03 ±0.01^{a}	0.02 ±0.01^{a}	0.03 ±0.02^{a}	Ns	0.02 ±0.01
14:1*c*-5	0.07 ±0.01^{a}	0.05 ±0.01^{b}	0.06 ±0.01^{a}	0.06 ±0.01^{a}	0.06 ±0.01^{ab}	*	0.06 ±0.01
15:0	0.05 ±0.01^{ab}	0.05 ±0.01^{a}	0.05 ±0.01^{a}	0.04 ±0.01^{b}	0.04 ±0.01^{b}	**	0.05 ±0.01
15:1*c*-5	0.25 ±0.07^{a}	0.18 ±0.07^{a}	0.25 ±0.05^{a}	0.25 ±0.06^{a}	0.21 ±0.04^{a}	Ns	0.23 ±0.06
15:1*c*-10	0.03 ±0.01^{a}	0.02 ±0.01^{a}	0.03 ±0.01^{a}	0.03 ±0.01^{a}	0.03 ±0.01^{a}	Ns	0.03 ±0.01
16:0	24.4 ±1.0^{a}	23.9 ±1.4^{a}	22.6 ±3.5^{a}	23.7 ±0.7^{a}	24.0 ±0.7^{a}	Ns	23.7 ±1.9
16:1*t*-9	0.23 ±0.03^{a}	0.22 ±0.02^{a}	0.26 ±0.05^{a}	0.23 ±0.04^{a}	0.21 ±0.02^{a}	Ns	0.23 ±0.04
16:1*c*-9	3.64 ±0.43^{a}	2.98 ±0.65^{b}	3.27 ±0.32^{ab}	3.30 ±0.65^{ab}	3.25 ±0.26^{ab}	*	3.29 ±0.51
17:0	0.27 ±0.08^{b}	0.33 ±0.05^{a}	0.28 ±0.07^{b}	0.23 ±0.06^{b}	0.22 ±0.05^{b}	***	0.27 ±0.07
17:1*t*-10	0.25 ±0.10^{a}	0.30 ±0.10^{a}	0.26 ±0.06^{a}	0.22 ±0.06^{a}	0.20 ±0.05^{a}	Ns	0.24 ±0.08
17:1*c*-10	0.04 ±0.01^{ab}	0.04 ±0.01^{b}	0.05 ±0.01^{a}	0.04 ±0.02^{b}	0.04 ±0.01^{b}	*	0.04 ±0.01
18:0	12.2 ±0.8^{a}	12.7 ±0.9^{a}	12.1 ±1.4^{a}	11.6 ±0.4^{a}	11.8 ±0.9^{a}	Ns	12.1 ±1.0
18:1*t*-9	0.11 ±0.01^{b}	0.11 ±0.01^{b}	0.11 ±0.02^{b}	0.13 ±0.02^{a}	0.11 ±0.02^{b}	*	0.11 ±0.02
18:1*c*-9	44.9 ±2.2^{a}	44.6 ±0.9^{a}	44.4 ±2.6^{a}	44.7 ±0.9^{a}	44.5 ±1.1^{a}	Ns	44.6 ±1.6
18:2*c*-9,12	7.85 ±1.56^{b}	8.63 ±0.80^{ab}	9.86 ±2.39^{a}	9.25 ±0.96^{a}	9.23 ±0.78^{a}	*	8.95 ±1.56
18:3*c*-6,9,12	0.00 ±0.01^{a}	0.01 ±0.01^{a}	0.00 ±0.01^{a}	0.01 ±0.01^{a}	0.00 ±0.01^{a}	Ns	0.01 ±0.01
20:0	0.16 ±0.03^{a}	0.17 ±0.03^{a}	0.16 ±0.02^{a}	0.16 ±0.02^{a}	0.16 ±0.03^{a}	Ns	0.16 ±0.03
18:3*c*-9,12,15	0.27 ±0.06^{c}	0.31 ±0.04^{bc}	0.62 ±0.24^{a}	0.40 ±0.18^{b}	0.32 ±0.05^{bc}	***	0.38 ±0.18
20:1*c*-11	0.72 ±0.05^{c}	0.95 ±0.05^{a}	0.81 ±0.07^{b}	0.70 ±0.15^{c}	0.87 ±0.09^{b}	***	0.81 ±0.12
21:0	0.11 ±0.12^{a}	0.07 ±0.05^{a}	0.04 ±0.04^{a}	0.08 ±0.07^{a}	0.07 ±0.09^{a}	Ns	0.07 ±0.08
20:2*c*-8,11	0.27 ±0.02^{b}	0.37 ±0.03^{a}	0.34 ±0.12^{a}	0.33 ±0.08^{a}	0.38 ±0.06^{a}	**	0.34 ±0.08
20:3*c*-8,11,14	0.06 ±0.02^{b}	0.05 ±0.01^{b}	0.06 ±0.01^{ab}	0.07 ±0.01^{a}	0.06 ±0.01^{ab}	*	0.06 ±0.01
20:3*c*-11,14,17	0.17 ±0.06^{b}	0.19 ±0.02^{ab}	0.19 ±0.02^{ab}	0.21 ±0.03^{a}	0.18 ±0.03^{b}	*	0.19 ±0.04
20:4*c*-5,8,11,14	1.52 ±0.52^{a}	1.54 ±0.22^{a}	1.68 ±0.31^{a}	1.91 ±0.37^{a}	1.57 ±0.19^{a}	Ns	1.63 ±0.36
22:1*c*-13	0.00^{a}	0.00 ±0.01^{a}	0.00^{a}	0.00^{a}	0.00^{a}	Ns	0.00
23:0	0.02 ±0.03^{a}	0.02 ±0.02^{a}	0.03 ±0.04^{a}	0.05 ±0.04^{a}	0.03 ±0.05^{a}	Ns	0.03 ±0.04
20:5*c*-5,8,11,14,17	0.04 ±0.0^{b}	0.04^{b}	0.07 ±0.01^{a}	0.07 ±0.02^{a}	0.01 ±0.02^{c}	***	0.05 ±0.03
24:0	0.00 ±0.01^{a}	0.01 ±0.01^{a}	0.01 ±0.01^{a}	0.01 ±0.02^{a}	0.02 ±0.04^{a}	Ns	0.01 ±0.02
24:1*c*-15	0.22 ±0.09^{b}	0.30 ±0.03^{a}	0.26 ±0.10^{ab}	0.31 ±0.07^{a}	0.31 ±0.05^{a}	*	0.28 ±0.08
22:5*c*-7,10,13,16,19	0.13 ±0.05^{c}	0.15 ±0.03^{c}	0.22 ±0.09^{ab}	0.26 ±0.07^{a}	0.17 ±0.04^{bc}	***	0.18 ±0.07
22:6*c*-4,7,10,13,16,19	0.07 ±0.03^{a}	0.07 ±0.01^{a}	0.07 ±0.05^{a}	0.05 ±0.01^{a}	0.08 ±0.04^{a}	Ns	0.07 ±0.03
SFAs	38.9 ±1.4^{a}	38.8 ±1.2^{a}	37.0 ±3.2^{b}	37.3 ±0.5^{ab}	38.1 ±1.5^{ab}	Ns	38.0 ±1.9
MUFAs	50.7 ±2.3^{a}	49.8 ±1.0^{a}	49.9 ±2.7^{a}	50.0 ±1.1^{a}	49.9 ±1.1^{a}	Ns	50.0 ±1.7
PUFAs	10.4 ±2.2^{c}	11.4 ±1.1^{bc}	13.1 ±2.9^{a}	12.6 ±1.3^{ab}	12.0 ±0.9^{abc}	*	11.8 ±2.0
P/S	0.27 ±0.06^{c}	0.29 ±0.03^{bc}	0.36 ±0.11^{a}	0.34±0.04^{ab}	0.32 ±0.04^{abc}	**	0.31 ±0.07
n-3	0.51 ±0.15^{c}	0.58 ±0.07^{c}	0.98 ±0.29^{a}	0.78 ±0.22^{b}	0.58 ±0.08^{c}	***	0.68 ±0.25
n-6	7.91 ±1.57^{b}	8.69 ±0.81^{ab}	9.92 ±2.38^{a}	9.33 ±0.95^{a}	9.30 ±0.78^{a}	*	9.02 ±1.56
n-6/*n*-3	16.1 ±2.6^{a}	15.1 ±1.3^{a}	10.4 ±1.5^{c}	12.7 ±3.6^{b}	16.1 ±1.5^{a}	***	14.1 ±3.1

Mean values ± standard deviation in each group. N, number of samples. V-S (Vipava hams, ripened 12 months), V-L (Vipava hams, ripened 18 months), Vs-GS (Vipava-style hams, German origin, ripened 12 months), Vs-GL (Vipava-style hams, German origin, ripened 18 months) and Vs-IS (Vipava-style hams, Italian origin, ripened 12 months). If SD < 0.01 g/100 g fatty acids, the values are not given. SFAs, saturated fatty acids. MUFAs, monounsaturated fatty acids. PUFAs, polyunsaturated fatty acids. P/S, PUFA/SFA. Sign., statistical significant: Ns, $P > 0.05$; * $P \leq 0.05$ and ** $P \leq 0.01$; *** $P \leq 0.001$.

The overall mean *n*-3 fatty-acid content in the hams was 0.68% and it was significantly influenced by the origin of the raw matter and the ripening time of the products ($P \leq 0.001$). Lower contents of *n*-3 PUFAs were seen for the V-S, V-L and Vs-IS samples (0.51% to 0.58%) than for those of Vs-GL (0.78%) and Vs-GS (0.98%). The extended ripening of hams had different influences on *n*-3 PUFA content: non-significant lower proportions in V-S *vs.* V-L sample (0.51 *vs.* 0.58%; $P > 0.05$), and significant higher proportion in Vs-GL *vs.* Vs-GS (0.98 *vs.* 0.78%; $P < 0.05$). Recommendations for the daily intake of *n*-3 PUFAs are 0.45 g for adults (FSA, 2004). Chapkin (1992) recommended 0.8 g of EPA and DHA daily for a healthy adult population, while Simopoulos (2002) recommended 0.65 g of EPA and DHA daily (calculated on an 8,400 kJ diet). All of this indicates that the hams included in our study are good sources of *n*-3 PUFAs, regardless of dry matter origin and ripening stage.

As an indicator of the lipid nutritional value, the P/S ratio did not reach the recommended minimal value of 0.4 (Wood *et al.*, 2003), with the mean value in the present study of 0.31. The German and Italian samples (Vs-GL, Vs-GS and Vs-IS) showed significantly higher P/S ratios (0.32–0.36; $P \leq 0.05$) compared with those Slovenian (V-L and V-S; 0.27–0.29). This appears to be a consequence of the different rearing conditions of the pigs (Lopez-Bote, 1998; Toldra and Flores, 1998). Similar P/S ratios have been reported for five different Spanish dry-cured hams (0.19–0.30; Fernandez *et al.*, 2007), with slightly higher, and nearer the recommended minimal value of above 0.4 reported for Carso dry-cured hams (Golob *et al*., 2006).

The Vipava-style hams produced from fresh pork legs of German origin contained significantly higher amounts of *n*-3 PUFAs (0.78%–0.98%) compared with the Slovenian and Italian hams (0.51%–0.58%). Extended ripening significantly decreased the content of the *n*-3 PUFAs only in the case of the Vs-GS hams ($P \leq 0.05$). The amounts of *n*-6 PUFAs were significantly lower in hams of Slovenian origin (7.9%–8.7%; $P \leq 0.05$) compared with German and Italian ones (9.3%–9.9%). Ripening did not affect the *n*-6 PUFA content, regardless of the origin of raw matter.

The ratios of *n*-6/*n*-3 PUFAs varied significantly across all of the groups, ranging from 10.4 (Vs-GS) to 16.1 (V-S and Vs-IS) ($P \leq 0.001$). Shorter ripened Vs-GS hams showed a nutritionally more favourable *n*-6/*n*-3 ratio of 10.4, with a similar result previously seen for Carso dry-cured ham (9.7) (Golob *et al*., 2006). This means that the samples were approaching the recommended values of *n*-6/*n*-3 ratio from 5 to 10 (WHO, 1994), although they did not reach the optimal ratio of 1 to 4 mentioned by Simopoulos (2002).

CONCLUSIONS

The intramuscular and intermuscular lipids of Vipava hams are composed of 38% SFAs, 50% MUFAs and 12% PUFAs. The origin of the raw matter has significant influence on the share of PUFAs in dry-cured hams, which is 2% lower in products from pigs of Slovenian (own) rearing. The extended ripening times of the hams decreased the contents of *n*-3 PUFAs in samples of German raw matter origin, differences are coincidental. The hams used in our study are relatively good sources of *n*-3 PUFAs (0.68%), regardless of the raw matter origin and ripening stage.

Important indicators of lipid nutritive values, such as the P/S ratio (0.31), the content of *n*-3 (0.68%) and *n*-6 (9.02%) PUFAs, and the ratio of *n*-6/*n*-3 PUFAs (14.1), on average did not reach recommended values for balanced (safe) nutrition, although they are similar to literature citations of other types of dry-cured hams from south European regions, produced from pigs reared under intensive systems.

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THE MICROBIOLOGICAL QUALITY OF RAW MILK AFTER INTRODUCING THE TWO DAY'S MILK COLLECTING SYSTEM

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ABSTRACT

This study was carried out to investigate the quality of raw milk after the decision of the dairies to collect the milk every two days and not daily as it has been performed till the year 2005. In wider central Slovenian production area we collected in winter and summer season 203 raw milk samples obtained from farm bulk milk tanks, collecting points and transportation tanks at the entrance to the dairy was studied. The total bacterial count, the number of coliforms, psychrotrophic micro-organisms, coagulase-positive staphylococci, yeasts and moulds were analysed using standard methods. The presence of mould species and production of aflatoxins were studied as well. The total bacterial count was higher than 100 000 cfu/ml in 48 (23.6%) out of all tested samples. Its mean value in all milk samples was 4.5 $\log_{10}$ cfu/ml*. The mean numbers of coliform bacteria, psychrotrophic micro-organisms, yeasts and moulds together, and coagulase-positive staphylococci were 2.1 $\log_{10}$ cfu/ml, 3.7 $\log_{10}$ cfu/ml, 2.3 $\log_{10}$ cfu/ml and 1.97 $\log_{10}$ cfu/ml, respectively. The yeasts were present in 95.0% of raw milk samples with the mean concentration of 1.7 $\log_{10}$ cfu/ml. Moulds were found in 63.3% of raw milk samples, their mean concentration was 0.6 $\log_{10}$ cfu/ml. Isolated mould strains belonged to genera *Geotrichum* (51.5%), *Aspergillus* (33.8%), *Mucor* (5.9%), *Fusarium* (2.9%) and *Penicillium* (2.9%). None of the isolated *Aspergillus* strains with typical growth on AFPA medium produced aflatoxin M_1 on YES or YGC medium supplemented with Methyl-β-cyclodextrin.

Key words: raw milk / microbiological quality / total bacterial count / moulds / aflatoxin M_1

MIKROBIOLOŠKA KAKOVOST SUROVEGA MLEKA PO UVEDBI SISTEMA DVODNEVNEGA ZBIRANJA

IZVLEČEK

Preučevali smo mikrobiološko kakovost odkupljenega surovega mleka po odločitvi mlekarn, da bodo mleko zbirale vsake dva dni in ne več dnevno, kot so to izvajale do konca leta 2005. V osrednjem proizvodnem področju Slovenije smo v zimskem in letnem obdobju odvzeli 203 vzorcev mleka iz hladilnih bazenov posameznih proizvajalcev, zbiralnic ter transportnih cistern na sprejemu mlekarne. S standardnimi mikrobiološkimi metodami smo ugotavljali skupno število aerobnih mezofilnih mikroorganizmov, koliformnih in psihrotrofnih mikroorganizmov, število kvasovk in plesni ter koagulaza-pozitivnih stafilokokov. Ugotavljali smo tudi prisotnost posameznih vrst plesni in njihovo sposobnost tvorbe aflatoksinov. Skupno število mikroorganizmov je presegalo 100 000 ke/ml† v 48 (23,6 %) od vseh preiskanih vzorcev.

* Abbreviations: cfu/ml = the number of colony forming units per millilitre of the sample.

† Kratice: ke/ml = število kolonijskih enot v ml vzorca.

Njihovo povprečno število v vseh vzorcih je bilo 4,5 $\log_{10}$ ke/ml. Povprečno število koliformnih mikroorganizmov je znašalo 2,1 $\log_{10}$ ke/ml, število psihrotrofnih mikroorganizmov 3,7 $\log_{10}$ ke/ml, skupno število kvasovk in plesni 2,3 $\log_{10}$ ke/ml in število koagulaza-pozitivnih stafilokokov 1,97 $\log_{10}$ ke/ml. Kvasovke s povprečno koncentracijo 1,7 $\log_{10}$ ke/ml smo ugotovili v 95 % vzorcev, plesni s povprečno koncentracijo 0,6 $\log_{10}$ ke/ml pa v 63,3 % vzorcev. Najpogosteje smo izolirali plesni iz rodov *Geotrichum* (51,5%), *Aspergillus* (33,8 %), *Mucor* (5,9%), *Fusarium* (2,9%) in *Penicillium* (2,9%). Nobeden izmed izoliranih sevov iz rodu *Aspergillus*, ki je kazal značilno rast na gojišču AFPA, ni na gojiščih YES in YGC z metil-β-ciklodekstrinom tvoril aflatoksina M_1.

Ključne besede: surovo mleko/ mikrobiološka kakovost / skupno število mikroorganizmov / plesni / aflatoksin M_1

INTRODUCTION

Milk is a complex biological fluid and by its nature, a good growth medium for many micro-organisms. Because of the specific production it is impossible to avoid contamination of milk with micro-organisms therefore the microbial content of milk is a major feature in determining its quality (Rogelj, 2003). Bacterial contamination of raw milk can originate from different sources: air, milking equipment, feed, soil, faeces and grass (Coorevits *et al*., 2008). The number and types of micro-organisms in milk immediately after milking are affected by factors such as animal and equipment cleanliness, season, feed and animal health (Rogelj, 2003). It is hypothesized that differences in feeding and housing strategies of cows may influence the microbial quality of milk (Coorevits *et al*., 2008). Rinsing water for milking machine and milking equipment washing also involve some of the reasons for the presence of a higher number of micro-organisms including pathogens in raw milk (Bramley, 1990).

After milking, milk is cooled, which additionally influence the dynamic of microbial process (Rogelj, 2003).

The conditions during storage and transport in refrigerated tanks cause the raw milk microbiota to change from predominantly Gram-positive to predominantly Gram-negative organisms as they grow. Gram-negative bacteria usually account for more than 90% of the microbial population in cold raw milk that has been stored. The Gram-negative flora is composed mainly of psychrotrophic species of *Pseudomonas*, *Achromobacter, Aeromonas, Serratia, Alcaligenes, Chromobacterium, Flavobacterium* and *Enterobacter* (Garcia-Armesto *et al*., 1997; Sørhaug *et al*., 1997; Ryser, 1999; Martins *et al*., 2006).

Frank (1997) also mentioned the presence of other genera: *Enterococccus*, Proteus, Lactococcus, *Streptococcus*, *Leuconostoc, Lactobacillus*, *Microbacterium*, *Propionibacterium*, *Micrococcus*, *Bacillus* and coliforms. Organisms unable to grow at refrigeration temperatures remain at low numbers, implying that temperature is an important factor contributing to the prevalence and proliferation of specific organisms in the milk (Jay, 1996; Frank, 1997).

Pasteurization of raw milk is effective in eliminating all but the thermoduric microorganisms of the genera *Microbacterium*, *Micrococccus*, *Streptococcus*, *Lactobacillus*, *Bacillus*, *Clostridium*, the coryneforms, and occasionally some Gram-negative rods (Jay, 1996). Psychrotrophs can grow at refrigeration temperatures below 7 °C, produce enzymes toxins and other metabolites (Jay, 1996) and contribute to high standard plate counts in both raw and pasteurised milk. Since milk is currently handled and stored at low temperatures, these organisms hinder efforts to increase the shelf life of pasteurised milk (Frank, 1997). Most of these bacteria produce extracellular proteolytic and lypolitic enzymes that are secreted into the milk. Many of these enzymes are not inactivated by pasteurizing at 72 °C for 15 s or by Ultra-High Temperature (UHT) treatment (Griffiths *et al*., 1981). The residual activities of these enzymes can reduce the organoleptic quality and shelf life of processed milk products (Fairbairn *et al*., 1986). Pasteurization cannot guarantee the absence of microorganisms, when they are

present in large numbers in raw milk or due to post-pasteurization contamination. (Salmeron *et al*., 2002).

Examination for the presence and number of specific micro-organisms is, therefore, an integral part of any quality control or quality assurance plan and it may be applied to a number of areas: raw materials, intermediate samples, finished products, or environmental/equipment sites. Milk-borne and milk-product borne outbreaks represent 2–6% of bacterial food-borne outbreaks reported by surveillance systems from several countries (De Buyser *et al*., 2001).

Yeasts and moulds in some cheese types can periodically cause problems, both economic and sensory. They usually present in raw milk do not survive pasteurization; their presence in pasteurized milk and other milk products is caused by re-infection during manufacturing (Nelson, 1981; Jodral *et al*., 1993). The contamination of milk products, particularly cheeses is caused by yeasts and moulds present in the environment of cheese factories, like walls and shelves of ripening rooms, air, equipment, water, milk, brine, etc. (Chapman and Sharpe, 1990; Jay, 1992). Of course, the presence of yeasts and moulds in raw milk is undesired, when in manufacturing unpasteurized milk is used.

Yeasts themselves are not commonly the cause of defect in dairy products unless they ferment lactose. In this case, they can grow rapidly and produce a characteristic yeasty or fruity flavour and obvious gas (Davis and Wilbey, 1990). They also produce metabolites, e.g. short-chain fatty acids and other compounds, with known toxic effects against undesired micro-organisms in the intestinal tract (Jakobsen and Narvhus, 1996).

Moulds are important in milk, which is used for the manufacture of cheese and other dairy products. The presence of wild types of moulds is undesirable as they may influence the organoleptic characteristics of the cheeses, they can produce mycotoxins and represent a potential health risk (Jodral *et al*., 1993; Wouters, *et al*., 2002). Mycotoxins are toxic metabolites produced by various fungi growing in a wide range of food and animal feedstuffs. The main mycotoxins that occur frequently are aflatoxins, ochratoxins, patulins, trichothecenes and zearalenones (Gilbert, 2002). The major toxigenic species of fungi belong to genera *Aspergillus, Fusarium, Acremonium* and *Phomopsis* (D'Mello and Macdonald, 1997). Aflatoxins are mycotoxins produced as carcinogenic, teratogenic and mutagenic secondary metabolites by some species of genus *Aspergillus* (Frisvad *et al*., 2005) Aflatoxin B_1 represents the highest degree of toxicity for animals, followed by aflatoxin M_1, G_1, B_2 and G_2 (Gourama and Bullerman, 1995). AFM_1 may be found in the milk of animals that have been fed with feed containing AFB_1.

The objective of this work was to evaluate the level of microbiological contamination of raw milk samples taken from farm cooling milk tanks, collecting points and from bulk raw milk from transportation tanks at the entrance to a dairy. This study was carried out to investigate the quality of raw milk after the decision of the dairies to collect the milk every two days and not daily as it has been performed till the year 2005.

We wished to found out the differences in number of micro-organisms between milk samples taken in winter and summer season and at different collecting stages.

Our interest was also the identification of moulds isolated from samples and the detection of their aflatoxin production.

MATERIALS AND METHODS

Sampling

The collection of the raw milk samples took place in January to February and May to June 2006 at the central part of Slovenia. A total of 203 raw milk samples were analysed, of which 100 samples were taken in winter and 103 of them in summer season. In both seasons 60 of

samples were obtained from collecting points and farm bulk milk tanks, while 40 and 43 of them were collected from transportation tanks at the entrance to the dairy in winter and in summer, respectively. The milk samples were taken in accordance with the instructions given in ISO/DIS 707 (1995). Samples were collected in sterile bottles and transported to the laboratory in cold chain under temperature 6 °C and analysed within 2 hours of sampling.

Mediums, reagents and reference strains

The samples were serially decimal diluted with Quarter strength Ringer's solution (Merck, Germany) and appropriate dilutions plated on media using the pour plate method (ISO/FDIS 8261 (E), 2001).

For the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) the Baird Parker with RPF supplement agar (Biokar Diagnostics, France) was used (SIST EN ISO 6888-2, 1999).

The enumeration of coliform micro-organisms was carried out on VRBL agar (Merck, Germany) according to the standard ISO 4832 (2006).

The presence and number of total bacterial count and psychrotrophic micro-organisms were evaluated on PCA agar (Merck, Germany) with the addition of 0.1% w/v (1 g per 1 l of medium) of skimmed milk powder. The plates were incubated at 30 °C for 72 hours for aerobic mesophilic counts (EN ISO 4833, 2003) and at 6.5 °C for 10 days for psychrotrophic micro-organisms (ISO 6730, 2005).

For the enumeration of yeasts and moulds the YGC agar (Merck, Germany) was used according to the standard ISO 6611(E) (2004).

Aspergillus flavus / parasiticus agar (AFPA base) (Oxoid, England), supplemented with chloramphenicol selective supplement (Oxoid, England) was used for isolation and partially identification of moulds. Yeast extract sucrose agar (YES) (Samson and Hoekstra, 2000) and also yeast glucose cloramphenicol agar (YGC), both supplemented with 0.3% methyl-β-cyclodextrin (Sigma, Germany) and 0.6% sodium deoxycholate (Sigma, Germany) were used for determination of aflatoxin production (Fente *et al*., 2001; Ordaz *et al*., 2003).

Quarter strength Ringer's solution (Merck, Germany) was used for decimal dilution of samples.

Reference strains: *A. flavus* EXF 523 and *A. flavus* EXF 438 were kindly given as a gift from University of Ljubljana, Biotechnical Faculty, Departement for Biology, Slovenia.

Inoculation and enumeration of micro-organisms

The milk samples were serially decimal diluted with Quarter strength Ringer's solution and appropriate dilutions were inoculated by pouring the plates with chosen medium (EN ISO 8261 (E), 2001).

Moulds isolation and identification

Each morphologically different mould colony from the plates with YGC medium was picked up, transferred to YGC and AFPA mediums and incubated for 5 days at 25 °C and for 42 hours at 30 °C, respectively.

Primary classification of colonies from solid mediums YGC and AFPA was based on colony characteristics (pigmentation, shape, background colour) and on microscopic examination of moulds using immersion objective magnifications 100/1.30 and 160/0.17, according to Samson and Hoekstra (2000). The identification of strains *A. flavus* and *A. parasiticus* was confirmed by reverse, yellow to orange pigmentation on AFPA medium (Pitt *et al*., 1983).

Aflatoxin production

For the examination of aflatoxin production at isolated moulds from the genus *Aspergillus*, the YES and YGC medium, supplemented with 0.3% methyl-β-cyclodextrin and 0.6% sodium deoxycholate were used (Fente *et al*., 2001; Ordaz *et al*., 2003). The plates with inoculated strains were incubated for 3 days at 28 °C and then observed under UV light (365 nm).

Statistical analyses

For statistical analyses, the SAS/STAT (2000) was used. Descriptive statistics including average, standard deviation, variability coefficient, minimum and maximum was done. The Pearson's correlation coefficients between variables log number of different groups of tested micro-organisms in milk samples were calculated.

RESULTS AND DISCUSSION

According the Regulatives EU (Regulation 853, 2004) the rolling geometric average of total number of micro-organisms should not exceed 100 000 per ml of raw cow's milk from primary production. The rolling geometric average should be calculated over a two month period, with at least two samples per month. In Slovenia, the microbiological quality of raw milk is very good and is comparable with milk quality in dairy developed countries. In the year 1994 only about 60% of milk, delivered to dairies, contained up to 100 000 micro-organisms per millilitre, while in the year 2005 already 99.5% out of all 448.5 millions litres of raw milk, delivered to dairies, contained less than 100 000 m.o./ml. Up to 50 000 micro-organisms per millilitre contained 93.6% of milk (Valjavec, 2006).

Since the year 2005 some Slovenian dairies have collected the milk from the farmers every two days and not daily any more. Such milk stays in the cooling pools or tanks two days and is mixed with warm milk after every new milking. The microbiological quality and the correlation between different groups of micro-organisms in such milk are different in comparison to the fresh milked milk, which is cooled immediately and take to the dairy daily.

In this study we aimed to investigate the microbiological quality of 203 raw milk samples, collecting in winter (100 samples) and summer season (103 samples). We determined the total bacterial count higher than 100 000 cfu/ml in 48 (23.6%) out of all tested samples.

The mean total aerobic mesophilic micro-organisms, coliform bacteria, psychrotrophic micro-organisms, coagulase-positive bacteria, and yeasts and moulds counts were determined as 4.5 $\log_{10}$ cfu/ml, 2.0 $\log_{10}$ cfu/ml, 3.7 $\log_{10}$ cfu/ml, 1.9 $\log_{10}$ cfu/ml and 2.3 $\log_{10}$ cfu/ml, respectively. The basic statistic parameters were determined in the Table 1. The highest mean values as well as minimal and maximal values were found for the number of total mesophilic aerobic count. The lowest number between all tested groups of micro-organisms was evaluated for coagulase-positive staphylococci (Table 1).

Aaku *et al*. (2004) and Arenas *et al*. (2004) reported on $5.5 \cdot 10^6$ cfu/ml and 10^6 to 10^7 cfu/ml of the total number of micro-organisms in pooled raw milk, respectively, which is higher than in our experiment ($3.2 \cdot 10^4$ cfu/ml).

The average number of coliforms, yeasts and moulds, and coagulase-postive staphylococci represented only 0.37%, 0.62% and 0.27% of the mean number of total bacterial count, respectively.

The mean number of almost all groups of micro-organisms was higher in summer season, but the correlations between their numbers and the total bacterial count stayed very similar in winter and summer season. The total bacterial count exceeded the 10^5 cfu/ml in 15% of winter samples and in 32% of summer samples. It decreased from summer to winter for 46.5%, the number of

coliforms was lower for 48.2%, the number of yeasts and moulds for 45.0% and the number of coagulase-positive staphylococci for 5.0%.

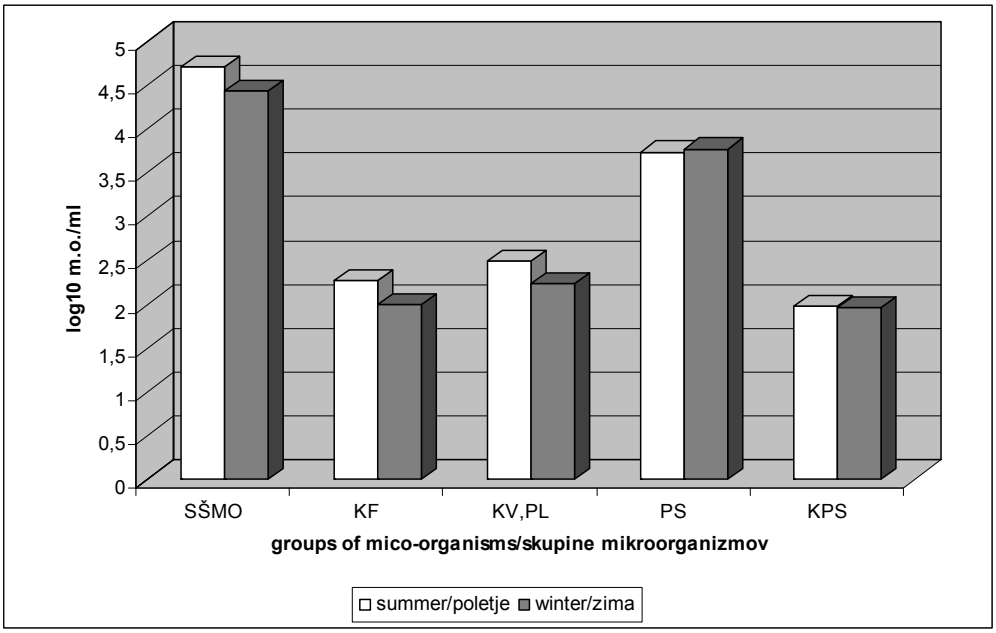
Table1. The basic statistical parameters for the log values of the number of individual groups of micro-organisms per millilitre of 203 tested samples

Preglednica 1. Osnovni statistični parametri za logaritemske vrednosti števila posameznih skupin mikroorganizmov v mililitru 203 preskušenih vzorcev

Groups of m.o. Skupine m.o.	Number of micro-organisms per ml of the sample, in $\log_{10}$ Število mikroorganizmov v ml vzorca, v $\log_{10}$					
	Average Povprečje	Median Mediana	Sd	KV	Min	Max
SŠMO	4.53	4.55	0.55	12.15	2.60	6.04
KF	2.09	2.17	1.07	51.69	0.00	4.53
KV-PL	2.32	2.44	0,71	30.95	0.30	4.18
PS	3.75	3.93	0.89	23.75	2.00	5.50
KPS	1.97	2.17	0.63	31.90	1.00	3.17

m.o.: micro-organisms / mikroorganizmi; Sd: standard deviation / standardni odklon; KV: coefficient of variation / koeficient variabilnosti; Min: minimum value / najmanjša vrednost; Max: maximum value / največja vrednost; SŠMO: total bacterial count / skupno število aerobnih mezofilnih mikroorganizmov; KF: coliform microorganisms / koliformni mikrooganizmi; KV, PL: yeasts and moulds / kvasovke in plesni; PS: psychrotrophic microorganisms / psihrotrofni mikroorganizmi; KPS: coagulase-positive staphylococci / koagulaza-pozitivni stafilokoki

The number of psychrotrophic micro-organisms was the exception in a few views. The mean of the psychrotrophs was 3.7 $\log_{10}$ cfu/ml and represented even 17.1% of the mean of total bacterial count. Their number was in winter higher than in summer for 6.9% (Fig. 1).

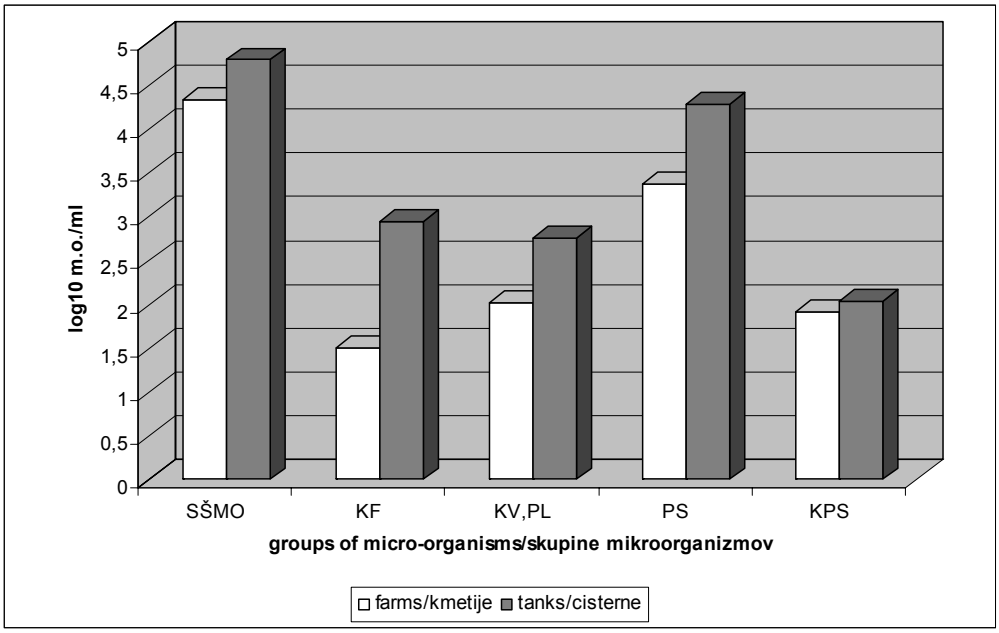


KF: coliform micro-organisms / koliformni mikroorganizmi; KV, PL: yeasts and moulds / kvasovke in plesni; PS: psychrotrophic micro-organisms / psihrotrofni mikroorganizmi; KPS: the coagulase-positive staphylococci / koagulaza-pozitivni stafilokoki

Figure 1. Average log numbers of the individual groups of micro-organisms per millilitre of milk samples in both seasons.

Slika 1. Povprečne logaritemske vrednosti posameznih skupin mikroorganizmov v mililitru vzorcev mleka v obeh sezonah.

The number of all tested groups of micro-organisms was higher in samples of raw bulk milk, collected from transportation tanks at the entrance of the dairy. The highest differences between samples taken from individual farms and from transportation tanks were in number of coliforms (about 1.44 $\log_{10}$ cfu/ml). As it is represented in the Fig. 2, the mean total bacterial count, number of coliforms, yeasts and moulds, psychrotrophic micro-organisms and coagulase-positive staphylococci were in milk from transportation tanks higher than in milk from individual farm tanks for 10.2%, 96.3%, 36.1%, 26.9% and 6.4%, respectively. The total bacterial count exceeded 100 000 cfu/ml in 21.7% of samples from farm bulk milk tanks and in 27.5% of samples from transportation tanks at the entrance of the dairy.



KF: coliform micro-organisms / koliformni mikroorganizmi; KV, PL: yeasts and moulds / kvasovke in plesni; PS: psychrotrophic micro-organisms / psihrotrofni mikroorganizmi; KPS: the coagulase-positive staphylococci / koagulaza-pozitivni stafilokoki

Figure 2. Average values of the log number of the individual groups of micro-organisms per millilitre of milk samples from farm bulk tanks and transportation tanks at the entrance of the dairy.

Slika 2. Povprečne logaritemske vrednosti posameznih skupin mikroorganizmov v mililitru vzorcev mleka iz hladilnih bazenov pri posameznih proizvajalcih in iz transportnih cistern na sprejemu mlekarne.

Almost all correlations between individually groups of micro-organisms were in both seasons highly statistically significant ($P \leq 0.0001$), except for coagulase-positive staphylococci there was no statistically significant correlation with any other tested group (Table 2).

The correlation of the number of coliforms with total bacterial count was understandable, because the colifirms represented a part of the total bacterial count. From the same reason there was also a correlation between the number of coliform and psychrotrophic micro-organisms, because a lot of coliform bacteria are capable to growth at law temperatures. Bramley (1990) reported that some species of the genera making up the coliform group of bacteria are psychrotrophic and constitute 10–30% of the whole group of micro-organisms, the majority of these coliforms are *Aerobacter* spp. In our experiment the number of coliforms represented 2.1% of the number of psychrotrophs.

The incidence of coliforms in raw milk has received considerable attention, partly due to their association with contamination of faecal origin and the consequent risk of more pathogenic

faecal organisms being present, partly because of the spoilage their growth in milk at ambient temperatures can produce, and not least due to the availability of sensitive and rapid tests for detecting and enumerating coliforms. Coliform counts regularly in excess of 100 cfu/ml are considered by some authorities as evidence of unsatisfactory production hygiene. Sporadic high coliform counts may also be a consequence of unrecognised coliform mastitis, mostly caused by *E. coli*. The coliform micro-organisms are found also on the surface of the underwashed or moisture milking equipment, (Bramley, 1990, Rogelj, 2003).

Table 2. The influence of the season and the place of sampling (farm bulk milk tanks and transportation tanks at the entrance of the dairy) on the log number of tested microorganisms from individual groups

Preglednica 2. Vpliv sezone in mesta odvzema vzorca (hladilni bazeni pri posameznih proizvajalcih ali transportne cisterne na sprejemu mlekarne) na logaritemsko število mikroorganizmov iz posameznih skupin

Groups of m.o.	Season / sezona		Sampling place / mesto vzorčenja	
Skupine m.o.	F	P	F	P
SŠMO	19.71	< 0.0001	59.42	< 0.0001
KF	7.04	0.0084	234.13	< 0.0001
KV-PL	10.91	0.0011	102.64	< 0.0001
PS	0.18	0.6679	68.07	< 0.0001
KPS	0.05	0.81	1.87	0.17

m.o.: micro-organisms / mikroorganizmi; SŠMO: total bacterial count / skupno število aerobnih mezofilnih mikroorganitmov; KF: coliform microorganisms / koliformni mikrooganizmi; KV, PL: yeasts and moulds / kvasovke in plesni; PS: psychrotrophic microorganisms / psihrotrofni mikroorganizmi; KPS: coagulase-positive staphylococci / koagulaza-pozitivni stafilokoki; F: variance ratio / vrednost variance; $P \leq 0.05$: statistically significant influence / statistično značilen vpliv; $P \leq 0.001$: highly stsatistically significant influence / visoko statistično značilen vpliv; $P > 0.05$: no statistically significant influence / ni statistično značilnega vpliva

In our case the average number of coliforms was about 120 cfu/ml. In about 10% of winter samples taken exclusively from farm tanks we did not detect any coliforms with standard plate count method.

Bramley (1990) also reported, that the number of psychrotrophs should represent about 10–50% of total bacterial count, which is in the agreement with our results, because the number of psychrotrophic micro-organisms represented 10.9% of total bacterial count in summer and 21.9% of them in winter. We suppose, that the cooling of raw milk in cooling tankers at individual milk producers or farms was very intensive and the environmental temperatures did not influence on the microbiological quality of milk, even if it was kept there two days. Villar *et al*. (1996) also reported about good correlation between the total bacterial count and psychrotrophic count ($r = 0.82$). The correlation coefficient between these two groups in our experiment was $r = 0.71$.

Leitner *et al*. (2008) established that refrigerated storage of good-quality milk from a single cow resulted in moderate deterioration of its quality, low level of bacterial growth (standard plate and psychrotroph counts), and low small losses of curd yield. When milk was collected from farm bulk milk tanks and from dairy silos, its quality deteriorated faster than that of single-cow milk resulting in high bacteria counts and high loss of curd yield, most of which was already apparent for the farm bulk milk tank. Statistical analyses in his study did not reveal any significant interaction between bacterial growth, milk composition, somatic cell count, and curd yield loss, indicating that other mechanisms such as enzymatic activity might be responsible. From the comparison between the high-quality milk from an uninfected cow's udder and the

commingled milk on the farm and in the dairy silos, it appears that introduction of milk coming from infected udders might cause curd yield loss (Leitner *et al*., 2008).

Our results agreed with the Leitner's comments (2008), because we established the statistically significant influence ($P \leq 0.05$) of the season and the place of sampling (individual farms, transportation tanks) on the total bacterial count, number of coliforms and yeasts and moulds. The influence of the place of sampling was determined also on the number of psychrotrophic micro-organisms (Table 2).

To avoid the increase of the number of micro-organisms the European Regulative 853 (2004) recommends that immediately after milking, milk must be held in a clean place designed and equipped to avoid contamination. It must be cooled immediately to not more than 8 °C in the case of daily collection, or not more than 6 °C if collection is not daily. During transport the cold chain must be maintained and on arrival at the establishment of destination, the temperature of the milk must not be more that 10 °C (Regulation EC 853, 2004).

The standard deviations and variability coefficients were in most cases high, because there were rather large differences in number of micro-organisms between samples (Table 1).

The number of coagulase-positive staphylococci was not in statistically significant correlation with other groups of micro-organisms and was not dependent on the season (Table 2).

Coagulase-positive staphylococci may cause human disease through the production of toxins. The formation of effective levels of toxin requires a high number of micro-organisms (approximately 10^5–10^6 micro-organisms per gram of food) at a pH value greater than 5 and so the presence of coagulase-positive staphylococci at a low level does not necessarily constitute a hazard. Dairy-related outbreaks of staphylococcal intoxication have been attributed to raw milk, dried milk, cheese and ice cream. Coagulase-positive staphylococci may be present in raw milk from the surfaces of animals, udder and teat canals of a cow, particularly if lesions are present. Also, the nasal area and hands of humans are recognised sites of contamination: poor personal hygiene can result in the contamination of milk and dairy products. Essential to the production of toxin is the growth of micro-organisms. In general, *Staphylococcus aureus* and the related species *Staphylococcus intermedius* and *Staphylococcus hyius* do not multiply at temperatures below 8 °C, and 10 °C is the minimum for toxin production. These micro-organisms are, however, resistant to salt. Pasteurisation will be effective against them but, if toxins are present, the toxins will not be inactivated. The higher counts of *Staphylococcus* recorded in spring, when milk yields are at their peak, are a cause for concern and mammary infections (Anonim., 1994; Bramley, 1990). De Buyser *et al*. (2001) reported that *Staphylococcus aureus* was by far the most frequent pathogen associated with food pathogen outbreaks (85.5% of the outbreaks) in France, followed by *Salmonella* (10.1%), *E. coli* (3%), *L. monocytogenes* (3%) and *C. jejuni* (1.5%) outbreaks.

In our experiment coagulase-positive staphylococci were found in all tested samples, but their number did not exceed 1300 bacteria per ml.

It is documented that yeasts occur in raw milk at insignificant numbers (Fleet, 1990) probably due to competitive utilization for the growth substrates by psychrotrophic bacteria of milk or owing to inhibition by metabolites excreted by bacteria (Viljoen, 2001).

The mean number of yeasts and moulds found in raw milk samples of our study was 2.3 $\log_{10}$ cfu/ml, which is comparable to the mean yeast count (2.64 $\log_{10}$ cfu/ml) in raw milk from farms located in different areas of Sardinia (Fadda *et al*., 2004). The maximum values of yeasts in raw milk samples from our producers were 4.1 $\log_{10}$ cfu/ml.

In all milk samples, two or more mould species were found, 210 of them were successfully isolated and classified to genera *Geotrichum* (51.5% of strains), *Aspergillus* (33.8% of strains), *Mucor* (5.9% of strains), *Fusarium* (2.9% of strains) and *Penicillium* (2.9% of strains). The results are close to results of Jodral *et al*. (1993) who reported that the genera most frequently isolated in the raw milk were *Geotrichum* (76.5%), *Fusarium* (45.3%) and *Aspergillus* (31,2%).

O'Brien *et al*. (2005) established about 91% of baled grass silage contaminated with moulds, which mostly belonged to genera *Penicillium, Geotrichum, Fusarium* and mucoraceous species. These genera were found in our raw milk samples to, so it could be expected that the feed was one of the possible sources of contamination of raw milk in spite of Finne Kure *et al*. (2004) adduced proofs that there are many possible sources of contamination of raw milk, beside the feed also the air and the environment.

We also expected the higher number of yeasts and moulds in milk sampled in winter, when the pasture or the hay was replaced by conserved or ensiled feed. Many authors namely reported on higher number of yeasts, moulds and consecutively the higher concentration of mycotoxins in ensiled feed, which was used mostly in winter season. These micro-organisms were very often transferred from feed to milk (Blanco *et al*., 1988; Lopez *et al*., 2003; Kamkar, 2005). Surprisingly, the average number of yeasts and moulds in raw milk samples was a little higher in summer (2.49 $\log_{10}$ cfu/ml) than in winter (2.23 $\log_{10}$ cfu/ml), while the minimal and maximal values were higher in winter, as we expected.

All 210 mould strains isolated from milk samples as well as standard strains *A. flavus* were inoculated on *A. flavus/ parasiticus* agar (AFPA). *Aspergillus* strains grew on the medium, but except reference strains *A. flavus* EXF 523 and *A. flavus* EXF 438 only one strain, previously classified as genus *Aspergillus* produced a distinctive bright orange yellow reverse colour on AFPA medium as described by Frädberg *et al*. (2003) and thus identified as *A. flavus* or *A. parasiticus* species.

Only about 70% out of 24 *Aspergillus* strains, growing on YGC medium, were able to form the colonies on YES medium supplemented with methyl-β-cyclodextrin and 0.6% sodium deoxycholate. None of isolated strains could cause a typical white fluorescent zone around the colonies, visible under UV light. We could conclude, that tested mould strains did not produce aflatoxins on these two media.

In our study it was found out that only one strain belonged to species *A. flavus / parasiticus* under typical growth on AFPA medium. *A. flavus* is not a common species on cheese. Most studies showed that aflatoxins could only be produced in milk and on cheese at temperatures higher than 10 °C and a limiting a_w of 0.79 was found for growth of *A. flavus* and aflatoxin production (Scott, 1989).

CONCLUSIONS

- In our study 76.4% out of total 203 tested raw milk samples contained less than 100 000 cfu/ml. There were statistical significant differences in total bacterial count between winter and summer samples and between samples taken from individual farm bulk milk tanks and transportation tanks at the entrance of the dairy.
- The total bacterial count exceeded 100 000 cfu/ml in 21.7% of samples from farm bulk milk tanks and in 27.5% of samples from transportation tanks at the entrance of the dairy.
- The number of all tested groups of micro-organisms was higher in samples of raw bulk milk, collected from transportation tanks at the entrance of the dairy. The highest differences between these samples were in number of coliforms.
- The average of the psychrotrophs represented even 17.1% of the average total bacterial count. Their number was in winter higher than in summer.
- All samples contained coagulase-positive staphylococci which may be an indicator of mastitical diseases of milking cows. The health care of milking cows should be more intensive.
- Isolated mould strains belonged to genera *Geotrichum*, *Aspergillus*, *Mucor, Fusarium* and *Penicillium.* None of the isolated *Aspergillus* strains produced aflatoxin M_1.

- The microbiological quality of milk samples mostly suited the requirements of the dairies, although that there was the percent of raw bulk milk from transportation tanks, contained less than 100 000 m.o./ml lower (72.5%) in comparison with the data for total bacterial count of raw milk in Slovenia in the year 2005.
- More attention should be focused on the cleaning of transportation tanks, appropriate handling with milk and its transportation at low temperatures from farms and collecting points to the dairies, particularly in summer season.

POVZETEK

Na osnovi uredbe EU (Regulation 853/2004) geometrijsko povprečje skupnega števila aerobnih mezofilnih mikroorganizmov v enem mililitru svežega kravjega mleka ne sme presegati 100 000. V Sloveniji je mikrobiološka kakovost surovega mleka dobra in je primerljiva s kakovostjo mleka mlekarsko razvitih držav. Ob koncu leta 2005 se je večina slovenskih mlekarn odločilo, da bodo mleko odvažale iz zbirnih hladilnih bazenov posameznih proizvajalcev ter zbiralnic vsake dva dni in ne več dnevno. Tako mleko stoji v hladilnih bazenih, ob vsaki molži pa se v bazen dolije k ohlajenemu še sveže pomolzeno toplo mleko. Mikrobiološka kakovost in razmerje med različnimi skupinami mikroorganizmov v takem mleku se razlikuje od sestave mikroflore v mleku, ki se takoj ohladi in zbira vsakodnevno.

V našem poskusu smo želeli preučiti mikrobiološko kakovost 203 vzorcev surovega mleka, od tega jih je bilo 100 odvzetih v zimskem in 103 vzorci v letnem obdobju. Vzorčili smo mleko iz zbirnih hladilnih bazenov pri posameznih proizvajalcih mleka, zbiralnicah in v transportnih cisternah ob sprejemu mleka v mlekarni. Ugotavljali smo skupno število mikroorganizmov, število koliformnih in psihrotrofnih mikroorganizmov, kvasovk in plesni ter število koagulaza-pozitivnih stafilokokov. Želeli smo ugotoviti prisotnost posameznih rodov plesni in tvorbo aflatoksinov pri vrstah rodu *Aspergillus*.

Skupno število mikroorganizmov je presegalo 100 000 ke/ml v 48 (23,6 %) od vseh preiskanih vzorcev. Njihova povprečna vrednost v vseh vzorcih mleka je bila 4,5 $\log_{10}$ ke/ml ($3.2 \cdot 10^4$ ke/ml). Povprečno število koliformnih mikroorganizmov je znašalo 2.1 $\log_{10}$ ke/ml, število psihrotrofnih mikroorganizmov 3.7 $\log_{10}$ ke/ml, skupno število kvasovk in plesni 2.3 $\log_{10}$ ke/ml in število koagulaza-pozitivnih stafilokokov 1.97 $\log_{10}$ ke/ml. Število vseh skupin mikroorganizmov, z izjemo psihrotrofov, je bilo višje v poletnem času odvzema vzorcev, čeprav je njihovo razmerje s skupnim številom mikroorganizmov ostalo podobno v obeh sezonah.

Število vseh skupin mikroorganizmov, posebno še koliformnih, je bilo višje v mleku iz transportnih cistern na sprejemu mlekarne.

Kvasovke smo ugotovili v 95 % vzorcev, plesni pa v 63,3 % vzorcev. Najpogosteje smo izolirali plesni iz rodov *Geotrichum* (51,5 %), *Aspergillus* (33,8 %), *Mucor* (5,9 %), *Fusarium* (2,9 %) in *Penicillium* (2,9 %). Nobeden izmed izoliranih sevov iz rodu *Aspergillus*, ki je kazal značilno rast na gojišču AFPA, ni na gojiščih YES in YGC z metil-β-ciklodekstrinom tvoril aflatoksina M_1.

Ugotovili smo, da je mikrobiološka kakovost surovega mleka kljub dvodnevnemu odvozu, še vedno v večini primerov ustrezna, čeprav je bil odstotek vzorcev v mlekarni sprejetega mleka, ki so vsebovali manj kot 100 000 mikroorganizmov/ml, v primerjavi z mikrobiološko kakovostjo mleka v Sloveniji v letu 2005, nekoliko nižji (72,5 %).

Iz rezultatov lahko povzamemo, da samo skupno število mikroorganizmov ni vedno realni pokazatelj mikrobiološke kakovosti mleka. Občasno je potrebno kontrolirati tudi prisotnost in število drugih skupin mikroorganizmov. Število koliformnih, psihrotrofnih in koagulaza-pozitivni mikroorganizmov je bilo v našem poskusu občasno visoko. Koliformni mikroorganizmi so namreč pokazatelj slabe higiene in možnega fekalnega onesnaženja, psihrotrofni

mikroorganizmi se množijo tudi pri nizkih temperaturah hlajenja in so pogosti kvarljivci mleka zaradi tvorbe termostabilnih proteolitičnih in lipolitičnih encimov, koagulaza-pozitivni stafilokoki pa so lahko prisotni v mleku mastitičnih krav. Tudi prisotnost gliv, kontaminentov iz okolja, je bila v vzorcih pogosta.

Mikrobiološka kakovost vzorcev mleka iz transportnih cistern na sprejemu mlekarne je bila, zlasti v letnem obdobju, opazno slabša v primerjavi z vzorci iz hladilnih bazenov posameznih proizvajalcev ali zbiralnic. Zato je večjo pozornost potrebno posvetiti čiščenju transportnih cistern, pravilnemu ravnanju z zbranim mlekom in njegovemu prevozu v mlekarno ob ustrezno nizkih temperaturah.

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DIFFERENCES IN MELTING TEMPERATURES OF DEGENERATED OLIGONUCLEOTIDES TARGETTING NITROUS OXIDE REDUCTASE (*NOSZ*) GENES

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ABSTRACT

One of the basic principles of molecular biology is the use oligonucleotides with comparable melting temperatures (Tm). To accommodate various evolutionary changes in target gene sequences in order to detect numerous variants of the same gene in complex microbial communities, the researchers were forced to design degenerated oligonucleotide probes and primers. In addition, recent studies suggested that relevant parameters influencing microbial activity should be included into models currently describing the final greenhouse gas emissions for public use. Further, data on microbial community structure and abundance should be included as well in near future. As one of the most potent greenhouse gases, nitrous oxide, results mainly from incomplete denitrification process, we chose nitrous oxide reductase gene (*nosZ*) as a model and surveyed published literature for *nosZ* gene oligonucleotides. We calculated *in-silico* Tm for each oligonucleotide degenerated variant and compared the resulting average Tm of both oligonucleotides used in pair. Degenerated oligonucleotides were found to contain variants differing in Tm for as much as 13 °C. More than 85% of oligonucleotides had difference in average Tm of paired oligonucleotide larger than 2 °C, more than 60% larger than 4 °C and more than 40% larger than 6 °C, 25% larger than 8 °C. By using such combinations at one annealing temperature or touch-down PCR or hybridization protocol, the full use of all degenerate variants could never be achieved thus bringing under the consideration the reaction chemistry. To increase the consistency of molecular results, a simple adjustment of Tm to at least comparable average Tm is recommended. In addition, critical evaluation of other methodological pitfalls should be regular practice in order to strengthen the value of molecular results as future public models parameters.

Key words: microbiology / molecular biology / melting temperature / oligonucleotides / *nosZ* / denitrification / models / greenhouse gases

RAZLIKE V TEMPERATURI TALJENJA ZAČETNIH OLIGONUKLEOTIDOV ZA ODKRIVANJE GENA *nosZ*

IZVLEČEK

Eden osnovnih principov molekularne biologije je uporaba oligonukleotidov s primerljivimi temperaturami taljenja (Tm). Da bi lahko z oligonukleotidi zajeli tudi evolucijske spremembe na tarčnih sekvencah istega gena znotraj kompleksnih mikrobnih združb, so se raziskovalci zatekli k uporabi degeneriranih oligonukleotidov. Nedavne študije predlagajo vključitev za mikrobe relevantnih parametrov, ki vplivajo na njihovo aktivnost, v modele, ki se trenutno uporabljajo za opis emisij toplogrednih plinov v javnosti. V bližnji prihodnosti pa se predvideva tudi vključitev

podatkov o strukturi mikrobnih združb in velikosti njihovih populacij. Ker je eden najmočnejših toplogrednih plinov, N_2O, rezultat v največji meri nepopolnega poteka denitrifikacije, sva izbrala gen za reduktazo N_2O (*nosZ*) kot model ter iz objavljene literature sestavila nabor uporabljanih oligonukleotidnih parov. Za vsako varianto degeneriranega oligonukleotida v paru sva izračunala predvideno Tm in primerjala povprečne Tm obeh oligonukleotidov v paru. Tm variant degeneriranih oligonukleotidov so se razlikovale do 13 °C. Več kot 85 % oligonukleotidov je imelo povprečno razliko Tm para > 2 °C, več kot 60 % > 4 °C in več kot 40 % oligonukleotidov je imelo Tm večjo od 6 °C. Z uporabo takih kombinacij pri eni temperaturi prileganja ali „PCR z zniževanjem temperature'' ali hibridizacijskih protokolih, je praktično nemogoče zagotoviti polno uporabo vseh degeneriranih variant. Našteto posledično različno vpliva na potek kemijskih reakcij prepoznave tarčnih mest. Da bi izboljšali konsistentnost molekularnih rezultatov, priporočava uskladitev povprečnih Tm para oligonukleotidov. Podobno pa je potrebno kritično oceniti druge metodološke šibke točke, da bi zagotovili uporabno vrednost rezultatov molekularnih tehnik kot bodočih parametrov v modelih.

Ključne besede: mikrobiologija / molekularna biologija / taljenje / temperatura / oligonukleotidi / *nosZ* / denitrifikacija / modeli / toplogredni plini

INTRODUCTION

Denitrification is a dissimilatory process in which oxidized nitrogen is used as an alternative electron acceptor for energy production when oxygen is limiting. As a part of the global nitrogen cycle, denitrification is believed to be responsible for the return of fixed nitrogen back to the atmosphere. Although responsible for nutrient loss in agriculture and a contribution to the greenhouse effect and the damage to the ozone layer, denitrification is also favourable in nutrient removal from wastewater and bioremediation (Tiedje, 1988). New generation of improved models describing the greenhouse gas emissions from long term ecological research field sites across Europe was initiated in 2006 (http://www.nitroeurope.eu/). At the same time, Schurgers *et al*. (2006) came up with an improved model describing anaerobiosis with water filled pore space and dynamic processes in various soils. Both studies suggested it was time to incorporate relevant parameters influencing microbial activity and on the long run, also data on microbial community structure and abundance into models currently describing the final greenhouse gas emissions.

While denitrification is considered a primarily bacterial respiratory process, it consists of four enzymatic steps carried out by nitrate, nitrite, nitric-oxide and nitrous oxide reductases. The latter is crucially involved in reduction of nitrous oxide (N_2O) to molecular dinitrogen (N_2). Generally, each enzyme is translated from mRNA transcribed from a genome or plasmid residing gene and once fully folded, contains a distribution of more conserved (active site) and more variable regions (neighbouring amino acid chains). However, complex microbial communities contain varieties of the same gene that differ slightly due to the fixation of various evolutionary events. Conserved regions of the same protein present in various bacterial lineages are preserved, but are not completely identical. This precludes the use of a single overall specific and covering oligonucleotide set that could be used in amplification or detection of all gene variants present in microbial community DNA. In this respect, researchers have come up with what appears to be an ideal solution – the use degenerate oligonucleotides to target as many different variants of the genes as possible, resulting in numerous different and degenerated oligonucleotide sets for the same gene. One of the basic principles of molecular biology is the use oligonucleotides with comparable melting temperatures (Tm) to enable their concomitant use at comparable Tm (Morris *et al*., 2002 and references herein). To verify whether published *nosZ* oligonucleotides were designed according to this principle, we calculated *in-silico* melting temperatures for all oligonucleotides, all their degenerated variants and compared the resulting average Tm of both oligonucleotides used in pair.

MATERIALS AND METHODS

Data selection

Literature on the molecular methods used for amplification of target denitrification genes from environmental samples was explored using available public databases: Medline (http://www.ncbi.nlm.nih.gov/PubMed), ScienceDirect (http://www.sciencedirect.com) and American Society for Microbiology (http://aem.asm.org/searchall). The following criteria for literature exploration and selection were adopted: **(i)** publication should be less than ten years old, **(ii)** it should report on the applied use of molecular tools to denitrifying microbial communities, **(iii)** the oligonucleotide sets should be directed towards *nosZ* in complex microbial communities, **(iv)** the oligonucleotide sets should be cited at least once as an indication of their impact.

As a result, the following publications were selected: Delorme *et al*. (2003), Henry *et al*. (2006), Horn *et al*. (2006), Nogales *et al*. (2002), Rich *et al*. (2003), Rösch *et al*. (2002); Rösch and Bothe (2005) Scala and Kerkhof (1998); Scala and Kerkhof (1999); Throbäck *et al*., (2004).

Data analysis

The orientation of oligonucleotides was tested using FunGene Repository / Pipeline (http://flyingcloud.cme.msu.edu/fungene/). Oligonucleotide pairs were organized according to their use in literature and their *in-silico* melting temperatures (Tm) were calculated according to SantaLucia (1998) using BioEdit 7.0.1 (Hall, 1999). The Tm module was set to calculate the theoretical melting temperature of each DNA oligonucleotide to its exact target site (exact complement) without any mismatches allowed. The environmental parameters during virtual annealing were 50 mM Na^{+}, 2.5 mM Mg^{++} and the concentration of each oligonucleotide was set to 100 nM. The calculation was done using the nearest neighbor thermodynamic model presented by SantaLucia (1998), which was based on the model by Borer *et al*. (1974):

$$Tm = \Delta H / (\Delta S + R*\ln(C/4)) - 273.15$$

where R is the molar gas constant and C is the concentration of oligonucleotide. A salt correction for ΔS is applied which is: $0.368 \times \ln([Na^{+}])*(P)$ (SantaLucia, 1998), where P is the number of phosphates and is equal to *length-1* for non-5'-phosphorylated oligonucleotides such as PCR oligonucleotides. Mg^{++} is assumed to have an effect roughly to 140X the sodium equivalent, according to Nakano *et al*. (1999) and von Ahsen *et al*. (1999).

The calculated Tm were organized to represent each oligonucleotide from a set and for each variant of degenerated oligonucleotide. Further, average Tm and corresponding standard deviations were calculated from the distribution and the differences in Tm among the oligonucleotides used in each set were determined as well. In addition, minimum and maximum Tm of each degenerate oligonucleotide were identified and sorted according to average Tm as primary criteria, and later according to minimum Tm, maximum Tm, fold degeneracy, Tm difference within oligonucleotide pair.

RESULTS AND DISCUSSION

In the present study elucidation of basic relationships between melting temperature, fold degeneracy, differences in melting temperatures of oligonucleotide pairs was conducted based on published oligonucleotide sets from ten studies published during the last decade. The differences in the respective average *in-silico* melting temperatures of oligonucleotides used in source

studies are depicted in Fig. 1. Large differences, up to 12 °C can be observed between predicted average melting temperatures of paired oligonucleotides. Also, three categories can be observed: **(i)** symmetric pairs of two degenerated oligonucleotides (A, I, K, L, M, N, O, P, R, S; n = 10 pairs), **(ii)** asymmetric pairs of degenerated and single variant oligonucleotide (B, D, F, G, H, J; n = 6 pairs) and **(iii)** two symmetric single variant oligonucleotides that were paired in just two cases (C, E). The more frequent use of two degenerated oligonucleotides in pair also reflects attempts of researchers to obtain as comprehensive collection of amplicons from microbial communities as possible.

However, one of the most common approaches during PCR or hybridization optimization procedures is to use developed oligonucleotides and test them on a set of different but defined DNAs. In order to make the two oligonucleotides that differ in average Tm be successful in detection of target genes, Tm of at least one of the oligonucleotides present in pair needs to be violated, either increased or decreased. In case the oligonucleotides are degenerated, this in turn favours binding of certain variants of oligonucleotides to their targets, while some of them can not or do so at much lower stringency, while other perform at optimal or too harsh conditions. This, however, is known to compromise specificity and efficiency of amplification of such approaches. Interestingly, these limitations are expressed in studies optimizing Real-Time PCR assays only. In addition, as the composition of degenerated target sets present in microbial community is not known a-priori, the variants of oligonucleotides may be differentially used up as a function of community structure or differences in chemistry or due to co-extracted impurities. As a result, concentrations of oligonucleotide variants binding to their targets may fall outside the optimal concentrations not allowing uncompromised reaction continuation but leaving only less competitive binding variants to interact with target DNA. As the concentration of most efficient oligonucleotide variant is decreasing during initial PCR cycles in exponential amplification this could in fact change the chemistry of PCR substantially and have impact on the final molecular results as well (Ausubel *et al*., 1999; Morris *et al*., 2002).

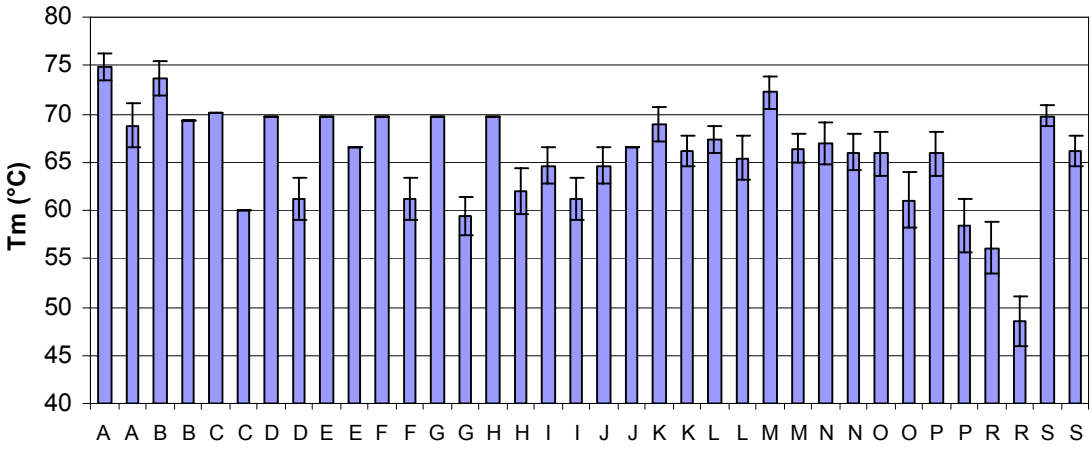

Figure 1. The distribution of average *in-silico* average melting temperatures of oligonucleotides (A-S) adopted from Delorme *et al*. (2003), Henry *et al*. (2006), Horn *et al*. (2006), Nogales *et al*. (2002), Rich *et al*. (2003), Rösch *et al*. (2002); Rösch and Bothe (2005) Scala and Kerkhof (1998); Scala and Kerkhof (1999); Throbäck *et al*. (2004). Tm (°C) – average melting temperature of oligonucleotide.

Slika 1. Porazdelitev povrečnih predvidenih temperatur taljenja začetnih oligonukleotidov (A-S) iz literature: Delorme *et al*. (2003), Henry *et al*. (2006), Horn *et al*. (2006), Nogales *et al*. (2002), Rich *et al*. (2003), Rösch *et al*. (2002); Rösch and Bothe (2005) Scala and Kerkhof (1998); Scala and Kerkhof (1999); Throbäck *et al*., (2004). Tm-povprečna temperatura taljenja začetnega oligonukleotida.

Fig. 2 shows that large differences among Tm are regular practice during oligonucleotide design in microbial ecology as only less than 12.5% of oligonucleotide pairs had predicted difference in average Tm smaller than 2 °C. More than 85% of oligonucleotides had difference in average Tm larger than 2 °C, more than 60% larger than 4 °C and more than 40% larger than 6 °C. Interestingly, 25% of oligonucleotides had Tm difference higher than 8 °C.

It could be argued, that our results are *in-silico* calculations that could hardly hold true if tried in PCR cycler. However, this approach was chosen deliberately in order to control Tm determination and avoid unsystematic technical biases during manipulations such as pipetting errors, unequal mixing, diffusion and chemical decomposition. Therefore, biases in our Tm calculations are systematic for all oligonucleotides. The calculated Tm values thus reflect the differences due to their DNA composition.

Further complicating issue is the generally incompatible chemical composition of PCR or hybridization buffers among different studies, suggesting existence of individual behaviour of oligonucleotide pairs in such mixtures. Surface plasmone resonance could be used to explore this issue (http://www.bf.uni-lj.si/bi/sprcenter/index.html).

Unfortunately, the outcomes of studies deploying different DNA extractions, oligonucleotide sets, PCR reaction conditions, have been subjected to (mis)interpretations many times. In many cases, results from various studies deploying different methodologies have been compared and then some general conclusions were proposed. To avoid comparing apples and oranges, all calculations used in this study applied the same criteria for all tested oligonucleotides.

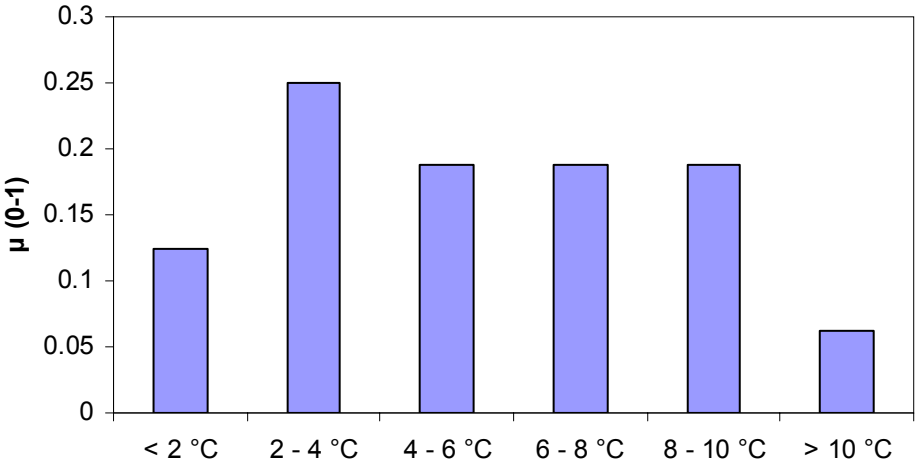

Figure 2. The fraction of oligonucleotide sets (μ) falling into average temperature difference classes: < 2 °C, 2–4 °C, 4–6 °C, 6–8 °C, 8–10 °C, > 10 °C.

Slika 2. Delež oligonukleotidnih setov (μ) porazdeljenih po razlikah v njihovih povprečnih temperaturah taljenja v temperaturne razrede: < 2 °C, 2–4 °C, 4–6 °C, 6–8 °C, 8–10 °C, > 10 °C.

Generally, degenerated oligonucleotide can contain variants with differing melting temperatures for as much as 15 °C. In addition, two degenerated oligonucleotides differing widely (> 8 °C) in their average Tm are routinely being used at one intermediate annealing temperature or using subsequent touch-down protocol (http://www.ncbi.nlm.nih.gov/PubMed). By doing this, the full use of all degenerate variants could never be achieved and consequently brings their specificity, detection limit, the existence and duration of exponential step, efficiency of amplification or hybridization and chemistry mass balances under question.

Of course one can suggest to circumvent PCR step in microbial ecology completely as metagenomic studies and direct reconstructions of genomes from the environment are already on

their course. However, as this is not applicable for majority of research labs in reality, much less drastic approaches can be easily adopted, such as simple adjustment of forward and reverse oligonucleotide melting temperatures or higher concentrations of degenerated oligonucleotides in PCR reactions. The actual sampling capability of each oligonucleotide for target sequences and their specificity were out of scope of our paper.

When the difference between average Tm of paired oligonucleotides (Δ Tm (A-B)) was plotted as a function of difference between minimum and maximum Tm of each oligonucleotide degenerated variants (Δ Tm (min-max)) no observable relationship was observed (Fig. 3). The shaded area designates the Δ Tm (min-max) space occupied by Tm calculated from degenerated variants of single oligonucleotides used in this study (Δ Tm (min-max) = 0 – 13 °C) (Fig. 4). In case all oligonucleotides used in this study were properly paired, their Tm difference between average Tm of each of them would be within 2 °C class and therefore below the horizontal line (Δ Tm (A-B) ≤ 2 °C).

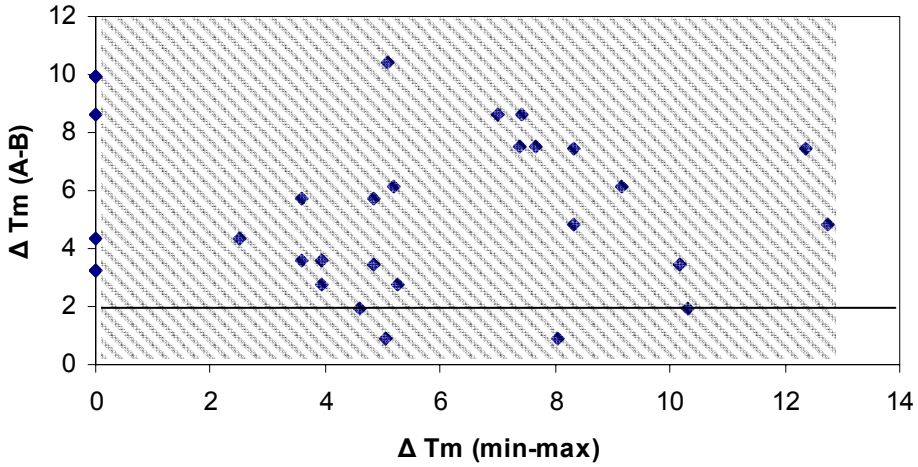

Figure 3. The relationship beween Δ Tm (A-B) (difference between *in-silico* average Tm of each oligonucleotides used in a pair) as a function of difference between maximum and minimum *in-silico* melting temperature of its degenerated variants (Δ Tm (min-max)) of single oligonucleotide.

Slika 3. Odnos med Δ Tm (pair) (razlika med *in-silico* Tm oligonukleotidov v paru) kot funkcija razlike med maksimalno in minimalno *in-silico* temperaturo posameznega od oligonukleotidov (Δ Tm (min-max)).

As this is not the case, this indicates that oligonucleotides are not paired accordingly and therefore violate the basic molecular prerequisites, and yet are in full scientific use. Further, we do not argue that such oligonucleotides would not produce detectable signal when applied to research. However, as shown in Fig. 4, Tm overlap is rarely encompassing hybridization Tm of all forward and reverse degenerate primer variants. Such oligonucleotides and their combinations have been and continue to being used to detect target sequences in complex mixtures of DNA. Therefore, the validity of the subsequent interpretation of molecular signal that is obtained in the form of the comparative diversity of clones sequenced from various environments, or T-RFLP patterns is questioned.

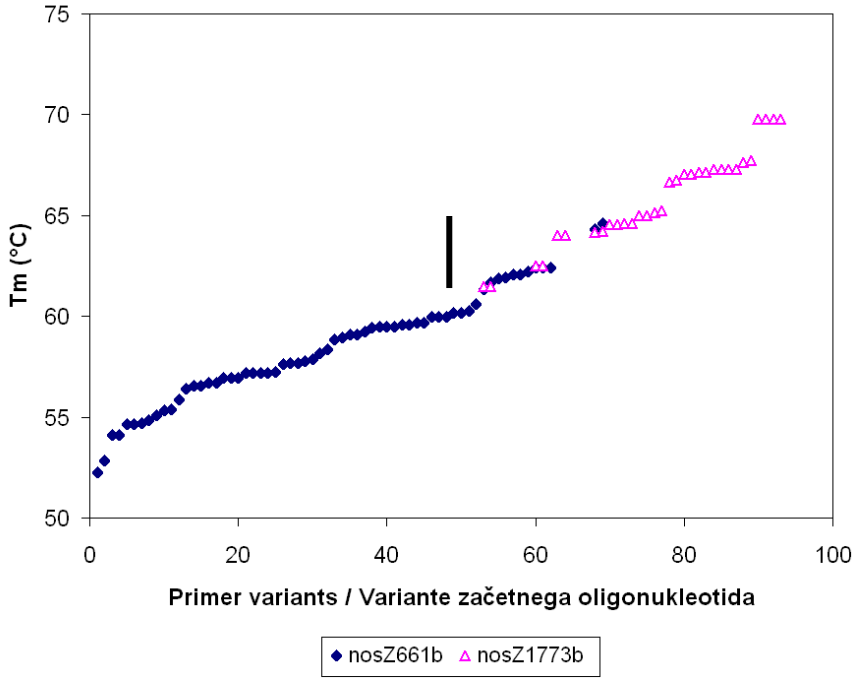

Figure 4. An example of narrow in-silico melting temperature overlap showing nosZ661b (`5′-cggytggggsmwkaccaa-3′`) and nosZ1773b (`5-atrtcgatcarytgntcrtt-3′`) degenerate primer variants. The vertical bar indicates melting temperature overlap of certain nosZ661b and nosZ1773b variants.

Slika 4. Primer ozkega ujemanje izračunanih temperature taljenja dveh degeneriranih začetnih oligonukleotidov nosZ661b (`5′-cggytggggsmwkaccaa-3′`) in nosZ1773b (`5′-atrtcgatcarytgntcrtt-3′`). Pokončna črta označuje ujemanje temperatur taljenja nekaterih variant začetnih degeneriranih oligonukleotidov nosZ661b and nosZ1773b.

CONCLUSIONS

Our analysis of *nosZ* targeting oligonucleotides and the oligonucleotide pairing schemes that are being used in modern microbial ecology all indicated that these approaches underestimate the complexity and vulnerability of research to incomplete, uncontrolled and unaccountable (false) conclusions. Our *in-silico* analysis showed that more than 85% of oligonucleotides had difference in average Tm larger than 2 °C, more than 60% larger than 4 °C and more than 40% larger than 6 °C. Interestingly, 25% of oligonucleotides had Tm difference higher than 8 °C, all indicating thermodynamically unfeasible annealing of a substantial portion of each degenerate primer variants to their respective targets when two highly degenerate primers are paired in single PCR. This issue is of central importance because the results of such studies are projected to be included into important models as novel parameters predicting greenhouse gas emissions that are going to be further delivered to public use for predictive purpose and directing future environmental and economic policies. Looking from that perspective, the issue of compatible melting temperatures should not be taken light-heartedly as rabbit's tail in our hands could hardly be confused for the dragon itself.

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ISOLATION OF *Escherichia coli, Staphylococcus aureus* AND *Listeria monocytogenes* FROM MILK PRODUCTS SOLD UNDER MARKET CONDITIONS AT AGRA REGION

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ABSTRACT

Escherichia coli, Staphylococcus aureus, and *Listeria monocytogenes* were isolated from milk products i.e. curd and cottage cheese, sold at shops in the unorganized sector of Agra region. Of the 116 bacterial isolates from cottage cheese, 15 were confirmed as *E. coli,* 12 as *S. aureus*, and two as *L. monocytogenes.* Fifty-eight isolates were obtained from curd samples of which five were confirmed as *E. coli,* eleven as *L. monocytogenes*, and no *S. aureus* contamination was found in curd. The result indicates that strict preventive measures should be adopted to ensure contamination free milk products for the good health of all consumers.

Key words: milk products / curd / cottage cheese / microbiology / *Escherichia coli* / *Staphylococcus aureus* / *Listeria monocytogenes* / public health / India

OSAMITEV BAKTERIJ *Escherichia coli, Staphylococcus aureus* IN *Listeria monocytogenes* IZ MLEČNIH PROIZVODOV, KI SO NA TRŽIŠČU V REGIJI AGRA (INDIJA)

IZVLEČEK

Iz skute in svežega sira, ki ju prodajajo v neorganiziranih prodajalnah na področju Agre (Indija), smo osamili bakterije *Escherichia coli, Staphylococcus aureus* in *Listeria monocytogenes.* Od 116 bakterijskih izolatov iz svežega sira smo identificirali 15 izolatov *E. coli,* 12 *S. aureus* in dva *L. monocytogenes.* Med oseminpetdesetimi izolati bakterij iz skute je bilo identificiranih pet izolatov *E. coli,* enajst *L. monocytogenes* in nobenega izolata *S. aureus.* Rezultati kažejo, da bi bilo za zagotavljanje zdravja potrošnikov nujno potrebno uvesti striktne ukrepe za preprečevanje okužbe mlečnih izdelkov.

Ključne besede: mlečni izdelki / skuta / sveži sir / mikrobiologija / *Escherichia coli* / *Staphylococcus aureus* / *Listeria monocytogenes* / zdravstveno varstvo / Indija

INRODUCTION

Milk is supposed to constitute a complex ecosystem for various microorganisms including bacteria. Milk products like cheese and curd are widely consumed and market for them has existed in many parts of the world for many generations. There is an increase demand by the consumer for high quality natural food, free from artificial preservatives, and contaminating microorganisms. Contamination of milk and milk products, with pathogenic bacteria is largely due to processing, handling, and unhygienic conditions. This paper describes the presence of

Escherichia coli, Staphylococcus Aureus, and *Listeria monocytogenes* in cottage cheese and curd available at shops comprising the unorganized sectors in Agra.

E. coli frequently contaminates food organism and it is a good indicator of fecal pollution (Diliello, 1982; Soomro *et al.,* 2002; Benkerroum *et al.,* 2004). Presence of *E. coli* in milk products indicates the presence of enteropathogenic microorganisms, which constitute a public health hazard. Enteropathogenic *E. coli* can cause severe diarrhoea and vomiting in infants, and young children (Anon., 1975).

Of late *L. monocytogenes* has been recognized as a food born pathogen (Kaclikova, *et al.,* 2001) that can contaminate dairy products (Menendez *et al.,* 2001). Its virulent strain can cause a serious disease called listeriosis, particularly the risk populations including pregnant women, newborns, the very old, and people who are immune compromised (Fleming *et al.,* 1985; Bille, 1989).

Illness through *S. aureus* range from minor skin infection such as pimples, boils, cellulites, toxic shock syndrome, impetigo, and abscesses to life threatening disease such as pneumonia, meningitis, endocarditis, and septicemia. (Soomro *et al.,* 2003; Masud *et al.,* 1988).

MATERIAL AND METHODS

Standard strains

Standard strains of *E. coli* (MTCC-443), *L. monocytogenes* (MTCC-1143), and *S. aureus* (MTCC-3381) were procured from MTCC Chandigarh. All the isolates were confirmed through biochemical tests by comparing with the results of standard strains.

Collection of samples

Samples of cottage cheese and curd were collected during the period of I year from different regions of Agra city and examined for the presence of *E. coli, L. monocytogenes*, and *S. aureus*. Samples of each milk product were collected aseptically, transferred to sterile plastic bags and were directly transported to the laboratory under cold conditions. They were stored at 4 °C and analyzed within 24 hours.

Microbiological analysis

A Portion (10 g or 10 ml) from the centre of each sample was extracted aseptically and homogenized with 90 ml sterile enrichment broth (lactose broth for *E. coli,* UVM-2 for *L. monocytogenes,* and peptone water for *S. aureus*) and incubated at 37 °C for 24 hours, for further biochemical analysis.

Table 1. Morphological and culture characteristics of isolated bacteria
Preglednica 1. Morfološke značilnosti kultur izoliranih bakterij

Isolated bacteria	Gram staining	Culture characteristics on selective media
E. coli	Gram negative rods	Colonies showing metallic sheen
S. aureus.	Gram positive cocci (in clusters)	Jet black colonies surrounded by white halo
L. monocytogenes	Gram positive rods	Greenish-yellow glistening, iridescent and pointed colonies surrounded by diffuse black zone

Media and growth conditions

For the isolation and identification of *E. coli*, the enriched sample was cultured on selective medium Levine Eosin Methylene Blue (EMB) Agar and incubated at 37 °C for 24 hours. Morphologically typical colonies (at least 4 / plate) producing metallic sheen were taken into nutrient broth for further identification. *S. aureus* was isolated by using the technique given by Baird Parker (1962). Enriched samples were streaked on Baird Parker Agar (BPA) and the plate was incubated at 37 °C for 24–48 hours. Appearances of jet black colonies surrounded by white halo were considered to be presumptive *S. aureus*.

For the plating of *L. monocytogenes* Dominguez-Rodriguez Isolation Agar (DRIA) (Dominguez-Rodriguez *et al.,* 1984) was used. The inoculum from enriched broth was streaked on DRIA plate and incubated at 37 °C for 24–48 hours. The greenish-yellow glistening, iridescent and pointed colonies surrounded by diffuse black zone were suspected to be listeriae (Table 1).

Physiological and biochemical examination

Four to five suspected colonies from each bacterial plate were picked, cultured and then identified by the various biochemical tests.

Biochemical tests were performed to confirm *E. coli* using Gram staining, Catalase test, Indole, Methyl red, Voges- Proskauer test, Nitrate reduction, Urease production, Simon citrate agar, and various sugar fermentation tests (Table 2).

Table 2. Biochemical characterization of *E. coli*
Preglednica 2. Biokemijska karakterizacija *E. coli*

Biochemical test		Reaction
Lactose fermentation		+
Catalase		+
Simmon's Citrate		–
Indole Production		+
Nitrate Reduction		+
Methyl Red		+
Voges- Proskauer		–
Urease		–
Acid from sugar	(a) Glucose	+
	(b) Mannitol	+
	(c) Lactose	+
	(d) Salicin	+
	(e) Sucrose	+

Confirmation of the genus, *Staphylococcus* was done by Gram staining and various biochemical tests including Catalase test, Oxidase test, Indole, Methyl red, Voges-Proskauer test, Nitrate reduction, acid from different sugars, and haemolysis on Sheep Blood Agar (S.B.A.) following the method of Cruickshank (1970), while the species, *S aureus* was confirmed by Coagulase test as described by Monica (1991) (Table 3).

Table 3. Biochemical characterization of *S. aureus*
Preglednica 3. Biokemijska karakterizacija *S. aureus*

Biochemical test		Reaction
Catalase		+
Oxidase		–
Indole Production		–
Nitrate Reduction		+
Methyl Red		+
Voges- Proskauer		+
Acid from sugar	(a) Glucose	+
	(b) Mannitol	+
	(c) Maltose	+
	(d) Lactose	+
	(e) Raffinose	–
	(f) Sucrose	+
Haemolysis		+
Coagulase		+

Morphologically typical colonies of *L. monocytogenes* were identified by Gram's staining. Catalase reaction, tumbling motility at 20–25 °C, Methyl red test, Voges-Proskauer test, Nitrate reduction, fermentation of sugars, and haemolysis on 5% Sheep Blood Agar were conducted (Table 4).

Table 4. Biochemical characterization of *L. monocytogenes*
Preglednica 4. Biokemijska karakterizacija *L. monocytogenes*

Biochemical test		Reaction
Catalase		+
Oxidase		–
Indole Production		–
Nitrate Reduction		–
Methyl Red		+
Voges- Proskauer		+
Haemolysis		+
Acid from sugar	(a) Rambose	+
	(b) α methyl d mannoside	+
	(c) Xylose	–

RESULTS

The present research findings pertain to the isolation of *E. coli, L. monocytogenes*, and *S. aureus* from milk products. Table 5 depicts the sampling data which consists of various numbers of samples analyzed and confirmed as *E. coli, L. monocytogenes*, and *S. aureus.* Out of 60 isolates 21 isolates were confirmed as *E. coli* (six from curd and 15 from cottage cheese); out of 63 isolates, 12 isolates were confirmed as *S. aureus* (from cottage cheese), out of 51 isolates, 13 isolates were confirmed as *L. monocytogenes* (11 from curd and two from cottage cheese) on the basis of morphological and biochemical characterization (Table 2, 3, and 4).

According to these results a higher contamination with *E. coli* and *S. aureus* was found in cottage cheese as compared to curd. No curd samples yielded *S. aureus* though incidence of *L. monocytogenes* was higher in curd rather than cottage cheese (Table 5).

Table 5. *E. coli, L. monocytogenes*, and *S. aureus* detected in the test sample
Preglednica 5. Število v vzorcih odkritih *E. coli*, *L. monocytogenes* in *S. aureus*

Organism	Number of sampling		Number of samples in which colonies appear		Number of isolates taken		Number of positive samples	
	curd	Cottage cheese	curd	Cottage cheese	curd	Cottage cheese	curd	Cottage cheese
E. coli	13	13	8	7	32	28	6	15
S. aureus	13	13	1	13	4	59	-	12
L. monocytogenes	13	13	5	8	22	29	11	2

DISCUSSION

E. coli, S. aureus, and *L. monocytogenes* occur frequently in milk products, such as curd and cottage cheese (Mary *et al.,* 1992; Oranusi *et al.,* 2007). These milk products are mostly prepared and consumed in the unorganized sector in Agra region. The method of their manufacturing, handling and sale is entirely based on the traditional systems. Such systems could provide a favorable environment for bacterial contamination. The unclean hands of workers, poor quality of milk, unhygienic conditions of the manufacturing unit and water supplied for washing the utensils could be the source for accelerating the bacterial contamination of milk products beside the post manufacturing contamination (Tariq Masud *et al.,* 1988; Johnson, 1961; Elmahmood *et al.,* 2007).

The incidence of the species of *E. coli* itself in milk and milk products, as a possible cause of food born disease, is not significant if *E. coli* is normally a ubiquitous organism (Hahn, 1996), yet the pathogenic strains if present could be harmful to consumers. The risk is magnified when the same samples of curd and cottage cheese are contaminated with *L. monocytogenes*, because *L. monocytogenes* can survive at temperatures of refrigeration where these milk products are normally stored (EI-Kest *et al.,* 1991).

S. aureus on the other hand releases a toxic chemical, enterotoxin. As little as 1.0 μg of the toxin in contaminated food produces symptoms of illness. This level of the toxin has been found at 10^5 cells /g of food (Ananthanarayna *et al.,* 2001).

Milk is one of the most important nutrients and protein dense food, because it is an excellent source of nine essential nutrients and casein, a major milk protein. Dairy products like curd and

cottage cheese made from milk and their consumption plays a significant role in the supply of important nutrients and protein required for good health. These milk products are very essential in the Indian diet so their contamination can cause varied health hazards. This can be of serious concern to the local consumers and also to the innumerable tourists flocking Agra from the different parts of India as well as the world.

The results of the present study indicate that strict preventive measures should be adopted to ensure contamination free milk products for the good health of all consumers. For this, consciousness and care is required from the point of generation to the point of consumption of these widely consumed milk products.

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PREDMETNO KAZALO PO DESKRIPTORJIH AGROVOC

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