ISSN 1580-4003

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA

# SLOVENIAN VETERINARY RESEARCH

## SLOVENSKI VETERINARSKI ZBORNIK

Slov Vet Res 2021; 58 (2): 43 – 90

Volume 58 2

Slov Vet Res • Ljubljana • 2021 • Volume 58 • Number 2 • 43 – 90

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA

# SLOVENIAN VETERINARY RESEARCH

## SLOVENSKI VETERINARSKI ZBORNIK

Volume
58 2

Slov Vet Res • Ljubljana • 2021 • Volume 58 • Number 2 • 43 – 90

The Scientific Journal of the Veterinary Faculty University of Ljubljana

**SLOVENIAN VETERINARY RESEARCH**
**SLOVENSKI VETERINARSKI ZBORNIK**

Previously: RESEARCH REPORTS OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA
Prej: ZBORNIK VETERINARSKE FAKULTETE UNIVERZA V LJUBLJANI

4 issues per year / izhaja štirikrat letno
Volume 58, Number 2 / Letnik 58, Številka 2

Editor in Chief / glavni in odgovorni urednik: Gregor Majdič
Co-Editor / sourednik: Modest Vengušt
Technical Editor / tehnični urednik: Matjaž Uršič
Assistants to Editor / pomočnici urednika: Valentina Kubale Dvojmoč, Klementina Fon Tacer

Published by / Založila: University of Ljubljana Press / Založba Univerze v Ljubljani
For the Publisher / Za založbo: Igor Papič, the Rector of the University of Ljubljana / rektor Univerze v Ljubljani

Issued by / Izdala: Veterinary Faculty University of Ljubljana / Veterinarska fakulteta Univerze v Ljubljani
For the Issuer / Za izdajatelja: Andrej Kirbiš, the Dean of the Veterinary Faculty / dekan Veterinarske fakultete

Editorial Board / uredniški odbor:
Vesna Cerkvenik, Robert Frangež, Polona Juntes, Tina Kotnik, Matjaž Ocepek, Ožbalt Podpečan, Ivan Toplak, Milka Vrecl, Veterinary Faculty University of Ljubljana / Veterinarska fakulteta Univerze v Ljubljani ; Simon Horvat, Janez Salobir, Biotechnical Faculty University of Ljubljana / Biotehniška fakulteta Univerze v Ljubljani; Andraž Stožer, Faculty of Medicine University of Maribor / Medicinska fakulteta Univerze v Mariboru

Editorial Advisers / svetovalca uredniškega odbora: Gita Grecs-Smole for Bibliography (bibliotekarka),
Leon Ščuka for Statistics (za statistiko)

Reviewing Editorial Board / ocenjevalni uredniški odbor:
Antonio Cruz, Institute Suisse du Medicine Equine (ISME), Vetsuisse Fakultat, University of Bern, Switzerland; Gerry M. Dorrestein, Dutch Research Institute for Birds and Exotic Animals, Veldhoven, The Netherlands; Sara Galac, Utrecht University, The Netherlands; Wolfgang Henninger, Veterinärmedizinische Universität Wien, Austria; Nevenka Kožuh Eržen, Krka, d.d., Novo mesto, Slovenia; Louis Lefaucheur, INRA, Rennes, France; Peter O'Shaughnessy, Institute of Comparative Medicine, Faculty of Veterinary Medicine, University of Glasgow, Scotland, UK; Peter Popelka, University of Veterinary Medicine, Košice, Slovakia; Dethlef Rath, Institut für Tierzucht, Forschungsbericht Biotechnologie, Bundesforschungsanstalt für Landwirtschaft (FAL), Neustadt, Germany; Phil Rogers, Grange Research Centre, Dunsany, Co. Meath, Ireland, Ireland; Alex Seguino, University of Edinburgh, Scotland, UK; Henry Staempfli, Large Animal Medicine, Department of Clinical Studies, Ontario Veterinary College, Guelph, Ontario, Canada; Frank J. M. Verstraete, University of California Davis, Davis, California, US; Thomas Wittek, Veterinärmedizinische Universität, Wien, Austria

Address: Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia
Naslov: Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
Tel.: +386 (0)1 47 79 100, Fax: +386 (0)1 28 32 243
E-mail: slovetres@vf.uni-lj.si

Sponsored by the Slovenian Research Agency
Sofinancira: Javna agencija za raziskovalno dejavnost Republike Slovenije

ISSN 1580-4003
Printed by/tisk: DZS, d.d., Ljubljana, June 2021
Number of copies printed / Naklada: 220
Indexed in/indeksirano v: Agris, Biomedicina Slovenica, CAB Abstracts, IVSI
Urlich's International Periodicals Directory, Science Citation Index Expanded,
Journal Citation Reports – Science Edition
https://www.slovetres.si/

This work is licensed under a Creative Commons Attribution-ShareAlike 4.0 International License / To delo je ponujeno pod licenco
Creative Commons Priznanje avtorstva-Deljenje pod enakimi pogoji 4.0 Mednarodna licenca

**SLOVENIAN VETERINARY RESEARCH**
**SLOVENSKI VETERINARSKI ZBORNIK**

**Slov Vet Res 2021; 58 (2)**

**Original Research Articles**

Habeeb GA, Durmuşoğlu H, İlhak Oİ. The combined effect of sodium lactate, lactic acid and acetic acid on the survival of *Salmonella* spp. and the microbiota of chicken drumsticks ....47
Sargious MAN, El-Shawarby RM, Abo-Salem ME, EL-Shewy EA, Ahmed HA, Hagag NM, Ramadan SI. Genetic diversity of Egyptian Arabian horses from El-Zahraa Stud based on 14 TKY microsatellite markers .... 55
Aloke C, Igwe ES, Obasi NA, Amu PA, Ogbonnia EC. Anti-diabetic effect of ethanol extract of Copaifera salikounda (Heckel) against alloxan-induced diabetes in rats ....63
Grilz-Seger G, Mesarič M, Brem G, Cotman M. Characterisation of coat colour in the Slovenian Posavje horse ....77

**Case Report**

Staji H, Tamai IA, Kafi ZZ. First report of *Paenibacillus cineris* from a Burmese python (*Python molurus bivittatus*) with oral abscess ....85

Slov Vet Res 2021; 58 (2): 47 – 54
DOI 10.26873/SVR-955-2020
UDC 579.62:637.51:636.5:579.842.1/.2:547.29

**Original Research Article**

# THE COMBINED EFFECT OF SODIUM LACTATE, LACTIC ACID AND ACETIC ACID ON THE SURVIVAL OF *Salmonella* spp. AND THE MICROBIOTA OF CHICKEN DRUMSTICKS

Goran Ali Habeeb[1], Halil Durmuşoğlu[2], Osman İrfan İlhak[3]*

[1]Sulaimani Veterinary Directorate, Veterinary Quarantine, International Border Compound of Bashmakh, Sulaimani, Iraq, [2]Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Dicle University, Diyarbakır, [3]Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Balikesir University, Balikesir, Turkey

*Corresponding author, E-mail: irfan.ilhak@balikesir.edu.tr

**Abstract:** The poultry processing industry has been investigating the new decontamination applications to prevent foodborne pathogens and extend the shelf life of poultry products. This study investigates the effects of lactic acid, acetic acid and sodium lactate, alone and in combination, on the survival of *Salmonella* spp. and the shelf life of chicken drumsticks. The fresh chicken drumsticks were inoculated with *Salmonella* Typhimurium and *Salmonella* Enteritidis and they were divided into groups as control (sterile tap water), 1% sodium lactate (SL), 1.5% lactic acid (LA), 1.5% acetic acid (AA), and their combinations. The drumstick samples were immersed into the treatment solutions for 5 minutes and stored at 4 ˚C for eight days, and they were analyzed for aerobic psychrotrophic bacteria (APB), *Pseudomonas* spp., lactic acid bacteria (LAB), *Salmonella* spp. and pH level. On day 5, APB, *Pseudomonas* spp. and LAB numbers exceeded 7.0 $log_{10}$ CFU $ml^{-1}$ in the control, SL, LA and LA+ SL groups. The reduction levels of *Salmonella* spp. were 1.2 and 0.9 $log_{10}$ CFU $ml^{-1}$ in the LA and AA+LA groups on day 0, and they were significantly different from the control group ($P<0.05$). The shelf life of the chicken drumsticks that were treated with the solutions containing 1.5% AA (AA, AA+ SL, AA+ LA and AA+ LA+ SL) was at least two days longer than the control group. It is concluded that the combinations of 1.5% LA, 1.5% AA and 1% SL can be used to reduce the number of *Salmonella* spp. and to extend the shelf life of chicken drumstick.

**Key words:** chicken drumstick; lactic acid; acetic acid; sodium lactate; Shelf life; *Salmonella* spp.

## Introduction

Chicken meat is one of the important animal protein sources in human diet. However, if raw chicken meat is not properly handled and preserved, it supports the growth of spoilage bacteria and foodborne pathogens such as *Salmonella* spp. (1, 2). Guran et al. (3) found that *Salmonella* spp. prevalence in the skin of chicken drumstick, chicken breast and thighs were 41%, 44.7% and 40.9%, respectively. In Turkey, the data from National *Salmonella* spp. Control Program, which was conducted between 2014 and 2017, revealed that 47% of the carcass samples (n= 691) taken from chicken slaughterhouses were contaminated with *Salmonella* spp. (4). The investigations to find effective applications to control *Salmonella* spp. in poultry carcasses and poultry products have still been continuing. On the other hand, there is increasing concern about the use of chemical preservatives in foodstuff by consumers. More and more people prefer minimally processed foods or foods treated with organic preservatives (5).

Lactic acid (LA) is one of the organic acids naturally occurring in muscles. Sodium lactate (SL) is the sodium salt of lactic acid. Acetic acid (AA), which is commonly known as vinegar, is one of the organic acids that occurs naturally during

Received: 29 August 2019
Accepted for publication: 13 November 2020

the spoilage of fruit and certain other foods by the bacteria of the genus *Acetobacter*. LA, SL and AA are substances affirmed as Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA), and they are allowed to be directly added to various foods to inhibit the microbial growth and to extend the shelf life of products (6). The antimicrobial efficacy of SL, LA and AA has been intensely studied by many researchers (7-10). However, there is limited information related to effect of organic acid blends on the shelf life of chicken meat parts and the survival of *Salmonella* spp. on chicken meat. Some researchers have studied the effects of various organic acid blends against the pathogenic microorganisms on poultry carcasses (11-15), and they have noted that the combinations of organic acids may improve the microbiological quality of poultry meat. In meat processing environments, the poultry meat microbiota consists mainly of *Pseudomonas* spp., lactic acid bacteria and many other psychrotrophic bacteria (16). There is limited information regarding the effect of organic acid blends on the microbiota in chicken meat.

The aims of this study were (i) to evaluate the antimicrobial efficacy of the combination of sodium lactate, lactic acid and acetic acid against aerobic psychrotrophic bacteria, *Pseudomonas* spp., lactic acid bacteria and *Salmonella* spp. on chicken drumstick, and (ii) to investigate the shelf life of chicken drumstick that are treated with the blends of sodium lactate, lactic acid and acetic acid.

## Materials and methods

### *Samples and preparation of Salmonella spp. inoculum*

In each of the three trials, 36 fresh chicken drumsticks with skin (each one weighed 100 - 150 gram) were used. Their production date was one or two days before the purchase date from a local supermarket. Throughout the experiments, a total of 108 chicken drumsticks were used.

Because of the differences in bacterial strains against antimicrobials, one salmonella cocktail culture, which was composed of one *Salmonella* Enteritidis (RSKK 92 (RSKK is a microorganism culture collection center in Turkish Public Health – Turkey)) and two *Salmonella* Typhimurium (NCTC 12416 and NCTC 74) strains, were used. Each of the *Salmonella* strains was grown in 10 ml of tryptic soy broth (TSB, Acumedia, Maryland) at 37°C for 18 h. Cultures were then centrifuged at 4192 × g for 10 minutes at 5°C, and the supernatant was discarded. The formed pellets were re-suspended in 10 ml of 0.1% sterile peptone water, and then they were centrifuged again to remove the organic residues. The supernatant was removed and the pellets of each strain were re-suspended in 1-2 ml of 0.1% sterile peptone water. These suspensions were combined in a single tube to obtain the salmonella cocktail, and the salmonella cocktail tube was completed to 10 ml with 0.1% sterile peptone water.

### *Inoculation of chicken drumstick*

Before the inoculation procedure, two randomly selected drumstick samples were taken, and tested for the existence of indigenous *Salmonella* spp.. For the inoculation, the *Salmonella* spp. cocktail of 0.25 ml was spread on each of the drumstick samples using a sterile L-shaped spreader. After inoculation, the drumsticks were kept at room temperature for 10 minutes for bacterial attachment. Then, the two samples were taken and analyzed to detect the initial inoculation numbers of *Salmonella* spp.

### *Decontamination treatments*

Sodium lactate solution (60% w/w) (CAS number 72-17-3), L(+) - Lactic acid solution (88-92%) (CAS number: 79-33-4), and Acetic acid (100%) (CAS number: 64-19-7) were used in this study, and they were purchased from Sigma (Sigma-Aldrich, Germany).

The chicken samples in each trial were divided into eight groups. Each group of drumstick samples was dipped into a sterile glass beaker containing 500 ml of one of the following sterile decontamination solutions (v/v) at ambient temperature for 5 minutes. Decontamination solutions (treatment groups) and their pH values were as follows:

1- Control (sterile tap water),

2- 1.5% Lactic acid (pH 2.3),

3- 1.5% Acetic acid (pH 2.75),

4- 1% Sodium lactate (pH 6.96),

5- 1.5% Lactic acid + 1% Sodium lactate (pH 3.33),

6- 1.5% Acetic acid + 1% Sodium lactate (pH 3.72),
7- 1.5% Lactic acid + 1.5% Acetic acid (pH 2.20),
8- 1.5% Lactic acid + 1.5% Acetic acid + 1% Sodium lactate (pH 3.28)

After the decontamination procedures, the chicken drumsticks were allowed to drain at room temperature for 10 minutes. After draining, each drumstick was individually placed in a separate foam plate, and it was wrapped with cling film and stored at 4°C for eight days.

### *Microbiological analysis*

Microbiological analyses were carried out on days 0 (after the dipping treatment), 3, 5 and 8. Briefly, 100 ml of 0.1% sterile peptone water (PW) was added into a sterile stomacher bag containing a chicken drumstick sample, and the stomacher bag was shaken manually for one minute. Then, 1 ml of the rinse solution was taken from the stomacher bag and serially diluted up to $10^{-6}$ in sterile tubes containing 9 ml of 0.1% PW, and they were used for the following microbial analyses.

Microbiological analyses were conducted by using the spread plate method and duplicate plates. Since a 1 ml rinse solution was used for microbiological analysis, the numbers of microorganisms were expressed as $\log_{10}$ CFU per ml rinse solution in all the samples.

Plate Count Agar (Merck, Germany) was used to enumerate Aerobic Psychrotrophic Bacteria (APB), and the plates were incubated at 6.5°C for 10 days. *Pseudomonas* Selective Agar (Merck, Germany) supplemented with *Pseudomonas* CFC Selective Supplement (Merck, Germany) was used to detect *Pseudomonas* spp., and the plates were incubated at 25°C for 2 days. After the incubation period, three colonies from each plate were randomly selected and subjected to oxidase test (Bactident Oxidase, Merck, Germany). According to the results obtained in the oxidase test (*Pseudomonas* spp. is oxidase positive), the numbers of *Pseudomonas* spp. were calculated. Lactic acid bacteria (LAB) were enumerated using de Man Rogosa and Sharpe (MRS) Agar (LAB-M, Merck, Germany), and the plates were incubated at 28°C for 2 days. *Salmonella* spp. was enumerated using Xylose Lysine Deoxycholate (XLD) agar (HiMedia, India), and the plates were incubated at 35°C for 24-36 h.

### *Determination of pH values of the samples*

After the microbiological analysis was completed, the pH values of the rinse solution of the samples were measured by using a pH meter (Selecta pH 2001, J.P., Spain).

### *Statistical analysis*

Analyses of the microbiological data and pH values of three independent trials were carried out using SPSS 22 software (IBM, SPSS Statistics, Version 22, USA). The numbers of bacteria were converted to logarithmic values (log CFU $ml^{-1}$ rinse solution) before calculating means and performing statistical analysis. The data were subjected to analysis of variance (ANOVA) appropriate to replicate × treatment groups × sampling times to determine fixed effects and interactions between variables. The Bonferroni test was used for multiple comparisons between the groups. Statistical significant level was expressed as $P<0.05$.

## Results

The decontamination treatment with 1% sodium lactate (SL) did not show bacteriostatic or bactericidal effect on *Salmonella* spp. or microbiota in the chicken drumsticks; however APB, *Pseudomonas* spp. and LAB counts in the groups that were treated with 1.5% lactic acid (LA), 1.5% acetic acid (AA) and their combination were lower than those in the control and SL groups (Table 1).

On day 0, the number of APB and *Pseudomonas* spp. counts in the groups that were treated with AA, AA+LA and AA+LA+SL were between 3.9 and 4.4 $\log_{10}$ CFU $ml^{-1}$ rinse solution, and they were statistically different from the control group (5.4 $\log_{10}$ CFU $ml^{-1}$) ($P<0.05$). On day 5, APB, *Pseudomonas* and LAB counts were below 7.0 $\log_{10}$ CFU $ml^{-1}$ rinse solution in the groups that were treated with AA, AA+LA and AA+LA+SL, and they were significantly different from the SL and control groups ($P<0.05$). During the five days storage, the results showed that *Pseudomonas* numbers of the groups containing AA were numerically lower than the other groups that did not contain AA (Table 1). The drumstick samples in the control, SL and LA+SL groups had a bad odor and a slight slime layer on day 5. Their APB and *Pseudomonas* counts were above 7.2 $\log_{10}$ CFU $ml^{-1}$ rinse solution. The control, SL and LA+SL groups were not analyzed on day 8 because of their apparent sensorial defect.

**Table 1:** The mean numbers of APB, *Pseudomonas* spp. and LAB of the drumstick samples immersed into decontamination solutions for 5 min and stored at 4°C ($log_{10}$ CFU $ml^{-1}$ rinse solution ± SD)

| Treatment Groups | Storage days | | | |
|---|---|---|---|---|
| | 0 | 3 | 5 | 8 |
| | Aerobic Psychrotrophic Bacteria | | | |
| Control | $5.4^{BCv}$ ± 0.2 | $6.9^{Cw}$± 0.2 | $7.8^{CDx}$ ± 0.2 | NA |
| SL | $5.7^{Cv}$ ± 0.1 | $6.9^{Cw}$ ± 0.2 | $8.1^{Dx}$ ± 0.2 | NA |
| LA | $4.9^{ABv}$±0.3 | $6.3^{BCw}$ ± 0.3 | $7.2^{ABCx}$ ±0.1 | $8.2^{Ay}$ ± 0.2 |
| AA | $4.4^{Av}$± 0.2 | $5.5^{Aw}$ ± 0.5 | $6.8^{ABx}$ ± 0.1 | $7.9^{Ay}$ ± 0.3 |
| LA+ SL | $4.9^{ABv}$±0.1 | $6.4^{BCw}$ ± 0.2 | $7.5^{BCDx}$ ±0.2 | NA |
| AA+ SL | $4.7^{ABv}$ ± 0.1 | $5.8^{ABw}$ ± 0.2 | $7.0^{ABCx}$ ±0.1 | $8.3^{Ay}$ ± 0.4 |
| AA+ LA | $4.2^{Av}$ ± 0.3 | $5.6^{ABw}$ ± 0.2 | $6.5^{Ax}$ ± 0.1 | $7.8^{Ay}$ ± 0.1 |
| AA+LA+ SL | $4.2^{Av}$ ± 0.3 | $5.6^{ABw}$ ± 0.1 | $6.6^{Ax}$ ± 0.2 | $7.8^{Ay}$ ± 0.2 |
| | *Pseudomonas* spp. | | | |
| Control | $5.4^{BCw}$ ± 0.4 | $6.8^{Cx}$ ± 0.4 | $7.9^{By}$ ± 0.4 | NA |
| SL | $5.7^{Cw}$ ± 0.5 | $6.9^{Cx}$ ± 0.4 | $7.9^{By}$ ± 0.2 | NA |
| LA | $4.2^{Aw}$ ± 0.2 | $5.6^{ABx}$ ± 0.3 | $7.0^{ABy}$ ± 0.2 | $7.5^{Ay}$ ± 0.1 |
| AA | $3.9^{Aw}$ ± 0.1 | $5.4^{ABx}$ ± 0.4 | $6.4^{Ay}$ ± 0.2 | $7.6^{Az}$ ± 0.2 |
| LA+ SL | $4.7^{ABw}$ ± 0.1 | $5.8^{Bx}$ ± 0.3 | $7.2^{ABy}$ ± 0.3 | NA |
| AA+ SL | $4.0^{Aw}$ ± 0.3 | $5.2^{ABx}$ ± 0.2 | $6.7^{Ay}$ ± 0.2 | $7.5^{Az}$ ± 0.2 |
| AA+ LA | $4.1^{Av}$ ± 0.4 | $5.2^{ABw}$ ± 0.2 | $6.6^{Ax}$ ± 0.17 | $7.3^{Ax}$ ± 0.2 |
| AA+LA+ SL | $3.9^{Av}$ ± 0.2 | $4.8^{Aw}$ ± 0.1 | $6.2^{Ax}$ ± 0.2 | $7.2^{Ay}$ ± 0.4 |
| | Lactic Acid Bacteria | | | |
| Control | $5.1^{Ax}$ ± 0.1 | $6.8^{CDy}$ ± 0.3 | $7.6^{Cz}$ ± 0.2 | NA |
| SL | $5.2^{Ax}$ ± 0.2 | $7.0^{Dy}$± 0.2 | $7.8^{Cz}$ ± 0.3 | NA |
| LA | $4.7^{Ax}$ ± 0.2 | $6.0^{ABCy}$ ±0.1 | $7.3^{BCz}$ ± 0.3 | $7.9^{Bz}$ ± 0.1 |
| AA | $4.9^{Ax}$ ± 0.1 | $5.5^{Ax}$ ± 0.4 | $6.6^{ABy}$ ± 0.2 | $7.7^{ABz}$ ± 0.4 |
| LA+ SL | $5.1^{Ax}$± 0.1 | $6,3^{BCDy}$ ±0.4 | $7.1^{ABCz}$± 0.4 | NA |
| AA+ SL | $5.1^{Ax}$ ± 0.1 | $5.9^{ABxy}$ ±0.4 | $6.3^{Ay}$ ± 0.1 | $7.4^{ABz}$ ± 0.4 |
| AA+ LA | $4.9^{Ax}$ ± 0.3 | $5.4^{Ax}$ ± 0.1 | $6.5^{ABy}$ ± 0.1 | $7.1^{AByz}$±0.1 |
| AA+LA+ SL | $5.0^{Aw}$ ± 0.1 | $5.8^{ABwx}$±0.4 | $6.5^{ABxy}$ ±0.4 | $7.0^{Ayz}$ ± 0.5 |

$^{ABC}$: Values with different superscripts within the same column are significantly different (P<0.05)
$^{wxyz}$:Values with the different superscript within the same row are significantly different (P<0.05)
NA: Not analyzed SL: Sodium Lactate LA: Lactic acid AA: Acetic acid

The samples which were analyzed for indigenous *Salmonella* spp. showed that the indigenous *Salmonella* spp. in the purchased drumstick samples was below the detection limit (<1 CFU $ml^{-1}$). The average inoculation level of *Salmonella* spp. colonies in the control samples was 5.4 $log_{10}$ CFU $ml^{-1}$ rinse solution (Table 2). After the decontamination treatments (on day 0), the counts of *Salmonella* spp. colonies of the samples reduced up to 1.2 $log_{10}$ CFU $ml^{-1}$ depending on the decontamination solutions when compared with the control sample (P<0.05). After day 0, the numbers of *Salmonella* spp. in the groups were almost stable during the storage time, and no significant differences was observed between the storage days (P>0.05). On day 5, the combination of AA+LA had the best antimicrobial efficacy on *Salmonella* spp. compared to the control group (P<0.05).

**Table 2:** The mean numbers of *Salmonella* spp. of the drumstick samples immersed into decontamination solutions for 5 min and stored at 4°C ($log_{10}$ CFU $ml^{-1}$ rinse solution ± SD)

| Treatment Groups | Storage days | | | |
|---|---|---|---|---|
| | 0 | 3 | 5 | 8 |
| Control | $5.4^{Cx}$ ± 0.1 | $5.3^{ABx}$ ± 0.2 | $5.3^{Bx}$ ± 0.1 | NA |
| SL | $5.2^{BCx}$ ± 0.1 | $5.4^{Bx}$ ± 0.2 | $5.3^{Bx}$ ± 0.1 | NA |
| LA | $4.2^{Ax}$ ± 0.2 | $4.8^{ABx}$ ± 0.2 | $4.8^{ABx}$ ± 0.3 | $4.6^{ABx}$ ±0.1 |
| AA | $4.7^{ABCx}$± 0.2 | $4.9^{ABx}$ ± 0.2 | $4.7^{ABx}$ ± 0.2 | $4.8^{ABx}$ ±0.1 |
| LA+ SL | $4.9^{ABCx}$± 0.2 | $4.9^{ABx}$ ± 0.1 | $5.1^{ABx}$ ± 0.1 | NA |
| AA+ SL | $5.4^{Cx}$ ± 0.1 | $5.3^{ABx}$ ± 0.2 | $5.4^{Bx}$ ± 0.1 | $5.1^{Bx}$± 0.2 |
| AA+ LA | $4.5^{ABx}$ ± 0.1 | $4.6^{Ax}$ ± 0.1 | $4.5^{Ax}$ ± 0.2 | $4.4^{Ax}$ ± 0.3 |
| AA+LA+ SL | $4.8^{ABCx}$± 0.3 | $4.8^{ABx}$ ± 0.1 | $4.9^{ABx}$ ± 0.2 | $4.8^{ABx}$ ±0.1 |

$^{ABC}$: Values with different superscripts within the same column are significantly different (P<0.05)
$^{x}$:Values with the same superscript within the same row are not significantly different (P>0.05)
NA: Not analyzed SL: Sodium Lactate LA: Lactic acid AA: Acetic acid

The initial pH of the control sample was 6.78. The pH of the samples that were treated with AA and LA decreased to 5.35 and 5.72 on day 0, respectively (Table 3), and the combination of AA and LA decreased the pH of the chicken drumstick to 5.02. Those groups were significantly different from the control and SL groups (P<0.05). However, the pH of the samples treated with organic acids, alone and in combination, dramatically increased and approached to the control group on day 3.

**Table 3:** The mean pH values of the rinse solutions of the drumstick samples immersed into decontamination solutions for 5 min and stored at 4°C (pH ± SD)

| Treatment Groups | Storage days | | | |
|---|---|---|---|---|
| | 0 | 3 | 5 | 8 |
| Control | $6.78^{Cxy}$± 0.1 | $6.67^{ABx}$±0.1 | $7.15^{ABy}$±0.1 | NA |
| SL | $7.00^{Cxy}$± 0.1 | $6.87^{Bx}$± 0.2 | $7.33^{By}$ ± 0.2 | NA |
| LA | $5.72^{Bx}$± 0.1 | $6.53^{ABy}$±0.2 | $7.15^{ABz}$±0.1 | $7.20^{Az}$± 0.0 |
| AA | $5.35^{ABx}$±0.2 | $6.39^{ABy}$±0.2 | $6.90^{ABz}$±0.2 | $6.91^{Az}$± 0.2 |
| LA+ SL | $5.76^{Bx}$ ± 0.3 | $6.68^{ABy}$±0.1 | $7.07^{ABy}$±0.1 | NA |
| AA+ SL | $5.53^{ABx}$±0.2 | $6.68^{ABy}$±0.2 | $6.90^{ABy}$±0.1 | $7.02^{Ay}$± 0.1 |
| AA+ LA | $5.02^{Ax}$ ± 0.1 | $6.27^{Ay}$± 0.2 | $6.72^{Ayz}$± 0.3 | $6.85^{Az}$± 0.1 |
| AA+LA+SL | $5.03^{Ax}$ ± 0.2 | $6.53^{ABy}$±0.1 | $6.83^{AByz}$±0.1 | $6.79^{Ayz}$±0.1 |

$^{ABC}$: Values with different superscripts within the same column are significantly different (P<0.05)
$^{xyz}$:Values with the same superscript within the same row are significantly different (P>0.05)
NA: Not analyzed SL: Sodium Lactate LA: Lactic acid AA: Acetic acid

## Discussion

Eliminating foodborne pathogens and reducing the number of microorganisms causing spoilage in poultry products are among the main goals of the poultry industry. Many researchers have reported that using solutions containing 1% to 3% organic acids have no negative effects on the sensory characteristics in poultry meat or may cause acceptable sensory changes such as insignificant differences in color and taste (7,15,17).

There was no statistical difference between the LA and AA treatments in point of antibacterial effect in the present study; however, the results showed that AA was more effective than LA on the APB and *Pseudomonas* spp. This may be due to the concentrations of undissociated acid molecules of LA and AA. Different acids have various impacts on bacterial survival because of their different dissociation degrees. Undissociated acid molecules penetrate into the bacterial cell and show antimicrobial properties (18). International

Commission on Microbiological Specifications for Foods (ICMSF) reported that un-dissociated proportions of LA and AA at pH 5 were 6.05% and 34.9%, and at pH 6.0 were 0.64% and 5.1%, respectively (19). In this research, the pH of the samples that were treated with AA and LA are 5.35 and 5.72 on day 0, respectively (Table 3). The high efficacy of AA on the APB and *Pseudomonas* spp. can be attributed to the low pH of the samples, because the concentration of undissociated acid molecules of AA is greater than that of LA at the same pH (18).

The drumstick samples in the control, SL and LA+ SL groups had a bad odor and slight slime layer on their skin on day 5 (Table 1). Their APB and *Pseudomonas* spp. counts were above 7.2 $log_{10}$ CFU $ml^{-1}$ rinse solution. Jay et al. (16) reported that a bad odor in chicken meat can be detected when the surface bacterial number (especially *Pseudomonas* spp.) is between 7.2 and 8.0 $log_{10}$ CFU $cm^{-2}$. As it is known, the initial bacterial load of a product has a great effect on the shelf life of the product. In this study, the initial APB counts of the control and 1% SL groups were 5.4 and 5.7 $log_{10}$ CFU $ml^{-1}$, respectively. Therefore, the control and 1% SL groups deteriorated before on day 5. There is limited information regarding the microbiota on chicken meat treated with organic acid blends. Zhu et al. (13) reported that the numbers of total viable bacteria and *Pseudomonas* spp. on the chicken drumsticks that were spray-washed with the combination of 0.5% LA+1% citric acid for 30 s were reduced 1.68 and 1.85 $log_{10}$ CFU/$cm^2$, respectively. Olaimat et al. (15) noted that the count of aerobic mesophilic bacteria on chicken breast meat that were immersed into 0.5% AA+0.5% malic acid (MA) blends for 5 minutes decreased 2 $log_{10}$ CFU/g.

In this study, none of the treatments showed bactericidal or bacteriostatic effects on LAB during the storage (Table 1). It was observed that LAB is more resistant to LA and AA compared to *Pseudomonas* spp.. In general, the groups treated with AA had a lower number of LAB than the groups treated with LA during the storage. Although there was no significant difference between the groups treated with AA, LA and their combination, it was observed that AA numerically caused more reduction in LAB compared to the LA. Olaimat et al. (15) reported that LAB counts on chicken breast meat treated with 0.5% AA for 5 min remained almost constant (approximately between 5 and 6 $log_{10}$ CFU/g), and 0.5%AA+0.5% MA blends solution resulted in approximately 2 $log_{10}$ CFU/g reduction by the end of 10 days of storage at 4°C.

Nagel et al. (20) and Lee et al. (21) reported that it was not easy to remove *Salmonella* spp. from the folded areas and follicles on chicken skin. İlhak et al. (9) observed that there was no significant reduction in the number of *Salmonella* spp. on chicken drumstick sprayed with 2% LA for 30 sec. Ramirez-Hernandez et al. (14) used the LA+AA blend at the concentration of 2-2.5% (v/v, pH 2.8) and spray method for 15 sec on *Salmonella* spp. in chicken thigh, and they did not find a significant reduction in *Salmonella* spp. counts compared to the control group. In this study, the counts of *Salmonella* spp. in the samples treated with LA and AA+LA combination were reduced 1.2 and 0.9 $log_{10}$ CFU $ml^{-1}$ respectively ($P<0.05$). Mani-López et al. (8) reported that the sensitivity of bacteria to the antimicrobial effect of acetic acid, lactic acid or sodium lactate increases with a decrease in the pH level of food. On the other hand, Tan et al. (22) noted that chicken skin and chicken meat have buffering capacity against changes in pH. This buffering capacity may help the survival of *Salmonella* spp. in chicken meat treated with organic acids.

In this study, the pH of the samples treated with organic acids was above 5 immediately after the treatments. After day 0, the pH of the samples reached above 6.0 (Table 3). The undissociated acid proportions of AA and LA are very few at pH 6.0 and above (19). The physical structure of chicken skin and the high buffering capacity of the chicken meat may have protected *Salmonella* spp. from the effect of organic acids (22). The reductions in the number of *Salmonella* spp. in the treated groups after day 0 were low. This may have been because of the protective effect of the physical structure of chicken skin and the high buffering capacity of the chicken meat and skin. However, Olaimat et al. (15) immersed chicken breast into 0.5 AA%+0.5% malic acid (MA) blends for 5 min, and they found that the acid blend resulted in more than 5.5 $log_{10}$ CFU/g reduction in viable *Salmonella* spp. at the end of 10 days storage at 4°C. Mikolajczyk (12) noted that immersing breast meat into a solution composed of equal parts of 1% acetic acid, 1% lactic acid, and 1% tartaric acid for 15 min resulted in 2 log Most Probable Number (MPN)/ml reduction in

the number of *Salmonella* spp. after storage at 4 °C for 6 days. When the findings of many studies regarding organic acids applications on poultry carcasses and carcass parts are examined (9, 14, 17, 20, 21), it is seen that the reducing effects of organic acids on *Salmonella* spp. change from insignificant level to about 2.5 $log_{10}$ CFU $ml^{-1}$ depending on acid concentration (1-5%), treatment time (15 sec to 20 min) and application methods (spraying, dipping). In our study, the reduction levels in the number of *Salmonella* spp. in the groups treated with organic acids, alone and in combined form, changed between 0 and 1.2 $log_{10}$ CFU $ml^{-1}$.

In each experiment, two drumsticks from the chicken drumstick samples, which were purchased from a supermarket, were randomly selected and analyzed for the presence of indigenous *Salmonella* spp.. It is expected that chicken meats purchased from supermarkets should not contain *Salmonella* spp. or should contain at very low concentrations (may be less than 1 $log_{10}$ CFU/g, at most 2 $log_{10}$ CFU/g). In this study, the initial inoculation level of *Salmonella* spp. was about 5.4 $log_{10}$ CFU/ml, which was at least 2000 times higher than the numbers likely to be found on the samples. Because of that, no significant interference was expected from indigenous *Salmonella* spp.

## Conclusion

Decontamination of chicken drumstick with 1% SL showed no advantage in extending the shelf life of the samples or in reduction of the number of *Salmonella* spp. The combination of 1% SL with 1.5% LA or 1.5% AA did not have a synergistic or additive antibacterial effect on the spoilage bacteria and *Salmonella* spp. when compared to LA and AA used alone.

In conclusion, the shelf life of chicken drumsticks that were treated with the decontamination solutions containing AA (alone or in combination) was extended at least 2 days when compared to the control group. *Salmonella* spp. was relatively resistant to 1.5% AA and 1.5% LA. This was probably because of the buffering capacity of chicken skin and meat. The buffering capacity of the product should not be neglected when organic acids are used as a decontamination agent. Increasing the acid concentrations would probably give better results in extension of shelf life of the product and elimination of *Salmonella* spp. However, the use of organic acid at high concentrations can negatively affect the organoleptic properties of the product. This study contributes to the poultry industry on choosing effective decontamination treatments to improve the shelf life and safety of poultry meats.

## Acknowledgement

This study was supported by Scientific Research Projects Coordination Unit of Firat University (FÜBAP) with the Project number of VF.17.11.

## References

1. Rouger A, Tresse O, Zagorec M. Bacterial contaminants of poultry meat: sources, species, and dynamics. Microorganisms 2017; 5: 50.

2. Silva F, Domingues FC, Nerín C. Trends in microbial control techniques for poultry products. Crit Rev Food Sci 2018; 58: 591–609.

3. Guran HS, Mann D, Alali WQ. *Salmonella* prevalence associated with chicken parts with and without skin from retail establishments in Atlanta metropolitan area, Georgia. Food Control 2017; 73: 462–7.

4. Republic of Turkey Ministry of Agriculture and Forestry. National *Salmonella* control program, 2018. (In Turkish) https://www.tarimorman.gov.tr/Duyuru/598/ulusal-salmonella-kontrol-programi-yayinlanmistir- (March, 2021)

5. Tajkarimi MM, Ibrahim SA, Cliver DO. Antimicrobial herb and spice compounds in food. Food Control 2010; 21: 1199–218.

6. FDA (Food and Drug Administration). CFR-Code of Federal Regulations, title 21, part 184. Direct food substances affirmed as generally recognized as safe. Subpart B-listing of specific substances affirmed as gras. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=184

7. Cosansu S, Ayhan K. Effects of lactic and acetic acid on survival of *Salmonella enteritidis* during refrigerated and frozen storage of chicken meats. Food Bioprocess Technol 2012; 5: 372–7.

8. Mani-López E, García HS, López-Malo A. Organic acids as antimicrobials to control *Salmonella* in meat and poultry products. Food Res Int 2012; 45: 713–21.

9. İlhak Oİ, İncili GK, Durmuşoğlu H. Evaluation of effect of thymol combined with lactic acid or sodium lactate on psychrophilic bacteria and *Sal-*

*monella* spp. on chicken drumstick. Ann Anim Sci 2017; 17: 271–80.

10. Radkowski M, Zdrodowska B, Gomolka-Pawlicka M. Influence of lactic acid on the survival of *Salmonella* on poultry carcass surface. Med Weter 2018; 74: 139–42.

11. Rajkovic A, Tomic N, Smigic N, Uyttendaele M, Ragaert P, Devlieghere F. Survival of *Campylobacter jejuni* on raw chicken legs packed in high-oxygen or high-carbon dioxide atmosphere after the decontamination with lactic acid/sodium lactate buffer. Int J Food Microbiol 2010; 140: 201–6.

12. Mikołajczyk A. Evaluation of the effects of a mixture of organic acids and duration of storage on the survival of *Salmonella* on turkey carcasses. J Food Protect 2015; 78: 585–9.

13. Zhu Y, Xia X, Liu A, et al. Effects of combined organic acid treatments during the cutting process on the natural microflora and quality of chicken drumsticks. Food Control 2016; 67: 1–8.

14. Ramirez-Hernandez A, Brashears MM, Sanchez-Plata MX. Efficacy of lactic acid, lactic acid-acetic acid blends, and peracetic acid to reduce *Salmonella* on chicken parts under simulated commercial processing conditions. J Food Protect 2018; 81: 17–24.

15. Olaimat AN, Al-Holy MA, Abu Ghoush MH, et al. The use of malic and acetic acids in washing solution to control *Salmonella* spp. on chicken breast. J Food Sci 2018; 83: 2197–203.

16. Jay JM, Loessner MJ, Golden DA. Modern food microbiology. 7th ed. New York : Springer Science+Business Media, 2005.

17. Lecompte JY, Collignan A, Sarter S, Cardinale E, Kondjoyan A. Decontamination of chicken skin surfaces inoculated with *Listeria innocua, Salmonella enteritidis* and *Campylobacter jejuni* by contact with a concentrated lactic acid solution. Br Poultry Sci 2009; 50: 307–17.

18. Feiner G. Meat products handbook. Practical science and technology. Cambridge : CRC Press, 2006.

19. IFT/FDA. Factors that influence microbial growth. IFT/FDA Report on task order 4. Comprehensive Reviews in Food Science and Food Safety 2003; 2: 21–32.

20. Nagel GM, Bauermeister LJ, Bratcher CL, Singh M, McKee SR. *Salmonella* and *Campylobacter* reduction and quality characteristics of poultry carcasses treated with various antimicrobials in a post-chill immersion tank. Int J Food Microbiol 2013; 165: 281–6.

21. Lee NY, Park SY, Kang IS, Ha SD. The evaluation of combined chemical and physical treatments on the reduction of resident microorganisms and *Salmonella* Typhimurium attached to chicken skin. Poultry Sci 2014; 93: 208–15

22. Tan SM, Lee SM, Dykes GA. Fat contributes to the buffering capacity of chicken skin and meat but enhances the vulnerability of attached *Salmonella* cells to acetic acid treatment. Food Res Int 2014; 66: 417–23.

## KOMBINIRANI UČINEK NATRIJEVEGA LAKTATA, MLEČNE KISLINE IN OCETNE KISLINE NA PREŽIVETJE *Salmonelle spp.* IN OSTALIH MIKROORGANIZMOV NA PIŠČANJIH BEDRIH

G. A. Habeeb, H. Durmuşoğlu, O. İ. İlhak

**Izvleček:** Perutninsko predelovalna industrija raziskuje nove prilagoditve za dekontaminacijo mesa, da bi preprečila prenašanje patogenih mikroorganizmov s hrano in podaljšala rok uporabnosti perutninskih izdelkov. V opisani raziskavi so avtorji proučevali učinke mlečne kisline, ocetne kisline in natrijevega laktata, samostojno ali v kombinaciji, na preživetje *Salmonelle spp.* in na rok uporabnosti piščančjih beder. Sveža piščančja bedra so inokulirali s *Salmonello typhimurium* in *Salmonella enteritidis* ter jih razdelili v skupine: kontrolna skupina (z dodano sterilno vodo iz vodovoda), skupina z 1% natrijevim laktatom (SL), skupina z 1,5% mlečne kisline (LA), skupina z 1,5% ocetne kisline (AA) in njihovih kombinacij. Bedra so za 5 minut potopili v raztopine za obdelavo in jih osem dni hranili pri 4° C ter jih analizirali za prisotnost aerobne psihrotrofne bakterije (APB), *Pseudomonas spp.*, mlečnokislinske bakterije (LAB), *Salmonella spp.* in pH-vrednosti. Peti dan so koncentracije APB, *Pseudomonas spp.* in LAB presegale 7,0 $\log_{10}$ CFU ml$^{-1}$ v kontrolni skupini, skupini SL, LA in LA + SL. Znižanje ravni *Salmonella spp.* so bile 0, 1 in $\log_{10}$ CFU ml$^{-1}$ v skupinah LA in AA + LA na dan 0 in so se bistveno razlikovale od kontrolne skupine ($P < 0{,}05$). Rok uporabnosti piščančjih beder, obdelanih z raztopinami, ki vsebujejo 1,5% AA (AA, AA + SL, AA + LA in AA + LA + SL), je bil vsaj dva dni daljši od kontrolne skupine. Ugotovljeno je bilo, da lahko kombinacije 1,5% LA, 1,5% AA in 1% SL uporabimo za zmanjšanje števila *Salmonella spp.* in podaljšajo rok uporabnosti piščančjih beder.

**Ključne besede:** piščančja bedra; mlečna kislina; ocetna kislina; natrijev laktat; rok uporabnosti; *Salmonella spp.*

Slov Vet Res 2021: 58 (2): 55 – 62
DOI 10.26873/SVR-1041-2020
UDC 636.1: 601.4:577.21

**Original Research Article**

# GENETIC DIVERSITY OF EGYPTIAN ARABIAN HORSES FROM EL-ZAHRAA STUD BASED ON 14 *TKY* MICROSATELLITE MARKERS

Mary A. N. Sargious[1, 2], Ragab M. El-Shawarby[2], Mohamed E. Abo-Salem[2], Elham A. EL-Shewy[2], Hanaa A. Ahmed[1], Naglaa M. Hagag[1], Sherif I. Ramadan[3]*

[1]Genome Research Unit, Animal Health Research Institute, Dokki, Giza, [2]Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Benha University, [3]Department of Animal Wealth Development, Faculty of Veterinary Medicine, Benha University, Moshtohor, Toukh 13736, Qalyubia, Egypt

*Corresponding author, E-mail: sherif.ramadan@fvtm.bu.edu.eg

**Abstract:** The objectives of this study were, firstly, to conduct genetic characterization of Egyptian Arabian horses based on 14 *TKY* microsatellite markers, secondly, to investigate the powerfulness of these 14 *TKY* markers for parentage assignment of Arabian horses. A total of 101 horse samples including (Arabian = 71, Thoroughbred = 19 and Nooitgedacht = 11) were analysed by 14 *TKY* microsatellite markers. The PCR products were electrophoresed on Genetic analyzer 3500 with the aid of Liz standard. The basic measures of the allele's size and genetic diversity were computed using bioinformatics software. The polymorphism of the *TKY* markers across the Arabian population showed moderate values for genetic diversity parameters; number of allele ($N_A$) =8.143, effective number of allele ($N_e$) = 3.694, observed heterozygosity ($H_O$) = 0.599, expected heterozygosity ($H_E$) = 0.691, polymorphic Information Content (PIC) = 0.636 and Inbreeding coefficient ($F_{IS}$) = 0.128. The combined probability of exclusion (CPE) value of the 14 *TKY* microsatellite loci of our Arabian horses was 0.9999. The results from current study confirm the applicability and efficiency of *TKY* microsatellite panel for evaluating the genetic diversity and parentage assignment of Egyptian Arabian horses.

**Key words:** Arabian horses; genetic diversity; microsatellite; TKY markers

## Introduction

Arabian horse is commonly believed to be one of the oldest and the most influential breed in the world. It is the most valuable and expensive breed as it combines the beauty of the body, fitness, agility, and intelligence as well (1). It is believed to be an immediate progenitor of many horse breeds including the Thoroughbred and Nooitgedacht (2-4). Nooitgedacht is a South African horse breed developed in 1951 as an attempt to save the endangered Basuto Pony (2). During the early colonial era in Southern Africa, the first imported horses were Arabs, Thoroughbreds, Javan, Persian and Spanish. These breeds had been mixed to form what is called the Cape horse and Basuto pony which later improved to become Nooitgedacht breed. Additionally, the Thoroughbred stallions were introduced to South Africa in the 1900's for the improvement of Basuto pony (2). Thoroughbred is one of the most valuable breed of horse in the world and is used mainly for racing, but is also bred for other riding disciplines (5). Thoroughbred was developed in United Kingdom and the pedigrees of all modern Thoroughbred individuals can be traced to three stallions namely; Darley Arabian, Godolphin Arabian and Byerley Turk which were originally imported into England during $17^{th}$ and $18^{th}$ centuries. Thoroughbreds are commonly used for

Received: 19 January 2020
Accepted for publication: 10 December 2020

crossbreding purposes to create new breeds or to improve existing ones, and have been influential in the creation of various warmblood breeds (6).

Egypt, although not an area of origin, it is considered a focal point for breeding Arabian horses for the past 200 years (7) . El-Zahraa Stud is the biggest Egyptian governmental farm having about 500 horses and is considered the main supplier of Arabian horses for other private farms all over the Egyptian country. Maintaining higher genetic diversity within horses is needed for long-term genetic improvement and to prevent the low performance (8-10) and expression of deleterious recessive genes due to inbreeding effect (11,12). Authorities for Arabian horses registry have adopted parentage testing programs for breed registration, studbook creation, and to maintain the purity of Arabians (7). Moreover, parentage test is important for global certification of our Arabian horses and to avoid breeding violations. In addition, keeping parentage records helps in prevention of animal adulteration during exportation and importation as well as detection of animal thefts. Microsatellites are considered the best markers for evaluation of genetic diversity and parentage testing in different animal species including horses (13,14). Genetic diversity and parentage studies of the Arabian horses reared in Egypt based on International Society of Animal Genetics (ISAG) microsatellite markers are scanty (15,14). Other microsatellite markers called *TKY* panel was isolated and give sufficient and reliable information for paternity testing and genetic diversity in Thoroughbred horses, which have much less heterozygosity than non-Thoroughbred horses (16). Moreover, *TKY* microsatellites showed multiple alleles as well as high heterozygosity among Japanese (17,18) and Bhutan (19) horses. Although the use of 12 microsatellite markers of ISAG panel is useful for genetic diversity evaluation, some markers showed few alleles, low heterozygosity and a lower value for probability of exclusion (20,16,21,13). So that the objectives of our study were, firstly, to conduct genetic characterization of Egyptian Arabian horses, secondly, to investigate the powerfulness of these 14 *TKY* markers for parentage assignment of Egyptian Arabian horses. We believe that this is the first report about the genetic diversity of Egyptian Arabian horse using TKY microsatellite panel.

## Materials and methods

### *Sample collection*

A total of 101 horse samples representing three different populations were examined, including 71 Arabian horse samples collected from El-Zahraa stud, Egyptian Agricultural Organization (EAO), Cairo, Egypt. In addition to 30 Equine DNA samples were brought for the ISAG Horse Comparison Test (HCT) 2017 and 2019 including 19 samples of Thoroughbred and 11 samples of Nooitgedacht breeds. The source of ISAG samples 2017 and 2019 are South Africa and Germany respectively. These thirty samples were used as a reference samples to compare and standardize allele sizes of *TKY* Panel and to represent a group of non-Arabian breeds.

### *DNA Extraction and Microsatellite Analysis*

Total DNA from the hair follicles of the Arabian horses were extracted using EZ-10 Spin Genomic DNA Minipreps purification kit following the manufacturer's protocol. A total of 14 TKY microsatellite markers (*TKY287, TKY294, TKY 297, TKY301, TKY312, TKY321, TKY325, TKY333, TKY 337, TKY341, TKY343, TKY344, TKY374 and TKY394*) specific to equine were used in this study (16). *TKY287, TKY321, TKY343*, and *TKY344* markers were labeled with FAM dye while *TKY297, TKY301, TKY312, TKY337, and TKY374* markers were labeled with HEX dye. Moreover, *TKY325, TKY333, TKY341, and TKY394* markers were labeled with NED dye and finally, *TKY394* marker was labeled with PET dye. These four dyes allow the amplification of the 14 markers in one multiplex PCR reaction without any overlapping between the markers. *TKY* Panel was adjusted by using known reference samples brought for the ISAG Horse Comparison Test 2017 and 2019. Alleles were designated with alphabetical symbols from the above allele sizes. A middle-sized allele was assigned as M. The 14 *TKY* microsatellites were amplified in one multiplex reaction using Amplitaq Gold DNA Polymerase with Gold Buffer and $Mgcl_2$ kit (Cat. No.: 4311816 – Applied Biosystems - USA). One multiplex PCR was performed in a total volume of 20 µl of the following mixture: 20–50 ng of equine genomic DNA, each forward/reverse primer at 0.3 µM,

200 µM of dNTPs, 2 µl of 10x reaction buffers; and 0.3 U of r*Taq* polymerase. PCR amplification entailed initial denaturation (95°C, 10 min), 30 cycles of 30 sec each at 95°C, 55°C and 60 sec at 72°C, and then 10 min at 72°C for final extension in a thermocycler (T100-BioRAD, UK). Fragment sizes of microsatellite alleles were determined using Genetic analyzer 3500 (Applied Biosystem-USA) with the aid of Liz standard (Cat. No.: 4322682 - Applied Biosystems - USA). The data obtained is further analyzed using Gene Mapper V 4.1 software (Applied Biosystem- USA).

### *Data analysis*

### *Marker polymorphisms and population diversity*

Number of alleles ($N_A$), effective number of alleles ($N_e$), private alleles, observed heterozygosity ($H_O$) and expected heterozygosity ($H_E$) were calculated by using GENALEX version 6 software (22). Polymorphic information content (*PIC*) was calculated by using CERVUS version 3 software (23). Hardy Weinberg equilibrium (*HWE*), fixation coefficient of an individual within a subpopulation ($F_{IS}$) were estimated by GENEPOP version 3.4 program (24). The power of exclusion (PE) and combined power of exclusion (CPE) of the 14 studied loci for Arabian population were calculated from allele frequencies using GENALEX version 6 software (22).

## Results

### *Marker polymorphisms and populations diversity*

The total numbers of alleles were 115, 88 and 78 for Arabian, Nooitgedacht and Thoroughbred, respectively. The comparison and standardization for alleles size and frequency of the 14 TKY loci in the Arabian and the two reference horse populations (Nooitgedacht and Thoroughbred) was shown in the supplementary figure 1. The polymorphism of 14 *TKY* markers across the Arabian population showed moderate values for genetic diversity parameters ($N_A$ =8.143, $N_e$ = 3.694, $H_O$, = 0.599, $H_E$ = 0.691 and *PIC* = 0.636). Arabian population showed high and positive value of $F_{IS}$ (0.129). Locus *TKY394* showed higher values for the most of diversity parameters ($N_A$ = 11, *Ne* = 7.638, $H_O$, = 0.676, $H_E$ = 0.875 and *PIC* = 0.855). The highest number of private alleles was recorded for *TKY344* locus, while *TKY301* and *TKY374* loci recorded non-private alleles. Locus *TKY337* showed the lowest values for all diversity indices ($N_A$ = 5, $N_e$ = 1.861, $H_O$, = 0.310, $H_E$ = 0.466 and PIC = 0.421). Moreover, it significantly deviated from *HWE* and recorded the highest $F_{IS}$ value (0.330). All loci except *TKY297* and *TKY325* showed a significant deviation from *HWE* (Table 1). The frequency of the five alleles of locus *TKY337* in the Arabian and in the two reference populations (Nooitgedacht and Thoroughbred) was shown in Figure 1. Allele 177 bp recorded very high frequency

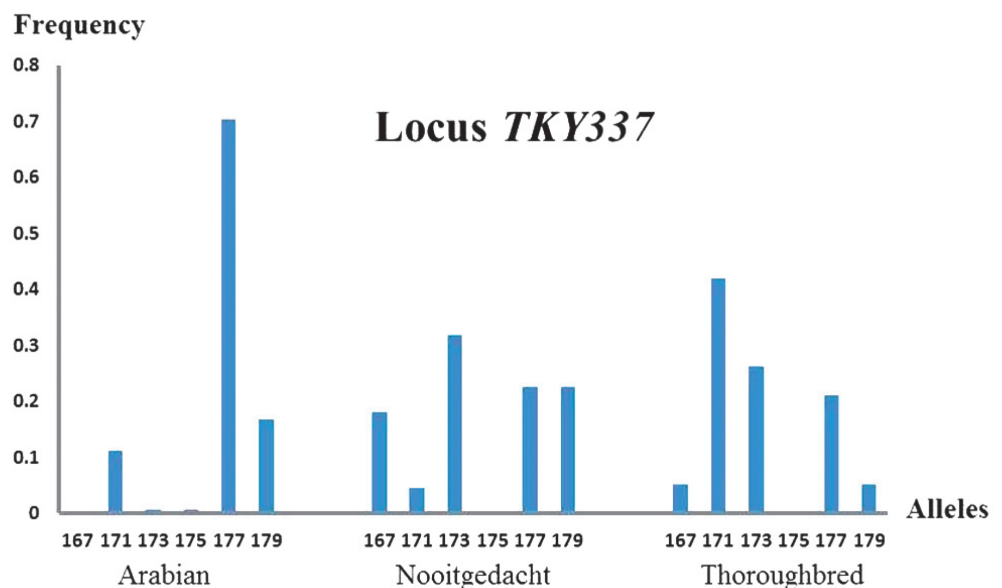



**Figure 1:** Allele's frequency of locus *TKY337* in the Arabian and the two reference horse populations (Nooitgedacht and Thoroughbred)

**Table 1:** Number of alleles (*Na*) effective number of alleles (*Ne*), Observed heterozygosity ($H_O$), expected heterozygosity ($H_E$), polymorphic information content (*PIC*), $F_{IS}$, and Hardy Weinberg equilibrium (*HWE*) across the Arabian population

| Locus | $N_A$ | Ne | Private alleles | $H_O$ | $H_E$ | PIC | PE | $F_{IS}$ | HWE |
|---|---|---|---|---|---|---|---|---|---|
| ***TKY287*** | 9 | 3.548 | 2 | 0.563 | 0.723 | 0.689 | 0.720 | 0.215 | *** |
| ***TKY294*** | 7 | 2.531 | 3 | 0.592 | 0.609 | 0.527 | 0.487 | 0.022 | *** |
| ***TKY297*** | 7 | 2.515 | 1 | 0.549 | 0.607 | 0.563 | 0.565 | 0.088 | ns |
| ***TKY301*** | 6 | 2.093 | 0 | 0.408 | 0.526 | 0.466 | 0.439 | 0.218 | *** |
| ***TKY312*** | 6 | 2.891 | 1 | 0.648 | 0.659 | 0.604 | 0.588 | 0.010 | *** |
| ***TKY321*** | 8 | 2.342 | 1 | 0.577 | 0.577 | 0.546 | 0.573 | -0.008 | *** |
| ***TKY325*** | 8 | 4.583 | 1 | 0.634 | 0.787 | 0.748 | 0.758 | 0.189 | ns |
| ***TKY333*** | 9 | 3.902 | 3 | 0.718 | 0.749 | 0.705 | 0.711 | 0.034 | *** |
| ***TKY337*** | 5 | 1.861 | 1 | 0.310 | 0.466 | 0.421 | 0.400 | 0.330 | *** |
| ***TKY341*** | 8 | 3.858 | 3 | 0.676 | 0.746 | 0.696 | 0.685 | 0.087 | *** |
| ***TKY343*** | 12 | 5.934 | 5 | 0.718 | 0.837 | 0.814 | 0.857 | 0.136 | ** |
| ***TKY344*** | 11 | 4.292 | 7 | 0.634 | 0.772 | 0.578 | 0.772 | 0.174 | *** |
| ***TKY374*** | 8 | 3.779 | 0 | 0.676 | 0.741 | 0.685 | 0.673 | 0.081 | *** |
| ***TKY394*** | 11 | 7.638 | 3 | 0.676 | 0.875 | 0.855 | 0.895 | 0.222 | *** |
| Mean | 8.143 | 3.694 | 2.214 | 0.599 | 0.691 | 0.636 | CPE >0.9999 | 0.128 | |
| ±SE | ±0.553 | ±0.425 | ±0.521 | ±0.031 | ±0.032 | ±0.31 | 0.006 | ±0.027 | |

ns = not significant, * = $P$<0.05, * * = $P$<0.01, *** = $P$<0.00

(0.704) in Arabian comparing to Nooitgedacht (0.227) and Thoroughbred (0.211) populations. The parentage assignment of the Arabian population using a combination of the 14 loci was very successful indicated by their high power of exclusion. The combined probability of exclusion (CPE) was 0.9999 in case of excluding putative parent pair. The highest PE value was reported for *TKY394* while the lowest for *TKY337* loci.

## Discussion

### *Marker polymorphisms and populations diversity*

This study presents the first description of genetic characterization of Egyptian Arabian horses based upon the *TKY* microsatellites panel. The number of alleles ($N_A$) and the frequency distribution of these alleles ($N_e$) in any population could tell us how informative a locus is. The number of alleles ($N_A$) of our Arabian population was higher than that of Thoroughbred breed of Tozaki study (16) for the most of studied *TKY* markers except for *TKY301* and *TKY325* loci (16). The means for $H_O$ (0.599) and $H_E$ (0.691) of our Arabian horses were higher than those of Kobayashi study for Misaki Japanese horses ($H_O$ = 0.509 and $H_E$ = 0.497) based on a combination of 32 microsatellites from ISAG and TKY panels (18). In contrast, the $H_O$ and $H_E$ of our Arabians were lower than both of Thoroughbred breed ($H_O$ = 0.731 and $H_E$ = 0.747) based on 15 TKY markers (16) and traditional horse breeds of Bhutan ($H_O$ = 0.790 and $H_E$ = 0.780) based on a combination of 29 microsatellite markers from ISAG and TKY panels (19).

The high mean of $N_A$ and lower mean of $N_e$ and the positive mean for $F_{IS}$ in addition to 12 out of 14 loci showed significant deviation from *HWE* might indicate that there was non-random mating among our Arabian horses in El-Zahraa stud and a selection program favoring some morphological characters might be practiced on this population. *TKY337* locus recorded the lowest values for all diversity indices and significantly deviated from *HWE* and showed the highest and positive value for $F_{IS}$ (0.330) in our Arabian horses. Moreover, only one allele (*177* bp) out of the five reported alleles of the *TKY337* locus recorded a very high frequency as shown in Figure 1. This might be attributed to that allele (177 bp) might be under some morphological or beauty related traits of

selective interest in our Arabian population in El-Zahraa stud. In respect to population genetic diversity, the Arabian population showed high inbreeding level indicated by the higher and positive value of $F_{IS}$ (0.129).

The CPE value of the 14 TKY microsatellite loci of our Arabian horses was 0.9999 which is higher than the required value by the International Stud Book Committee (0.9995), So the 14 TKY microsatellites are suitable for evaluation of genetic diversity not only for Thoroughbred populations (16), but also for our Egyptian Arabian horses. In this study, seven microsatellite loci (*TKY287, TKY312, TKY325, TKY333, TKY341, TKY343, TKY374* and *TKY394*) had high PIC values (>0.600). High level of CPE (>0.9999) can be achieved using only six of the 14 loci (*TKY287, TKY325, TKY333, TKY343, TKY344* and *TKY394*) as shown in (Figure 2), which makes these six markers highly valuable for parentage testing in Arabian horses. Ten microsatellite loci at least was suggested to be used for achieving maximum exclusion in horses (25), but our results showed that fewer loci can achieve relatively high power of exclusion, similar result was recorded by Sereno et al. (26). Two markers, *TKY301* and *TKY337* were found to have a PIC value lower than 0.500 for the studied Arabian population. As these two markers are considered less informative, they can easily be excluded from parentage testing for the Arabian horses with no significant loss of exclusion power.

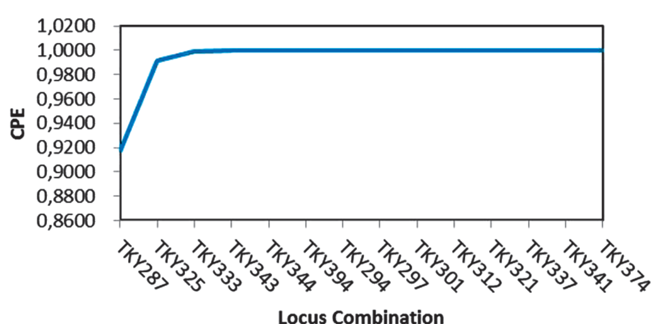



**Figure 2:** Combined probability of exclusion (CPE) as a function of the number of 14 TKY microsatellite loci for the Arabian horse population

## Conclusion

The 14 *TKY* markers provide a powerful and efficient tool for genetic diversity and parentage studies in the Arabian horses of El-Zahraa stud. Locus *TKY337* should be interpreted with caution and should be analyzed in further studies in different Arabian horse populations to test if it is linked to any morphological traits or not. Finally, the high $F_{IS}$ (0.129) of Arabian horses in El-Zahraa stud should be corrected by modifying mating system through avoiding excessive use of certain sires. The data presented in our study will provide the horse breeders with an effective tool for confirming parentage and lineages. Genetic diversity analysis will also generate valuable data necessary for the conservation of this valuable horse breed.

## Acknowledgment

The current work was conducted according to the protocols that were approved by the Committee of Animal Care and Welfare, Benha University, Egypt with an approval number: BUFVTM 03-09-2019.

RM-E, ME-A, EA-E and HA-A planned and designed the study. MA-S collected the hair samples and extracted the DNA. MA-S, HA-A and NM-H performed the microsatellite genotyping. SI-R, HA-A and MA-S performed the analysis and interpretation of the data. SI-R and MA-S drafted the article and revised it critically for important intellectual content. All authors read and approved the manuscript final version to be published. The authors declared that they have no conflict interests.

We thank the Egyptian Agriculture Organization for providing Arabian horse samples of El-Zahraa stud. This work was financially supported by the Animal Health Research Institute (AHRI) of Agricultural Research Centre, Dokki, Giza, Egypt.

## References

1. Khanshour A, Conant E, Juras R, Cothran EG. Microsatellite analysis of genetic diversity and population structure of Arabian horse populations. J Hered 2013; 104: 386–98.

2. Cothran E, Van Dyk E. Genetic analeysis of three South African horse breeds. J S Afr Vet Assoc 1998; 69: 120–5.

3. Bower MA, McGivney BA, Campana MG, et al. The genetic origin and history of speed in the Thoroughbred racehorse. Nat Commun 2012; 3: art. 643. doi: 10.1038/ncomms1644

4. Wallner B, Palmieri N, Vogl C, et al. Y chromo-

some uncovers the recent oriental origin of modern stallions. Curr Biol 2017; 27: 2029–35.

5. Bower MA, McGivney BA, Campana MG, et al. The genetic origin and history of speed in the Thoroughbred racehorse. Nat Commun 2012; 3: 1–8.

6. Kay J, Vamplew W. Encyclopedia of British horse racing. Routledge. 2012.

7. Hudson W. Whole-loop mitochondrial DNA D-loop sequence variability in Egyptian Arabian equine matrilines. PloS One 2017; 12: e0184309. doi: 10.1371/journal.pone.0184309

8. Vicente A, Carolino N, Gama L. Genetic diversity in the Lusitano horse breed assessed by pedigree analysis. Livest Sci 2012; 148: 16–25.

9. Curik I, Ferenčaković M, Sölkner J. Genomic dissection of inbreeding depression: a gate to new opportunities. Rev Bras Zootec 2017; 46: 773–82.

10. Todd ET, Ho SY, Thomson PC, Ang RA, Velie BD, Hamilton NA. Founder-specific inbreeding depression affects racing performance in Thoroughbred horses. Sci Rep 2018; 8: art. 6167. doi: 10.1038/s41598-018-24663-x

11. Tarr CJ, Thompson PN, Guthrie AJ, Harper CK. The carrier prevalence of severe combined immunodeficiency, lavender foal syndrome and cerebellar abiotrophy in Arabian horses in South Africa. Equine Vet J 2014; 46: 512–4.

12. Ela NAA, Khalid A, Ahmed HA, Brooks SA. Molecular detection of severe combined immunodeficiency disorder in Arabian horses in Egypt. J Equine Vet Sci 2018; 68: 55–8.

13. Khanshour AM, Conant EK, Juras R, Cothran EG. Microsatellite analysis for parentage testing of the Arabian horse breed from Syria. Turk J Vet Anim Sci 2013; 37: 9–14.

14. Sargious MA, Bakry H, El-Shawarby R, Ahmed HA. Parentage testing of Arabian horse in Egypt using microsatellite DNA typing. Benha Vet Med J 2014; 1: 100–8.

15. Mahrous KF, Hassanane M, Mordy MA, Shafey HI, Hassan N. Genetic variations in horse using microsatellite markers. J Genet Eng Biotechnol 2011; 9: 103–9.

16. Tozaki T, Kakoi H, Mashima S, et al. Population study and validation of paternity testing for Thoroughbred horses by 15 microsatellite loci. J Vet Med Sci 2001; 63: 1191–7.

17. Tozaki T, Takezaki N, Hasegawa T, et al. Microsatellite variation in Japanese and Asian horses and their phylogenetic relationship using a European horse outgroup. J Hered 2003; 94: 374–80.

18. Kobayashi I, Akita M, Takasu M, et al. Genetic characteristics of feral Misaki horses based on polymorphisms of microsatellites and mitochondrial DNA. J Vet Med Sci 2019; 81: 707–11.

19. Dorji J, Tamang S, Tshewang T, Dorji T, Dorji TY. Genetic diversity and population structure of three traditional horse breeds of Bhutan based on 29 DNA microsatellite markers. PloS One 2018; 13: e0199376. doi: 10.1371/journal.pone.0199376

20. Achmann R, Huber T, Wallner B, Dovc P, Müller M, Brem G. Base substitutions in the sequences flanking microsatellite markers HMS3 and ASB2 interfere with parentage testing in the Lipizzan horse. Anim Genet 2001; 32: e52. doi: 10.1046/j.1365-2052.2001.0647k.x

21. Monies D, Abu Al Saud N, Sahar N, Meyer B. Population studies and parentage testing for Arabian horses using 15 microsatellite markers. Anim Genet 2011; 42: 225–6.

22. Peakall R, Smouse PE. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 2012; 28: 2537–9.

23. Huang K, Mi R, Dunn DW, Wang T, Li B. Performing parentage analysis in the presence of inbreeding and null alleles. Genetics 2018; 210: 1467–81.

24. Rousset F. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J Hered 1995; 86: 248–9.

25. Ellegren H, Johansson M, Sandberg K, Andersson L. Cloning of highly polymorphic microsatellites in the horse. Anim Genet 1992; 23: 133–42.

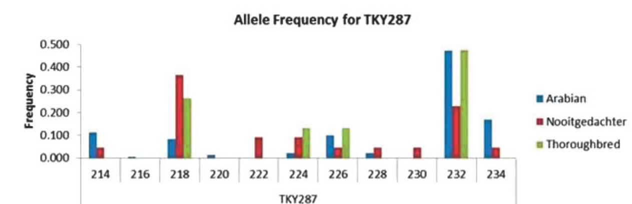
26. Sereno FTPdS, Sereno JRB, Vega-Pla JL, Delgado JV. DNA testing for parentage verification in a conservation nucleus of Pantaneiro horse. Genet Mol Biol 2008; 31: 64–7.

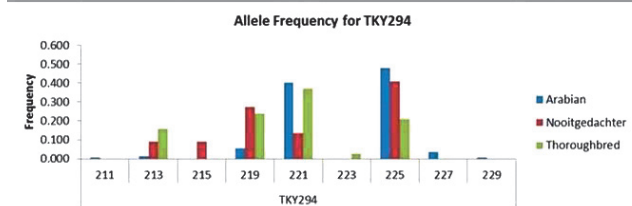
# GENSKA RAZNOVRSTNOST EGIPČANSKIH KONJ ARABSKE PASME IZ KOBILARNE EL-ZAHRAA NA PODLAGI 14 MIKROSATELITSKIH OZNAK *TKY*

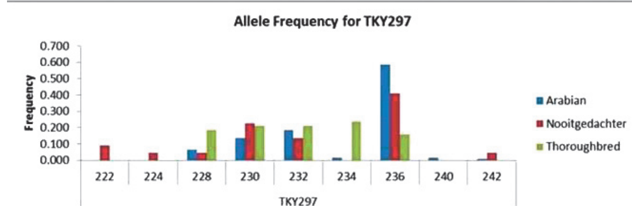
M. A. N. Sargious, Ragab M. El-Shawarby, Mohamed E. Abo-Salem, E. A. EL-Shewy, H. A. Ahmed, N. M. Hagag, S. I. Ramadan

**Izvleček:** Nameni raziskave so bili genetska karakterizacija egipčanskih konj arabske pasme na podlagi 14 mikrosatelitskih označevalecv *TKY* ter raziskava moči 14 označevalcev *TKY* za dodelitev staršev arabskih konj. S pomočjo 14 mikrosatelitskih označevalcev *TKY* je bilo analiziranih 101 vzorcev konj (arabski = 71, čistokrvni = 19 in konji Nooitgedacht = 11). Produkte PCR so analizirali s pomočjo elektroforeze na genskem analizatorju 3500 s pomočjo Liz standarda. Osnovne mere velikosti alela in genske raznovrstnosti so bile izračunane s pomočjo programske opreme za bioinformatiko. Polimorfizem označevalcev *TKY* v arabski populaciji je pokazal zmerne vrednosti za parametre genske raznolikosti; število alelov ($N_A$) = 8,143, efektivno število alelov ($N_e$) = 3,694, opazovana heterozigotnost ($H_O$) = 0,599, pričakovana heterozigotnost ($H_E$) = 0,691, polimorfna informacijska vsebina (*PIC*) = 0,636 in Inbriding koeficient ($F_{IS}$) = 0,128. Skupna vrednost verjetnosti izključitve (CPE) 14 mikrosatelitskih lokusov *TKY* njihovih arabskih konj je bila 0,9999. Rezultati te raziskave potrjujejo uporabnost in učinkovitost mikrosatelitske plošče *TKY* za oceno genetske raznovrstnosti in starševske pripadnosti egipčanskih arabskih konj.

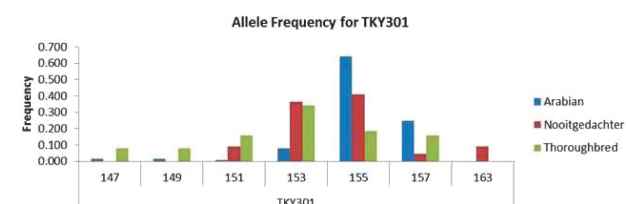
**Ključne besede:** arabski konji; genska raznolikost; mikrosatelit; markerji *TKY*

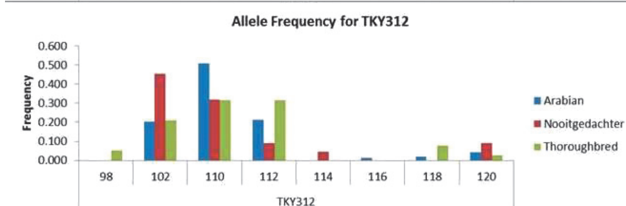


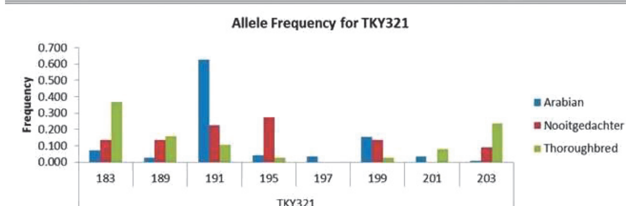



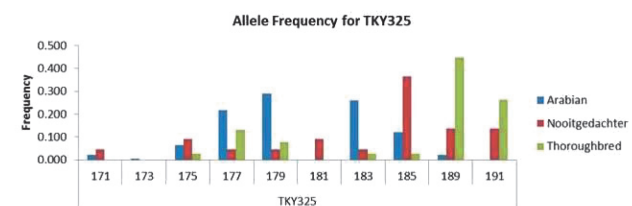

**Supplementary figure 1 (a)**

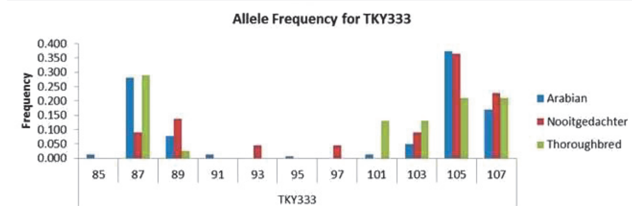


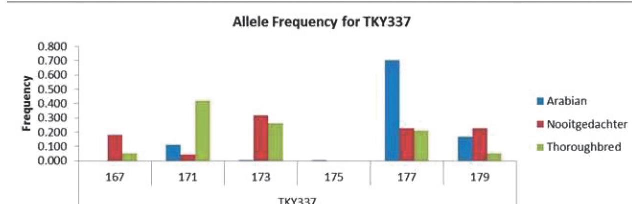



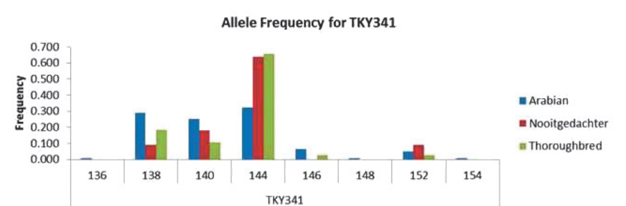

**Supplementary figure 1 (b)**

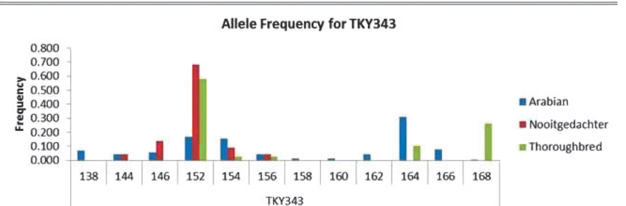


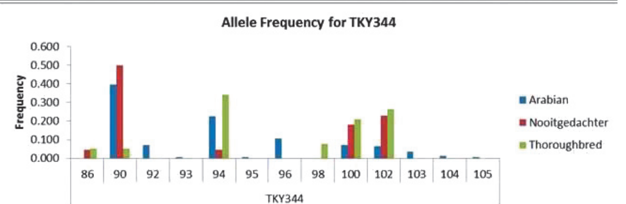



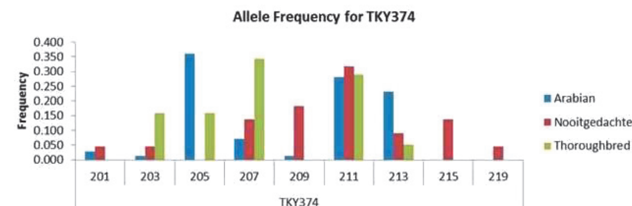

**Supplementary figure 1 (c)**

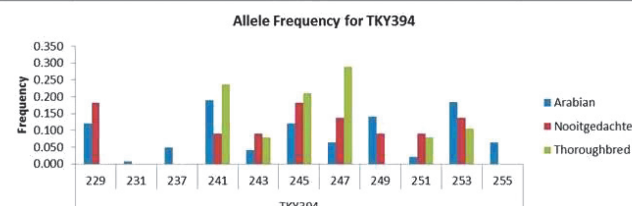



**Supplementary figure 1 (d)**




**Supplementary figure 1 (e)**

**Supplementary Figure 1** Allele's size and frequency of the 14 TKY loci in the Arabian and the two reference horse populations (Nooitgedacht and Thoroughbred)

a) Allele's size and frequency of *TKY287, TKY294* and *TKY297* loci
b) Allele's size and frequency of *TKY301, TKY312* and *TKY321* loci
c) Allele's size and frequency of *TKY325, TKY333* and *TKY 337* loci
d) Allele's size and frequency of *TKY341, TKY343* and *TKY344* loci
e) Allele's size and frequency of *TKY374* and *TKY394* loci

Slov Vet Res 2021; 58 (2): 63 – 75
DOI 10.26873/SVR-1072-2020
UDC 616.379-008.64:633.88: 599.323.45

Original Research Article

# ANTI-DIABETIC EFFECT OF ETHANOL EXTRACT OF *Copaifera salikounda* (HECKEL) AGAINST ALLOXAN-INDUCED DIABETES IN RATS

Chinyere Aloke[1]*, Emmanuel Sunday Igwe[1], Nwogo Ajuka Obasi[1], Pascal Anyaegbunam Amu[2], Egwu Chinedu Ogbonnia[1]

[1]Department of Medical Biochemistry, Alex Ekwueme Federal University Ndufu-Alike, Ebonyi State, [2]Department Science Laboratory Technology, Institute of Management and Technology, Enugu, Enugu State, Nigeria

*Corresponding author, E-mail: alokec2002@yahoo.com

**Abstract:** Accumulating evidences have reinforced the use of medicinal plants in the treatment of various ailments as a result of negative side effects associated with conventional drugs. Plant components such as phenols and flavonoids with antioxidant potential have confirmed protective roles against oxidative stress-induced degenerative diseases like diabetes mellitus (DM). The current study was carried out to investigate the effect of seed pod ethanol extract from *Copaifera salikounda* (SPEECS) in alloxan-induced diabetic rats. SPEECS was obtained by maceration of seed pod powder in absolute ethanol for 72 h, filtered, concentrated and dried *in-vacuo*. Gas chromatography-mass spectrometry (GC–MS) technique was used to quantitatively elucidate the chemical constituents of SPEECS. Twenty-four male albino rats were randomly allocated into four groups (n=6): normal control, DM control, DM + 200 mg/kg SPEECS and DM + 400 mg/kg SPEECS groups. DM was induced in the Wistar albino rats through intraperitoneal injection of 200 mg/kg body weight of alloxan. After 14 days of treatment, the body weight changes and the fasting blood glucose level were determined in the different groups. Also, serum biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin (ALB), total protein (TP), malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) were estimated. The GC-MS results confirm nine bioactive compounds with 9-octadecenoic acid (55.75%) being most abundant. SPEECS (200 and 400 mg/kg) administration significantly (*P < 0.05*) caused gain in weight, decreased fasting blood glucose and reversed the elevated liver function enzymes (ALT, AST, ALP) while total TP and ALB were markedly elevated relative to DM control group. Furthermore, SPEECS attenuated the activities of SOD and CAT while the level of MDA was significantly (*P < 0.05*) decreased in dose dependent manner in comparison to the DM control. This study indicated that SPEECS can alleviate hyperglyceamia of DM.

**Key words:** *Copaifera salikounda*; oxidative stress; medicinal plants; diabetes mellitus; phytochemicals; orthodox

## Introduction

Diabetes mellitus (DM) is an important world health challenge and is associated with elevated level of blood glucose. This condition is orchestrated by total or relative lack of insulin or inability of the cells to respond to insulin produced by the β−cells of the islets of Langerhans in the pancreas, manifesting as hyperglycemia and abnormalities in carbohydrate, protein and fat metabolism (1, 2). The use of animal models is one of the best methods for the elucidation of the disordered physiological processes of any ailment in order to draw a blueprint for the manufacture of drugs for its treatment (3). Alloxan has been widely acknowledged to selectively damage the pancreatic insulin-producing β-cells, thus employed for DM induction in experimental animals. It produces injurious effect on the β-cells of the pancreas by the oxidation of sulphydryl group (SH-group), production of free radicals; glucokinase enzyme inhibition and interference in the intracellular calcium balance (4). Thus, it induces oxidative

The mitochondria serve as the primary source of reactive oxygen species (ROS) in cells resulting from imperfectly coupled electron transport. It has been reported that oxidative stress contributes substantially in the development and continuation of DM and its complications, as a result of elevated formation of free radicals and

Received: 23 March 2020
Accepted for publication: 24 November 2020

compromised antioxidant defenses (6). Oxidative stress in diabetic subjects is mediated by different mechanisms particularly chronic exposure to elevated blood glucose. Previous studies reported that hyperglycemia can elicit the generation of ROS and reactive nitrogen species (RNS) by the mitochondrial respiratory system (7), autoxidation of glucose (8), polyol pathway activation (9), production of advanced glycation end products (AGEs) (10), compromised enzyme antioxidant system (11) and disproportionate glutathione reduction/oxidation status (12). Also, high blood glucose can promote an important oxidative disparity, favoring the production of free radicals and the reduction of antioxidant defenses. Increased concentrations of ROS/RNS can culminate in the impairment of major components of the cellular structure, nucleic acids, proteins, amino acids, and lipids (13). These oxidative alterations in the DM condition would impact several cell functions, metabolism, and gene expression, which will consequently bring about other disease conditions (14).

The use of folklore medicine in the management of DM is of immense importance due to their relative safety and low cost (2). Scientific exploration into the use of these herbal medicines demonstrated that they enhance secretion of insulin, facilitate the intake of glucose by adipose or muscle tissues and prevent the absorption of glucose from the intestine and its production by the hepatocyte (15). A nutritional diet rich in antioxidants is helpful to circumvent oxidative stress-induced diabetes mellitus (16). Some synthetic drugs with the potential to lower blood glucose such as metformin, rosiglitazone, sulphonylureas and thiazolidinediones are available for the management of DM (17). However, prolonged usage of these orthodox anti-diabetic agents poses potential negative side effects. The high cost and negative side effects associated with these drugs limit their usage in developing countries including Nigeria in the management of DM (18). Thus, identification of novel, potent and efficacious natural remedies for treatment of DM has drawn the attention of many researchers (19).

*Copaifera salikounda* (*C. salikounda*) (Heckel) is a deciduous medium-sized to large tall tree (up to 50 m tall) that belongs to the family *Caesalpiniaceae* (*Leguminosae - Fabaceae*). The plant has seed pod which contains only one seed. It is a timber tree of commercial importance which is limited because of its restricted distribution and scattered occurrence. It is distributed from Guinea Bissau eastward to Ghana. The wood is used in production of veneer and in furniture making. Aromatic resin obtained from the wood and bark is used locally in making a scented unguent for cosmetic use. The pounded bark is applied on the body as a perfume in Liberia. The pulped leaves of *C. salikounda* have been reported to be employed in treatment of sores or as hot poultice while the dried leaves and bark blended with baked and powdered clay are employed in ulcer management. Furthermore, an infusion of the fruit valve is employed as blood cleanser whereas a cold extract of the seed is used in vertigo treatment (20). The seed pod of *C. salikounda* is used as food ingredient and in treatment of rheumatoid arthritis (21). Aloke et al. (21) had reported the presence of the following phytochemicals in the seed pod of *C. salikounda* in order of their abundance as follows: total phenolics > alkaloids> terpenoids> carbohydrates> flavonoids> reducing sugar> tannins> glycosides> steroids. There is paucity of scientific information on the use of the seed pod ethanol extract from *Copaifera salikounda* (SPEECS) for the management of DM. The current study was done based on personal communications and interviews from different traditional medicine practitioners that affirm the ethno-medicinal use of SPEECS in treating hyperglycemia. This work therefore, is the first documented proof of the anti-diabetic potential of SPEECS.

This study investigated the ability of SPEECS produced in a process similar to the practice of folklore medicine practitioners, to alleviate hyperglycemia and oxidative stress in diabetic rats. The findings are anticipated to support the traditional usage and claim of efficacy and scientifically rationalize the traditional therapy in the management of diabetic conditions.

## Materials and methods

### *Materials*

Dried samples of seed pods of *C. salikounda* were collected in a farmland at Umuigboke Ugwulangwu in Ohaozara Local Government of Ebonyi State, Nigeria. The plant was identified and authenticated by a taxonomist, Mr. Alfred Ozioko at the International Centre for Ethnomedicine and Drug Development Nsukka, Enugu State with voucher specimen no:InterCEDD/16281.

All chemicals used were of analytical grade and were purchased from Sigma Aldrich, Saint Louis, U.S.A.

### *Seed pod extract preparation*

The seed pods of *C. salikounda* were further dried in the shade after washing with water. They were then ground using mortar and pestle. The ground seed pods obtained were passed through the sieve to obtain its powdered form. The ethanol extract was prepared by maceration of 103.3 g of seed pod powder in 500 ml of absolute ethanol for 72 hours. Then, the extract was filtered, concentrated, and dried *in-vacuo*. The percentage yield of the extract was 38.9%. See Figure 1 for the plate showing the seed pod *C. salikounda*.

**Figure 1:** Plate showing the seed pod of *C. salikounda* with some seeds

Seven weeks old male Wistar albino rats (110 – 150 g) obtained from Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Enugu State, Nigeria were used for this study. The rats were housed in the Animal House, Alex Ekwueme Federal University Ndufu-Alike, Ikwo, Ebonyi State, Nigeria. They were kept in well-ventilated cages at room temperature. The rats were fed a standard rat pellet (Vital Feed Nig., Ltd) and water. The rats were acclimatized for a period of seven days prior to the induction of experimental diabetes mellitus. The present study was performed according to international, national and institutional rules considering animal experiments, clinical studies and biodiversity rights. Approval for this study was given by the Academic Board of Department of Medical Biochemistry and Institutional Animal Ethical Committee of Alex Ekwueme Federal University Ndufu-Alike Ikwo, Nigeria with ethical code no: FBMS/EC/AE/2352 prior to the commencement of the experiments in line with the National Institute of Health's ethical guidelines for the care and usage of laboratory animals.

### *Gas Chromatography – Mass spectrometry (GC – MS) analysis*

Using gas chromatographic mass spectrometry, the phytochemical compounds of seed pod ethanol extract from *C. salikounda* were determined using GC-MSQP2010 Shimadzu system linked with a GC column (2010) coated with polymethyl silicon (0.25nm x 50m). Using programmed temperature from 80 to 200oC and held for 80oC for 1 min at rate of 5oC/min and 200oC for 20 mins, the detector and injector were set to a temperature of 300oC and 220oC respectively while the flow rate of the nitrogen carrier gas was 1 ml/min and the split ratio 1:75. Gas chromatography mass spectrum was performed using GC-MS QP 2010 in conjunction with Shimadzu Japan with the injector temperature of 220oC and pressure of the carrier gas at 116.9 kpa while the column length and diameter were 30m and 0.25mm respectively and the flow rate was 50ml/min. The elutes were accordingly run into a mass spectrometer with a dictator voltage set at 1.5kv and sampling rate of 0.2 sec. The mass spectrum was also furnished with a computer fed mass spectra data bank. Hermlez 233 M-Zcentrifuge (Germany) was used. Components identification was verified based on the relative retention time and mass spectra with computer Wiley Libraries and thus confirmed by mass spectra comparison of the peaks and those from literature (22).

### *Acute toxicity test*

The acute toxicity test was conducted to determine the median lethal dose ($LD_{50}$) of SPEECS and to choose the safe dose for the treatment. Acute toxicity study of SPEECS was carried out based on Lorke method with slight modifications (23). Thirty-six (36) rats were used for the acute toxicity test. Prior to the acute toxicity study, the rats were weighed and fasted overnight and were assigned into two experimental groups, A and B. Group A with four rats served as the normal control group and was administered normal saline. The Group B received SPEECS. Group B was further sub-divided into eight groups with

each group having four rats. The sub-groups were orally given SPEECS at 200, 400, 800, 1200, 1800, 2000, 3000 and 5000 mg/kg body weight respectively and the test animals were observed for 24 hours. Furthermore, the rats in the different groups were monitored for the first 2 hours for behavioral changes and morbidity and up to 72 hours for mortality.

*Experimental design*

*Induction of DM*

DM was induced in the Wistar albino rats after depriving them food overnight by intraperitoneal (IP) injection of 200 mg/kg body weight of freshly prepared alloxan dissolved in normal saline (24). Thereafter, the rats were fasted overnight after three days of injection of alloxan and their blood glucose determined using glucometer (Accu-Chek, Boerhinger Mannheim, Germany). Rats with fasting blood glucose ≥ 200 mg/dl on the third day after injection of alloxan were selected for the study.

*Experimental groups.*

A total of 24 male Wistar albino rats were used. The rats were randomly allocated into 4 groups of 6 rats each as follows:

– Normal Control = Normal control (received only normal saline 1.0mL/kg body weight).
– DM = Diabetic rats not treated, received only normal saline 1.0mL/kg body weight.
– DM + SPEECS (200 mg/kg) = Diabetic rats treated with 200mg/kg body weight SPEECS *per os* daily for 14 consecutive days
– DM + SPEECS (400 mg/kg) = Diabetic rats treated with 400mg/kg body weight SPEECS *per os* daily for 14 consecutive days

*Body weight determination*

The rats were weighed at three different times using a digital weighing balance. These measurements were done before induction of experimental DM, 72 hours after induction of DM and after the 14 days of SPEECS administration.

*Collection of blood sample*

Fourteen days after administration of SPEECS, the rats were fasted overnight and five rats from each group were humanely euthanized by cervical dislocation and their blood samples were collected through cardiac puncture into labeled plain sample tubes. The samples were centrifuged using a standard centrifuge at 4000 x g for 10 minutes and the sera were obtained for biochemical analysis.

*Determination of fasting blood glucose*

The fasting blood glucose levels of the rats were determined using “Accu-Chek Active Glucometer” and blood glucose test strips (Roche Diagnostics, Mannheim, Germany) before induction of experimental DM and 72 hours after induction of DM. The fasting blood glucose levels were also measured after the 14-day administration of SPEECS before the rats were sacrificed.

*Determination of biochemical parameters*

The serum activities of ALT and AST were determined spectrophotometrically (25) while ALP activity was determined using previously established method (26). The level of total protein was determined using colorimetric Biuret method (27) while serum albumin was determined using bromcresol green method (28).

*Assessment of lipid peroxidation and antioxidant status.*

Malondialdehyde (MDA) in serum was determined spectrophotometrically by measuring thiobarbituric acid reactive substance (TBARS) (29). Serum activities of superoxide dismutase (SOD) was assessed by the method outlined by Fridovich and Mc-Cord (30) and catalase (CAT) was determined by colorimetric method (31)

*Statistical analysis*

All the data were expressed as mean ± standard deviation. The means were compared using one-way analysis of variance (ANOVA) and significant variation were determined by Duncan multiple range test using SPSS software version 20. Value of $p < 0.05$ was considered to be statistically significant.

## Results

### *GC-MS analysis of SPEECS*

The results of GC-MS analysis of SPEECS gave nine bioactive constituents: n-hexadecanoic acid (8.97%), 7-octadecanoic acid methy ester (4.73 %), 9-octadecenoic acid (55.75 %), Octadecanoic acid (4.43 %), Phenol, 2-methyl (8.72 %), (E) 8- methyl-7-dodecen-1-ol acetate (3.62 %), Phthanic acid (1.81 %), Cyclopropaneundecanal, 2-Nonyl (4.82 %) and 1,4-benzenediol, 2-methyl (7.15 %) as shown in Table 1.

**Table 1:** Results from GC-MS analysis of SPEECS

| S. NO | Retention Time | Name of compound | Molecular formula | Molecular weight (g/mol) | Area % |
|---|---|---|---|---|---|
| 1 | 17.320 | n-hexadecanoic acid | $C_{16}H_{32}O_2$ | 257.422 | 8.97 |
| 2 | 38.290 | 7-octadecanoic acid methy ester | $C_{19}H_{36}O_2$ | 296.495 | 4.73 |
| 3 | 38.534 | 9-octadecenoic acid | $C_{18}H_{34}O_2$ | 282.468 | 55.75 |
| 4 | 38.659 | Octadecanoic acid | $CH_3(CH_2)_{16}COOH$ | 284.48 | 4.43 |
| 5 | 40.254 | Phenol, 2-methyl | C7H8O | 108.14 | 8.72 |
| 6 | 40.461 | (E) 8- methyl-7-dodecen-1-ol acetate | $C_{15}H_{28}O_2$ | 240.382 | 3.62 |
| 7 | 40.723 | Phthanic acid | $C_8H_6O_4$ | 166.14 | 1.81 |
| 8 | 41.812 | Cyclopropaneundecanal, 2-Nonyl | $C_{23}H_{44}O$ | 336.595 | 4.82 |
| 9 | 42.675 | 1,4-benzenediol, 2-methyl | $C_7H_8O_2$ | 124.13 | 7.15 |

### *Acute toxicity study*

In acute toxicity testing, no abnormal behavior and mortality were recorded. This indicated that the $LD_{50}$ was greater than 5000 mg/kg. This formed the basis for the selection of the doses of the extracts administered.

### *Effect of SPEECS on body weight of alloxan-induced diabetic rats*

The results of alteration in body weight of the experimental rat groups are presented in Table 2. The average weight of the normal control group was found to be relatively stable but that of DM control group showed significant ($p< 0.05$) reduction in body weight during the 14 days. The results showed that injection of alloxan caused sharp reduction in body weight of diabetic rats which was reversed by the administration of SPEECS at 200 and 400 mg/kg after 14 days of treatment. The weight gain in rats after 14 days treatment was higher in group fed with 400 mg/kg of SPEECS compared to those fed with 200 mg/kg of SPEECS as shown in Table 2.

### *Effect of SPEECS on liver function markers of alloxan-induced diabetic rats*

The serum concentrations of ALB and TP were low and the activities of ALT, AST and ALP were significantly higher in the DM rats group in comparison with the normal control group. TP level was significantly ($p< 0.05$) elevated in DM group treated with 200 mg/kg while there was non-signification elevation of ALB concentration when compared with DM control group. The serum activities of ALT, AST and ALP were significantly ($p< 0.05$) lower while the levels of ALB and TP were significantly ($p< 0.05$) higher in DM group treated with 400 mg/kg relative to DM model rats (Table 3). However, there was significant increase (p< 0.05) in the levels of ALB, TP and significant reduction in the actions of AST and ALP in DM + SPEECS (400 mg/kg) when compared with DM + SPEECS (200 mg/kg) while the activity of ALT reduced but not statistically significant (Table 3).

**Table 2:** Effect of SPEECS on body weight of alloxan-induced diabetic rats

| Group | Body weight (g) | | |
|---|---|---|---|
| | Day 1 | Day 7 | Day 14 |
| Control | 137.9 ± 1.87 | 147.7 ± 2.30 | 148.1 ± 2.42 |
| DM | 125.2 ± 3.22* | 115.4 ± 2.71* | 116.1 ± 2.69* |
| DM + SPEECS (200 mg/kg) | 125.7 ± 4.82# | 128.5 ± 2.78# | 128.5 ± 2.78# |
| DM + SPEECS (400 mg/kg) | 120.6 ± 2.10& | 133.9 ± 1.43# | 134.1 ± 1.45# |

Results are expressed as mean ± SEM (n=5). DM: Diabetes mellitus; P< 0.05 was considered significant. *p< 0.05: the difference is significant compared to control group in the same column; #p < 0.05: the difference is significant compared to DM group in the same column; &p< 0.05: the difference is significant compared to DM + SPEECS (200 mg/kg) group in the same column. DM + SPEECS (200 mg/kg): Diabetic rats treated with 200mg/kg b.w seed pod ethanol extract from *C. salikounda*; DM + SPEECS (400 mg/kg): Diabetic rats treated with 400mg/kg b.w seed pod ethanol extract from *C. salikounda*; SPEECS: Seed pod ethamol extract from *C. salikounda*

### *Effect of SPEECS on liver function markers of alloxan-induced diabetic rats*

The serum concentrations of ALB and TP were low and the activities of ALT, AST and ALP were significantly higher in the DM rats group in comparison with the normal control group. TP level was significantly ($p< 0.05$) elevated in DM group treated with 200 mg/kg while there was non-signification elevation of ALB concentration when compared with DM control group. The serum activities of ALT, AST and ALP were significantly ($p< 0.05$) lower while the levels of ALB and TP were significantly ($p< 0.05$) higher in DM group treated with 400 mg/kg relative to DM model rats (Table 3). However, there was significant increase ($p< 0.05$) in the levels of ALB, TP and significant reduction in the actions of AST and ALP in DM + SPEECS (400 mg/kg) when compared with DM + SPEECS (200 mg/kg) while the activity of ALT reduced but not statistically significant (Table 3).

**Table 3:** Effect of SPEECS on biochemical parameters of alloxan-induced diabetic rats

| Groups | ALT (U/L) | AST (U/L) | ALP (U/L) | ALB (g/L) | TP (g/L) |
|---|---|---|---|---|---|
| Normal Control | 37.5 ± 0.69 | 59.6 ± 0.86 | 31.5 ± 0.86 | 3.47 ± 0.17 | 5.3 ± 0.11 |
| DM | 42.8 ± 1.72* | 75.4 ± 0.77* | 57.7 ± 1.11* | 3.19 ± 0.21 | 5.1 ± 0.08 |
| DM + SPEECS (200 mg/kg) | 33.4 ± 1.22# | 61.6 ± 0.82# | 45.9 ± 0.60# | 3.22 ± 0.11 | 5.5 ± 0.09# |
| DM + SPEECS (400 mg/kg) | 30.5 ± 0.56# | 51.6 ± 1.11#& | 38.8 ± 0.84#& | 3.91 ± 0.10#& | 5.9 ± 0.06#& |

Results are expressed as mean ± SEM (n=5). DM: Diabetes mellitus; P< 0.05 was considered significant. *p< 0.05: the difference is significant compared to control group in the same column; #p < 0.05: the difference is significant compared to DM group in the same column; &p< 0.05: the difference is significant compared to DM + SPEECS (200 mg/kg) group in the same column. DM + SPEECS (200 mg/kg): Diabetic rats treated with 200mg/kg b.w seed pod ethanol extract from *C. salikounda*; DM + SPEECS (400 mg/kg): Diabetic rats treated with 400mg/kg b.w seed pod ethanol extract from *C. salikounda*; SPEECS: Seed pod ethamol extract from *C. salikounda*. ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; TP: total protein; ALB: albumin.

### *Effect of SPEECS on fasting blood glucose level of alloxan-induced diabetic rats*

The glucose lowering effect of the ethanol extract of seed pod *C. salikounda* is shown in Figure 2. The fasting blood glucose of alloxan-induced diabetic rats three days after injection of alloxan were significantly increased ($p< 0.05$) in comparison with the normal control rats. The blood glucose level of the groups treated with 200 and 400 mg/kg of the ethanol extracts seed pod of *C. salikounda* decreased significantly ($p< 0.05$) after 14 days of treatment in comparison to the DM control group. However, there was significant blood glucose level reduction in the group treated with 400 mg/kg of the extract in comparison to the group treated with 200 mg/kg. This showed that the effect of the extract was dose dependent.

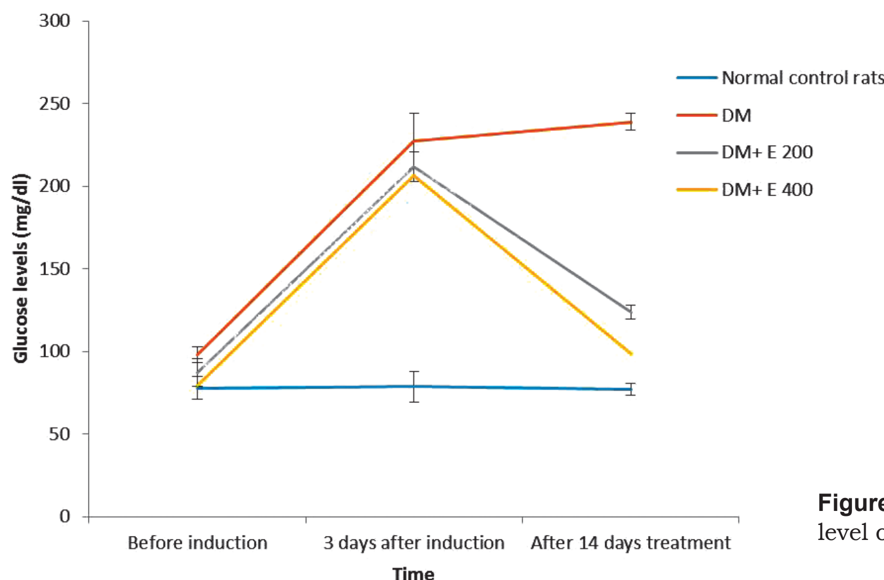



**Figure 2:** Effect of SPEECS on blood glucose level of alloxan-induced diabetic rats

SPEECS: seed pod ethanol extract from *C. salikounda*; DM: Diabetes mellitus group without any treatment. DM + E 200: Diabetic rats treated with 200 mg/kg b.w SPEECS; DM + E 400: Diabetic rats treated with 400mg/kg b.w SPEECS.

### *Effect of SPEECS on antioxidant status of alloxan-induced diabetic rats*

The serum activities of SOD and CAT were markedly ($p < 0.05$) higher in DM control group compared to the normal control group (Figure 3 A-B). The activities of SOD and CAT were significantly ($p < 0.05$) lower in diabetic groups treated with 200 and 400 mg/kg SPEECS. Besides, the activities of SOD and CAT were significantly lower in diabetic rats treated with 400 mg/kg when compared with those treated with 200 mg/kg. This is an indication that 400 mg/kg of the extract is more potent and efficacious (Figure 3 A-B)

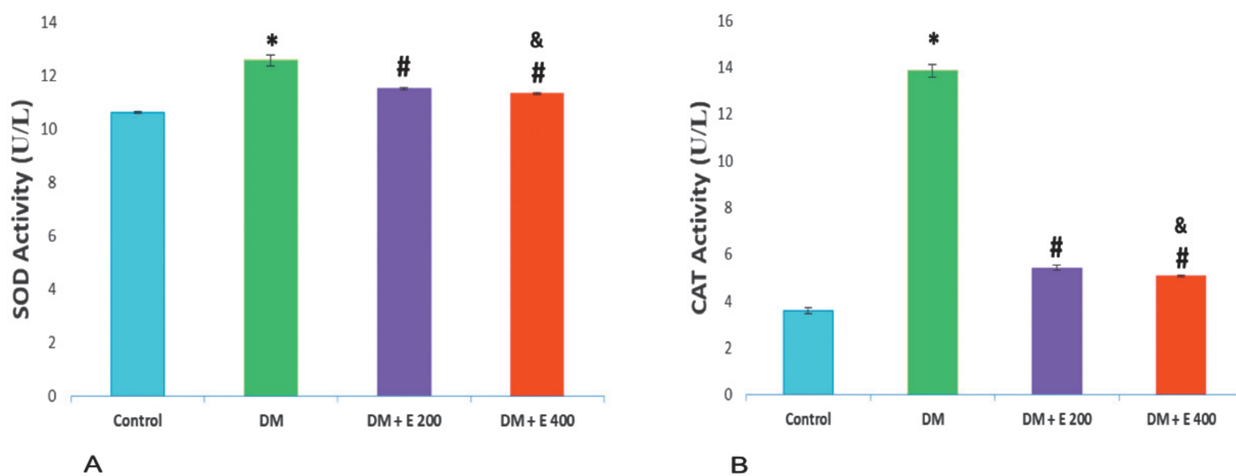



**Figure 3:** (A-B) Effect of SPEECS on antioxidant status in alloxan-induced diabetic rats (n=5)

SPEECS: seed pod ethanol extract from *Copaifera salikounda*; $p<0.05$ was considered significant. *$p<0.05$: the difference is significant compared to control group; #$p<0.05$: the difference is significant compared to DM group; &$p<0.05$: the difference is significant compared to DM + Extract (200 mg/kg) group; DM: Diabetes mellitus group without any treatment; DM + E 200: Diabetic rats treated with 200 mg/kg b.w SPEECS; DM + E 400: Diabetic rats treated with 400mg/kg b.w SPEECS; SOD: Superoxide dismutase; CAT: Catalase.

### *Effect of SPEECS on serum MDA level in alloxan-induced diabetic rats*

The serum concentration of MDA at the end of the experiment in the DM model rats was significantly (*p<0.05*) higher in comparison with the normal control group. However, this effect was reversed following 14 days treatment with 200 and 400 mg/kg of the extract in the treated groups. Additionally, the MDA level was significantly lower in the group treated with 400 mg/kg of the extract in comparison to diabetic rats that received 200 mg/kg of the extract (Figure 4) showing that the extract is more effective at 400 mg/kg dose.

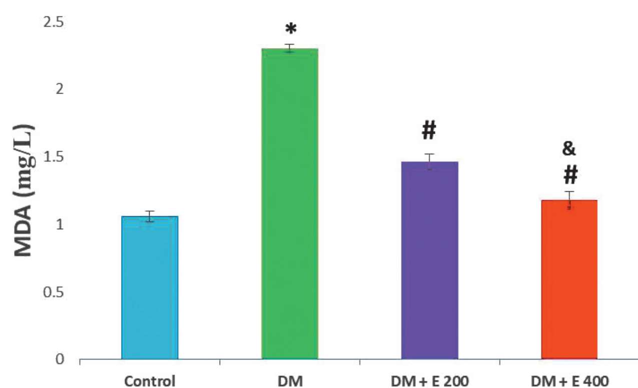



**Figure 4:** Effect of SPEECS on serum MDA level in alloxan-induced diabetic rats (n=5).

SPEECS: seed pod ethanol extract from *Copaifera salikounda*; DM: Diabetes mellitus; *p<0.05* was considered significant. **p<0.05*: the difference is significant compared to control group; #*p<0.05*: the difference is significant compared to DM group; &*p<0.05*: the difference is significant compared to DM + Extract (200 mg/kg) group; DM: Diabetes mellitus group without any treatment; DM + E 200: Diabetic rats treated with 200 mg/kg b.w SPEECS; DM + E 400: Diabetic rats treated with 400mg/kg b.w SPEECS; MDA: Malondialdehyde

## Discussion

Robust evidence has shown that plants are exemplary source of drugs. Currently, several available drugs have been produced from medicinal plants either directly or indirectly (32). Some studies have shown that a wide variety of plant extracts profoundly lowered blood glucose level in alloxan-induced diabetic animals (32, 33, 34, 35). The results of this study showed that SPEECS has antihyperglycemic effect on alloxan-induced DM in Wistar albino rats

Report on the quantification of the chemical components in the seed pod of *C. salikounda* indicated that it is predominantly rich in total phenolics, alkaloids, terpenoids, flavonoids, tannins, steroids, glycosides, reducing sugar and carbohydrates (21). In this study, the GC-MS analysis of SPEECS revealed the presence of known α-glucosidase inhibitors such as 9-octadecenoic acid and octadecanoic acid in the ethanol extracts. The inhibition of α-glucosidase has been reported to be a possible mechanism in treating DM by enzyme inhibition (36) and this affirms possible anti-hyperglycemic effects of SPEECS in this study. Other reports have shown the anti-diabetic property as well as release of insulin stimulation potential of hexadeconoic acid and octadecenoic (37, 38). Thus, the GC-MS identified compounds in this study: 9-octadecenoic acid, octadecanoic acid and hexadecanoic acid may be the potential anti-diabetic agents in the extract which may have exerted their effect via stimulation of insulin release and α-glucosidase inhibition. The findings in this study revealed that SPEECS contains bioactive compounds which mitigated hyperglyceamia and oxidative stress in alloxan-induced diabetic rats and restored the alteration in their body weights. The administration of alloxan produced a significant oxidative impact as evidenced by elevation of lipid peroxidation (MDA) and disturbances in antioxidant enzymes status.

The ability of alloxan to induce weight loss in untreated rats (DM control group) mimics what is commonly seen in clinical diabetes mellitus (39). This loss in weight could be as result of destruction of the pancreatic cells by alloxan leading to insulin deficiency which causes increased ketone bodies production. Consequently, elevated ketone bodies with increased lipolysis result in loss of body weight (40). The gain in body weight observed in diabetic rats treated with SPEECS could be attributed to better modulation of hyperglycemia in the diabetic rats and reduction in fasting blood glucose which could improve body weight in alloxan-induced diabetic rats (41). Additionally, the ability of SPEECS to improve body weight may be due to its ability to lower the elevated blood glucose via increased glucose metabolism, and this may be attributed to the protective effect of the extract in preventing muscle wasting (42).

The results of this study have shown that SPEECS at a dosage of 200 and 400 mg/kg body weight significantly (*P< 0.0001*) decreased the elevated blood glucose levels compared to normal rats. The glucose lowering effect of SPEECS (Figure 2) may be attributed to the enhanced secretion of insulin from the beta cells of pancreas or may be due to increased tissue uptake of glucose by enhancement

of insulin sensitivity (43). Additionally, other mechanisms that could have accounted for reduction in blood glucose level include: 1) the ability of the extract to function as an astringent enhancing the precipitation of intestinal mucus membrane protein and formation of a bilayer that shield the intestine and prevent glucose uptake, 2) the ability of the extract to speed up glucose liberation from circulation by promoting its filtration and renal excretion, and 3) promotion of glucose liberation via enhanced metabolism and inclusion into fats depots, an action requiring the pancreas to make insulin (44). Flavonoids have been reported as strong bioactive antioxidant and anti-diabetic agents that can regulate insulin secretion. Flavonoid hinders the action of hepatic glucose-6-phosphatase thereby preventing gluconeogenesis and breakdown of glycogen and consequently brings down elevated blood glucose (45). The presence of flavonoids and saponins in SPEECS (21) could have led to the revival of the non-functional beta cells by the flavonoid and prevention of the glucose transport by inhibiting intestinal sodium glucose co-transporter-1(S-GLUT-1) by the saponins (46, 47). Also, saponins have been shown to exhibit blood glucose lowering potential (48). The blood glucose lowering potential of SPEECS may be due to the synergy of its phytochemical constituents.

Additionally, hyperglycemia increases the generation of free radicals by glucose auto-oxidation and the increment of free radicals may lead to liver damage (49). The liver is necrotized in diabetic rats. This leads to increased activities of ALT, AST and ALP enzymes as they leak from the liver membrane into the blood stream and as such serves as indicators of the hepatotoxicity of alloxan (50). The significant ($P < 0.05$) elevation of serum enzymes such as ALT, AST and ALP observed in diabetic mellitus control rats as compared to normal control rats might be due to the leakage of these enzymes from the liver membrane into the blood stream (51). However, the oral administration of SPEECS significantly ($P < 0.0001$) decreased the elevated serum enzymes and implied heptato-protective effects (Table 3).

The functional capacity of the hepatocyte is measured by total protein content as the liver is provided with mechanism for the production of serum protein excluding γ-globulins. Hence, injury to the liver is marked by decrease in the level of protein in the blood and reduced Alb which can affect the body functions of animals (52). Disruption of protein synthesis is a consequence of decline in liver function (53). The observed decrease in Alb level in diabetes mellitus control group in this study could be a result of a decline in the number of cells responsible for Alb synthesis in the liver through necrosis by diabetogenic agent, alloxan. However, treatment with SPEECS at doses 200 and 400 mg/kg significantly ($P < 0.05$) increased serum TP and Alb in a dose dependent manner suggesting increased protein synthesis probably due to the regeneration of the damaged liver cells by the SPEECS.

Physiochemical processes in the body produce ROS which can participate in various reactions that when not controlled will lead to some clinical signs (54). Oxidative stress caused by excessive production of superoxide and compromised antioxidant enzymes has been associated with development of clinical conditions attributed to DM. DM is one of the disease conditions that is linked with immoderate generation of ROS like hydrogen peroxide ($H_2O_2$), hydroxyl radical (HO-) and superoxide anions ($O^{2-}$). Consequently, cells must be safeguarded from this oxidative damage by antioxidant enzymes (52). This study found a significantly elevated activity of SOD and CAT in diabetic rats when compared with the normal control. Excessive generation of ROS particularly in DM cannot be completely mopped up by antioxidant enzymes. Thus, when oxidative stress occurs as a result of disease condition, the body system enhances the induction or up regulation of these enzymes (55). The alteration in the activity of these enzymes might be due to altered metabolism occasioned by induction of DM with alloxan. However, treatment with SPEECS attenuated the activity of SOD and CAT at the tested concentrations.

MDA is a breakdown product of lipid peroxidation of polyunsaturated fatty acid in the membrane of cells. In this study, the presence of elevated level of MDA in diabetic rats when compared with control is suggestive of induced peroxidation of lipid and oxidative stress which has been revealed to be implicated in DM (56). Treatment with the SPEECS significantly reduced the MDA level relative to the DM control group (Figure 4). The total anti-diabetic effect of SPEECS may be due to the combined action of their phytcomponents irrespective of their amount. Consequently, the significant antidiabetic action of SPEECS in this

work may be ascribed to the presence of alkaloids, flavonoids, phenols, steroids or terpenoids and other constituents that could act independently or in synergy to scavenge free radicals, hence safeguarding the islet of Langerhans from injury and death.

This study, however, has limitations due to financial constraints as the experiment leading to the exact mechanism of the extract could not be done. We did not include a reference drug in the study to compare its effect on the diabetic rats with that of the extract. Besides, a longer period of three weeks would have been better to evaluate the efficacy of the extract.

In conclusion the results of this study indicated that SPEECS has anti-diabetic and anti-oxidative potentials. These findings corroborate its usage in the management of diabetes mellitus in traditional medicine and advocate for more research to harness fully the potentials of *C. salikounda* for treatment of DM.

## Acknowledgements

The authors declare there is no conflict of interest.

We declare that this work was fully funded by the authors and no grant was received by the authors.

The authors are grateful to Prof. Ejike C.E.C.C. for his invaluable comments and contributions to this work.

## References

1. Ionut V, Amorin RP. Epidemiology of diabetes mellitus: a current review. Rom J Diabetes Nutr Metab Dis 2012; 19(4): 433–40. doi: 10.2478/v10255-012-0050-0

2. Osadebe PO, Uzor PF, Omeje E O, Agbo MO, Obonga WO. Hypoglycemic activity of the extract and fractions of *Anthocleista vogelii* (Planch) stem bark. Trop J Pharm Res 2014; 13: 1437–43. doi: 10.4314/tjpr.v13i9.9

3. Chatzigeorgiou A, Halapas A, Kalafatakis K, Kamper E. The use of animal models in the study of diabetes mellitus. In Vivo 2009; 23: 245–58.

4. Dhanesha N, Joharapurkar A, Shah G, et al. Exendin-4 ameliorates diabetic symptoms through activation of glucokinase. J Diabetes 2012; 4(4): 369–77. doi: 10.1111/j.1753-0407.2012.00193.x

5. Bhat AH, Dar KB, Zargar MA, Masood A, Ganie SA. Modulation of oxidative stress and hyperglycemia by *Rheum spiciformis* in alloxan induced diabetic rats and characterization of isolated compound. Drug Res (Stuttg). 2018; 69(4): 218–26. doi: 10.1055/a-0665-4291

6. Ceriello A. New insights on oxidative stress and diabetic complications may lead to a “causal” antioxidant therapy. Diabetes Care 2003; 26(5): 1589–96. doi:10.2337/diacare.26.5.1589

7. Nishikawa T, Araki E. Impact of mitochondrial ROS production in the pathogenesis of diabetes mellitus and its complications. Antioxid Redox Sign 2007; 9(3): 343–53. doi:10.1089/ars.2007.9.ft-19

8. Yorek MA. The role of oxidative stress in diabetic vascular and neural disease. Free Radic Res 2003; 37(5): 471–80.

9. Cameron NE, Cotter MA, Hohman TC. Interactions between essential fatty acid, prostanoid, polyol pathway and nitric oxide mechanisms in the neurovascular deficit of diabetic rats. Diabetologia 1996; 39(2): 172–82. doi: 10.1007/BF00403960

10. Monnier VM. Intervention against the Maillard reaction in vivo. Arch Biochem Biophys 2003; 419(1): 1–15.

11. Maritim AC, Sanders RA, Watkins JB. Diabetes, oxidative stress, and antioxidants: a review. J Biochem Mol Toxicol 2003; 17(1): 24–38. doi:10.1002/jbt.10058

12. Kaneto H, Fujii J, Suzuki K, et el. DNA cleavage induced by glycation of Cu,Zn-superoxide dismutase. Biochem J 1994; 304(1): 219–25. doi: 10.1042/bj3040219

13. Valko M, Leibfritz D, Moncol J, et al. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007; 39(1): 44–84. doi:10.1016/j.biocel.2006.07.001

14. Young IS, Woodside JV. Antioxidants in health and disease. J Clin Pathol 2001; 54(3): 176–86. doi:10.1136/jcp.54.3.176

15. Hui H, Tang G, Go VLW. Hypoglycemic herbs and their action mechanisms. Chin Med 2009; 4: e11. doi: 10.1186/1749-8546-4-11

16. Berraaouan A, Abid S, Bnouham M, Berraaouan A. Antidiabetic oils. Curr Diabetes Rev 2013; 9: 499–505.

17. Nathan DM, Buse JB, Davidson MB, et al. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus

statement of the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care 2009; 32: 193–203. doi: 10.2337/dc08-9025.

18. Gupta RC, Chang D, Nammi S, Bensoussan A, Bilinski K, Roufogalis BD. Interactions between antidiabetic drugs and herbs: an overview of mechanisms of action and clinical implications. Diabetol Metab Syndr 2017; 9: e59. doi:10.1186/s13098-017-0254-9

19. Xu X, Shan B, Liao CH, Xie JH, Wen P W, Shi JY. Antidiabetic properties of *Momordica charantia* L. polysaccharide in alloxan-induced diabetic mice. Int J Biol Macromol 2015; 81: 538–543. doi: 10.1016/j.ijbiomac.2015.08.049

20. Oteng-Amoako AA, Obeng EA. *Copaifera salikounda* Heckel. Record from PROTA4U. In: Lemmens RHMJ, Louppe D, Oteng-Amoako AA, eds. PROTA (Plant Resources of Tropical Africa / Ressources végétales de l'Afriqutropicale), Wageningen, Netherlands: Wageningen University, 2012. https://prota4u.org/database/protav8.asp?g=pe&p=Copaifera+salikounda+Heckel (February, 2021)

21. Aloke C, Ibiam UA, Obasi NA, et al. Effect of ethanol and aqueous extracts of seed pod of *Copaifera salikounda* (Heckel) on complete Freund's adjuvant induced rheumatoid arthritis in rats. J Food Biochem 2019; 43(7): e12912. doi: 10.1111/jfbc.12912

22. Okwu ED, Ighodaro UB. GC-MS evaluation of bioactive compounds and antibacterial activity of the oil fraction from the leaves of *Alstonia boonei* De Wild. Pharma Chem 2010; 2(1): 261–2.

23. Lorke D. A new approach to practical acute toxicity tests. Arch Toxicol 1983; 54(4): 275–87.

24. Bell RH, Hye RJ. Animal models of diabetes mellitus: physiology and pathology. J Surg Res 1983; 35: 433–60.

25. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamate oxaloacetate and pyruvate transaminase. Am J Clin Pathol 1957; 28: 56–63. doi:10.1093/ajcp/28.1.56

26. Englehardt VA. Measurement of alkaline phosphatase. Aerztl Labour 1970; 16: 42–3.

27. Weichselbaum TE. An accurate and rapid method for the determination of protein in small amount of blood, serum and plasma. Am J Clin Pathol 1946; 10: 40–9.

28. Doumas BT, Watson WA, Biggs HG. Albumin standard and measurement of albumin with bromcresol green. Clin Chem Acta 1971; 31(1): 87–96.

29. Buege JA, Aust SD. Microsomal lipid peroxidation. In: Flesicher S, Packer L, eds. Methods in enzymology. Vol. 52. New York : Academic Press, 1978: 302–10.

30. Fridovich I, Mc-Cord JM. Superoxide dismutase: an enzymatic function for erythrocuperin. J Biol Chem 1969; 244(22): 6045–55.

31. Sinha AK. Colorimetric assay of catalase. Anal Biochem 1972; 47(2): 389–94.

32. Yakubu MT, Akanji MA, Nafiu MO. Antidiabetic activity of aqueous of *Cochlospermum planchonii* root in alloxan-induced diabetic. Cameroon J Exp Biol 2010; 6: 91–100.

33. Ananda PK, Kumarappan CT, Sunil C, Kalaichelvan VK. Effect of *Biophytum sensitivum* on streptozotocin and nicotinamideinduced diabetic rats. Asian Pac J Tropical Biomed 2012; 11: 31–5.

34. Alamgeer, Rashid M, Bashir S, et al. Comparative hypoglycemic activity of different extracts of *Teucrium stocksianum* in diabetic rabbits. Bangladesh J Pharmacol 2013; 8: 186–93.

35. Nagappa AN, Thakurdesai PA, Venkat RN, Singh J. Antidiabetic activity of *Terminalia catappa* Linn fruits. J Ethnopharmacol 2003; 88: 45–50.

36. Channabasava GM, Chandrappa CP, Sadananda TS. In Vitro antidiabetic activity of three fractions of methanol extracts of *Loranthus Micranthus*, identification of phytoconstituents by GC-MS and possible mechanism identified by GEMDOCK method. Asian J Biomed Pharm Sci 2014; 4 (34): 34–41.

37. Parker SM, Moore PC, Johnson LM, Poitout V. Palmitate potentiation of glucose-induced insulin release: a study using 2-bromopalmitate. Metabolism 2003; 52 (10): 1367–71.

38. Zuraini A, Zamhuri KF, Yaacob A, et al. In vitro anti-diabetic activities and chemical analysis of polypeptide-k and oil isolated from seeds of *Momordica charantia* (Bitter gourd). Molecules 2012; 17(8): 9631–40. doi:10.3390/molecules17089631

39. World Health Organisation. Diet, nutrition, and the prevention of chronic diseases. World Health OrganisationTechnical Report Series 2003; 916: 1-149.

40. Singh SK, Kesari AN, Gupta RK, Jaiswal D, Watal G. Assessment of antidiabetic potential of *Cynodon dactylon* extract in streptozotocin diabetic rats. J Ethnopharmacol 2007; 114: 174–9. doi:10.1016/j.jep.2007.07.039

41. Ebong PE, Atangwho IJ, Eyong EU, Egbung GE. The antidiabetic efficacy of combined extracts from two continental plants: *Azadirachta indica* (A.

Juss) (Neem) and *Vernonia amygdalina* Del. (African bitter leaf). Am J Biochem Biotechnol 2008; 4(3): 239–44. doi: 10.3844/ajbbsp.2008.239.244

42. Ezejiofor AN, Okorie A, Orisakwe OE. Hypoglycaemic and tissue-protective effects of the aqueous extract of *Persea americana* seeds on alloxan-induced albino rats. Malays J Med Sci 2013; 20(5): 31–9.

43. Maniyar Y, Bhixavatimath P. Antihyperglycemic and hypolipidemic activities of aqueous extract of *Carica papaya* Linn. leaves in alloxan-induced diabetic rats. J Ayurveda Integr Med 2012; 3(2): 70–4. doi: 10.4103/0975-9476.96519

44. Muhtadia M, Primariantia AU, Sujonoa TA. Antidiabetic activity of durian (*Durio zibethinus Murr.*) and rambutan (*Nephelium lappaceum* L.) fruit peels in alloxan diabetic rats. Procedia Food Sci 2015; 3: 255–61. doi: 10.1016/j.profoo.2015.01.028

45. Chen PY, Csutora P, Veyna-Burke NA, Marchase RB. Glucose-6 phos-phate and Ca2+sequestration are mutually enhanced in microsomes from liver, brain, and heart. Diabetes 1998; 4 (6): 874–81. doi:10.2337/diabetes.47.6.874

46. Hakkim FL, Girija S, Kumar RS, Jalaluddeen MD. Effect of aqueous and ethanol extracts of *Cassia auriculata* L. flowers on diabetes using alloxan – induced diabetic rats. Int J Diabetes Metab 2007; 15: 100–6.

47. Tiwari AK, Rao JM. Diabetes mellitus and multiple therapeutic approaches of phytochemicals: present status and future prospects. Curr Sci 2002; 83: 30–8.

48. Patel DK, Prasad SK, Kumar R, Hemalatha S. An overview on antidiabetic medicinal plants having insulin mimetic property. Asian Pac J Trop Biomed 2012; 2(4): 320–30. doi: 10.1016/S2221-1691(12)60032-X.

49. Singh R, Seherawat A, Sharma P. Hypoglycemic, antidiabetic and toxicological evaluation of *Momordica dioica* fruit extracts in alloxan-induced rats. J Pharmacol Toxicol 2011; 6(5): 454–67. doi: 10.3923/jpt.2011.454.467

50. Saeed MK, Deng Y, Dai R. Attenuation of biochemical parameters in streptozotocin-induced diabetic rats by oral administration of extracts and fractions of *Cephalotaxus sinensis*. J Clin Biochem Nutr 2008; 42: 21–8. doi: 10.3164/jcbn2008004

51. Mansour HA, Newairy AS, Yousef MI, Sheweita SA. Biochemical study on the effects of some Egyptian herbs in alloxan-induced diabetic rats. Toxicology 2002; 170: 221–8.

52. Kanchana N, Mohamed AS. Hepatoprotective effect of *Plumbago zeylanica* on paracetamol induced liver toxicity in rats. Int J Pharm Pharm Sci 2011; 3: 151–4.

53. Afaf A, Faras EI, Elsawaf AL. Hepatoprotective activity of quercetin against paracetamol-induced liver toxicity in rats. Tanta Med J 2017; 45: 92–8. doi: 10.4103/tmj.tmj_43_16

54. Kesavulu MM, Giri R, Kameswara-Rao B, Apparao C. Lipid peroxidation and antioxidant enzyme levels in type 2 diabetics with microvascular complications. Diabetes Metab 2000; 26: 387–92.

55. Qujeq D, Rezvani T. Catalase (antioxidant enzyme) activity in streptozotocin-induced diabetic rats. Int J Diabetes Metab 2007; 15: 22–4.

56. Ceriello A. Oxidative stress and glycaemic regulation. Metabolism 2000; 49(2 suppl 1): 27–9.

# ANTIDIABETIČNI UČINEK EKSTRAKTA ETANOLA *Copaifera salikounda* (HECKEL) NA SLADKORNO BOLEZEN, SPROŽENO Z ALLOXAN-om, PRI PODGANAH

C. Aloke, E. S. Igwe, N. A. Obasi, P. A. Amu, E. C. Ogbonnia

**Izvleček:** Obstaja vedno več dokazov, ki poudarjajo uporabnost zdravilnih rastlin pri zdravljenju različnih bolezni, tudi zaradi različnih negativnih stranskih učinkov, povezanih s konvencionalnimi zdravili. Rastlinske sestavine kot so fenoli in flavonoidi z antioksidativnim potencialom, imajo po nekaterih raziskavah zaščitno vlogo pred degenerativnimi boleznimi, ki jih povzroča oksidativni stres, kot je sladkorna bolezen *diabetes mellitus* (DM). Študija je bila izvedena z namenom raziskovanja učinka etanolnega semenskega ekstrakta iz rastline *Copaifera salikounda* (SPEECS) pri podganah s sladkorno boleznijo, ki jo je povzročil alloxan. SPEECS je bil pridobljen z maceracijo praška semen v prahu v absolutnem etanolu 72 ur ter nadaljnjo filtracijo, koncentracijo in sušenjem v vakuumu. Za kvantitativno ugotavljanje kemijskih sestavin SPEECS je bila uporabljena tehnika plinske kromatografije in masne spektrometrije (GC-MS). Štiriindvajset samcev podgan Wistar je bilo naključno razporejenih v štiri skupine (n=6): normalna kontrola, kontrola DM, DM + 200 mg/kg SPEECS in DM + 400 mg/kg SPEECS. DM je bil pri podganah sprožen z intraperitonealno injekcijo 200 mg/kg telesne mase alloxana. Po 14 dneh zdravljenja so bile pri različnih skupinah določene spremembe telesne teže in nivo glukoze v krvi (na tešče). Poleg tega so avtorji raziskave izmerili še nekatere serumske biokemične parametre kot so ravni alaninske aminotransferaze (ALT), aspartatne aminotransferaze (AST), alkalne fosfataze (ALP), albumina (ALB), skupnih proteinov (TP), malondialdehida (MDA), superoksiddismutaze (SOD) in katalaze (CAT). Rezultati GC-MS so v izvlečku SPEECS pokazali devet bioaktivnih spojin, v katerih je največ 9-oktadecenojske kisline (55,75%). SPEECS (200 in 400 mg/kg) je povzročil znatno ($P < 0{,}05$) povečanje telesne mase, znižanje glukoze v krvi na tešče in znižal raven encimov pokazateljev jetrne funkcije (ALT, AST, ALP), medtem ko je bila raven TP in ALB pri podganah, ki so prejemale SPEECS izrazito povišana v primerjavi z DM kontrolno skupino. Zdravljenje s SPEECS je tudi oslabilo aktivnosti SOD in CAT, medtem ko se je raven MDA znatno zmanjšala ($P < 0{,}05$) v primerjavi s kontrolno skupino DM. Ta študija je pokazala, da lahko SPEECS ublaži hiperglikemijo pri sladkorni bolezni pri podganah.

**Ključne besede:** *Copaifera salikounda*; oksidativni stres; zdravilne rastline; sladkorna bolezen; fitokemikalije; ortodoksni

Slov Vet Res 2021: 58 (2): 77 – 84
DOI 10.26873/SVR-1091-2020
UDC 636.1.022:677.3:675.046.1

**Original Research Article**

# CHARACTERISATION OF COAT COLOUR IN THE SLOVENIAN POSAVJE HORSE

Gertrud Grilz-Seger[1]*, Matjaž Mesarič[2]*, Gottfried Brem[1], Marko Cotman[3]

*the authors equally contributed to this work

[1]Institute of Animal Breeding and Genetics, University of Veterinary Sciences Vienna, Veterinärplatz 1, A-1210 Vienna, Austria, [2]Clinic for Reproduction and Large Animals, University of Ljubljana, Veterinary Faculty, Cesta v Mestni log 47, [3]Institute for Preclinical Sciences, University of Ljubljana, Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia

*Corresponding author, E-mail: gertrud.grilz@vetmeduni.ac.at

**Abstract:** Different approaches and classification systems have been established to describe equine coat colour, which varies between breeds and countries. In the present study, we first characterised the coat colour variability in the Slovenian Posavje Horse applying colour spectrophotometry following the CIE L*a*b system. As derived from the classification system of Sponenberg (light bay, bay, mahogany bay, brown and seal brown), the phenotype categories could be confirmed by spectrophotometric data. Furthermore, L*a*b values revealed comparable high phenotypic variability of bay coat colour in the Posavje breed, and the darker shades of bay coat colour were associated with the *ASIP* and *MC1R* genotype combination *A/a E/E*. CIE L*a*b colour spectrophotometry represents an effective tool to characterise and quantify coat colour in horses, especially in chestnut horses, for which the underlying genetic background of coat colour variation remains unknown.

**Key words:** Posavje Horse; MC1R; ASIP; coat colour; spectrophotometry; CIE L*a*b

## Introduction

Breeding for coat colour has been a part of horse breeding throughout history and represented a central objective since prehistoric times (1- 4). Due to the high economic, aesthetic, and health impacts, coat colour has been in the focus of genetic research in recent decades. The genetic background of the base coat colours bay, black and chestnut, predominantly occurring in Posavje Horses has been determined. Marklund et al. (5) identified a missense mutation in the gene melanocyte-stimulating hormone receptor (*MC1R*), responsible for chestnut coat colour. Rieder et al. (6) detected an 11-bp deletion in exon 2 of the ASIP (Agouti signalling protein) gene, resulting in black coat colour. Nevertheless, the background of the bright variety of shades within bay and chestnut is not fully resolved. In addition to studying environmental effects influencing colour shades (7, 8), researchers have investigated genotype interactions of *ASIP* and *MC1R* (6, 9, 10); in these publications, the genotype combination *A/a E/E* was associated with darker shades of bay.

In the studies of Rieder et al. (6) and Sakamoto et al. (10), the classification of the different coat colour shades was derived by visual inspection, whereas Druml et al. (9) used a colour-spectrophotometer to define exact coat colour groups.

Received: 23 April 2020
Accepted for publication: 23 December 2020

For the quantification of coat colour, the use of a spectrophotometer and the standardised L*a*b* colour system as defined by the Commission Internationale de l'Eclairage (CIE), has proven to be helpful in a series of studies. For example, this procedure was applied to investigate the greying process in Lipizzan (11) and Kladrub horses (7). It was further utilised for the estimation of additive genetic effects of coat colour in Noriker horses (12), Lipizzan, Nonius, Arabian Purebred, Shagya Arabian, Gidran (13), and Black Kladrub horses (7) and for quantifying genotype interactions in Noriker and Shagya Arabian horses (9).

This study aimed to characterise base coat colour variation in the Slovenian Posavje Horse with particular focus on bay coat colour, using spectrophotometric measurements. In the Posavje Horse, a small autochthonous draught horse breed, a wide spectrum of base colour shades exists. We quantified and validated the classification categories derived by visual inspection, and compared the measurements with those of Noriker horses and Shagya Arabians, two horse breeds for which darker phenotypes are preferred by the breeders. Furthermore, we investigated the association between genotype combinations of *ASIP* and *MC1R* and coat colour variation.

## Materials and methods

The Posavje Horses included in this study were selected to represent the whole spectrum of coat colour variability in this breed. We measured 70 adult Posavje Horses (<3 years of age) during the summer months of 2018 using a Chroma Meter CL-100 (Konica Minolta, Japan). From these 70 horses, 55 were bay, ten were chestnut, and five were black. All animals were phenotyped by visual inspection according to the system of Sponenberg (14).

Measurements were taken from five different body areas, including the neck, shoulder, axillary area, belly, and croup (Figure 1). Colour, as defined by the CIE L*a*b* colour space, consists of three axes defining variation from black to white (L*) at a scale from 0 to +100, blue to yellow (b*) at a scale from -100 to +150, and green to red (a*) at a scale from -170 to +100. Additionally, we used published data (3) from 24 Noriker (12 bay, 12 black) and 32 Shagya Arabians (30 bay and two black), which were measured using the same Chroma Meter CL-100 (Konica Minolta, Japan) and methodology previously described. In total, data of 126 horses were used for this study.

To determine the *ASIP* and *MC1R* genotypes, genomic DNA was isolated from 300 μl whole blood with a Wizard Genomic DNA Purification Kit (www.promega.com) according to manufacturer's instructions. The coat colour gene loci were genotyped using restriction fragment length polymorphism (RFLP) methods for *MC1R* (15) and polymerase chain reaction (PCR) techniques for *ASIP* (6).

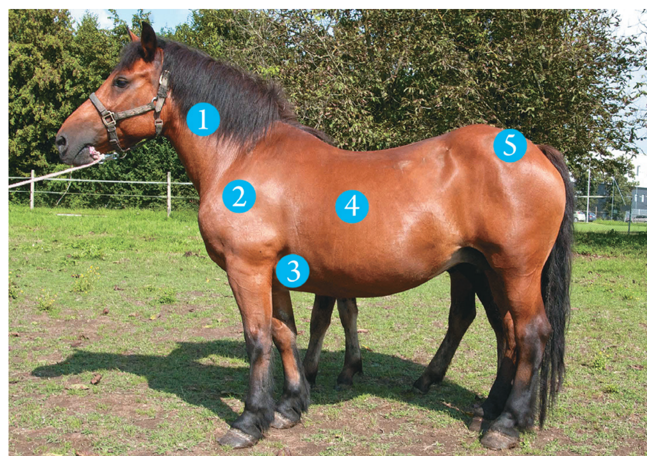



**Figure 1:** Coat colour was measured at five different body areas: 1=neck,2=shoulder, 3= axillary area, 4=belly, and 5=croup (Foto: Matjaž Mesarič)

We applied MANOVA and canonical discriminant analysis in order to analyse differences in colour measurements between a) classification categories of bay coat colour and b) genotype combinations of *ASIP* and *MC1R* comprising horses of all base colours. For the MANOVA, we applied two generalised linear models, which included a) coat colour classification categories as a fixed effect ($y_{ik}$ = classification$_i$ + $e_k$) and b) *ASIP/MC1R* genotype as a fixed effect ($y_{ik}$ = genotype$_i$ + $e_k$). In order to test the differences of L*a*b measurements between the four genotype combinations in bay horses, we performed a comparison of LSMeans correcting for multiple level using the Tukey-Kramer test.

Canonical discriminant analysis is a multivariate technique that can be used to determine relationships among categorical variables and groups of independent variables.

The canonical discriminant function searches for linear combinations of independent variables in a data set to achieve maximum separation of classes (populations), in this case, *ASIP/MC1R* genotypes, in a lower dimensional discriminant

space. A major task of this method is to test and visualise the discrimination power based upon the canonical variables. The resulting discrimination plots assist in analysing and discussing the underlying data and results derived from other statistical procedures. In this study, we performed a canonical discriminant analysis to determine the colour measurements of all Posavina horses (bay, black, chestnut) according to their *ASIP/MC1R* genotypes. All statistical analyses and graphical representations were performed using the SAS software package (16).

## Results

Phenotypic classification by visual inspection revealed a high variability in shades of bay and chestnut Posavje Horses. In chestnut horses, both classification categories, chestnut and sorrel (light mane and tail), were present, and phenotypic variability ranged from light sorrel to dark chestnut (Figure 2). Comparable variation was observed in bay horses, for which coat colour categories ranged from light bay up to seal brown. Most of the horses were classified bay (41.8%), followed by 23.7% mahogany bay. Dark shades were represented by 16.3% brown horses, of which 1.8% were seal brown. Light shades (light bay, red bay) were present in 16.4% of horses (Figure 3). Black horses were predominantly summer black.

According to the CIE L*a*b measurements, the mean L-values describing the brightness from white to black, ranged in black horses from 19.9 (belly) to 22.9 (axillary are). The mean a-values, defining the colour axis from green to red, were lowest in the neck area (a=1.7) and highest in the axillary area (a = 3.2). Finally, in black horses the mean b-values, characterising the blue to yellow axis, varied between 2.5 (belly) and 4.1 (axillary area) (Tab. 1).

The mean L-values in bay horses ranged from 26.3 (neck) to 37.0 (axillary area), mean a-values varied from 7.4 (neck) to 9.5 (belly) and mean b-values were in between 9.5 (neck) and 17.6 (axillary area) Table 1). Highest individual L-values were measured in the axillary area (L=52.9), and the darkest points were measured in the neck area (L=16.2). The darkest bay colour categories (seal brown, brown and dark mahogany bay) exhibited partially comparable measurements on the L-axis in neck and croup area (L from 16.2 to 27.1) as observed in black horses.

In chestnut horses, individual L-values ranged from 26.1 (neck) to 61.2 (axillary area), whereas the means covered the range from 29.3 (neck) to 42.7 (axillary area) and thus overlapped with the measurements of bay horses from medium to light colour categories (bay to light bay).

**Table 1:** Mean values, standard deviation (SD) for L*, a*, b* measured at five different body ares for black, bay and chestnut Posavje Horses

| Measuring point | Variable | black | | bay | | chestnut | |
|---|---|---|---|---|---|---|---|
| | | mean | SD | mean | SD | mean | SD |
| Neck | | 20.42 | 2.08 | 26.26 | 3.94 | 29.29 | 3.18 |
| Shoulder | | 22.30 | 1.48 | 30.29 | 4.00 | 33.20 | 2.15 |
| Axillary area | L* | 22.92 | 1.95 | 37.01 | 6.13 | 42.72 | 8.41 |
| Belly | | 19.92 | 4.12 | 32.25 | 4.30 | 34.63 | 3.97 |
| Croup | | 22.24 | 1.23 | 27.86 | 4.17 | 30.55 | 2.82 |
| Neck | | 1.70 | 1.05 | 7.42 | 2.75 | 9.37 | 1.57 |
| Shoulder | | 2.46 | 1.50 | 8.55 | 2.12 | 8.84 | 1.13 |
| Axillary area | a* | 3.16 | 1.26 | 9.03 | 1.98 | 8.03 | 2.20 |
| Belly | | 2.26 | 0.96 | 9.51 | 2.27 | 9.06 | 1.23 |
| Croup | | 2.48 | 1.57 | 7.69 | 2.51 | 8.89 | 1.42 |
| Neck | | 2.74 | 2.57 | 9.47 | 4.41 | 12.49 | 2.95 |
| Shoulder | | 2.54 | 1.84 | 12.58 | 4.13 | 14.94 | 2.56 |
| Axillary area | b* | 4.12 | 1.78 | 17.61 | 4.00 | 18.11 | 1.40 |
| Belly | | 2.46 | 1.86 | 14.68 | 3.80 | 15.57 | 3.05 |
| Croup | | 3.04 | 2.61 | 10.60 | 4.04 | 13.28 | 2.68 |

Measurements of a-values (red-green axis) generally overlapped with those of bay horses (a=8.0 (axillary area) to a=9.4 (neck)), whereas the colour variation in the direction of yellow was more pronounced with mean b-values ranging from 12.5 (neck) to 18.1 (axillary area). On an individual level, the b-values were between 9.0 (neck) and 19.2 (axillary aera).

Multiple comparisons of means adjusted according to the method of Tukey and Kramer revealed that the L*a*and b* measurements resulted in significant differences between the colour classification categories of bay coat colour. Accordingly, light bay differed from mahogany bay and brown ($p<0.05$). In the axillary area area, light bay, seal brown, and mahogany bay could not be distinguished, only brown horses were more darkly pigmented in this area. Light bay horses exhibited the most similarity to chestnut horses expressed in L- and b-values measured at neck, belly, and croup. Slight deviations were identified in the a-values of the measuring areas of the shoulder and axillary.

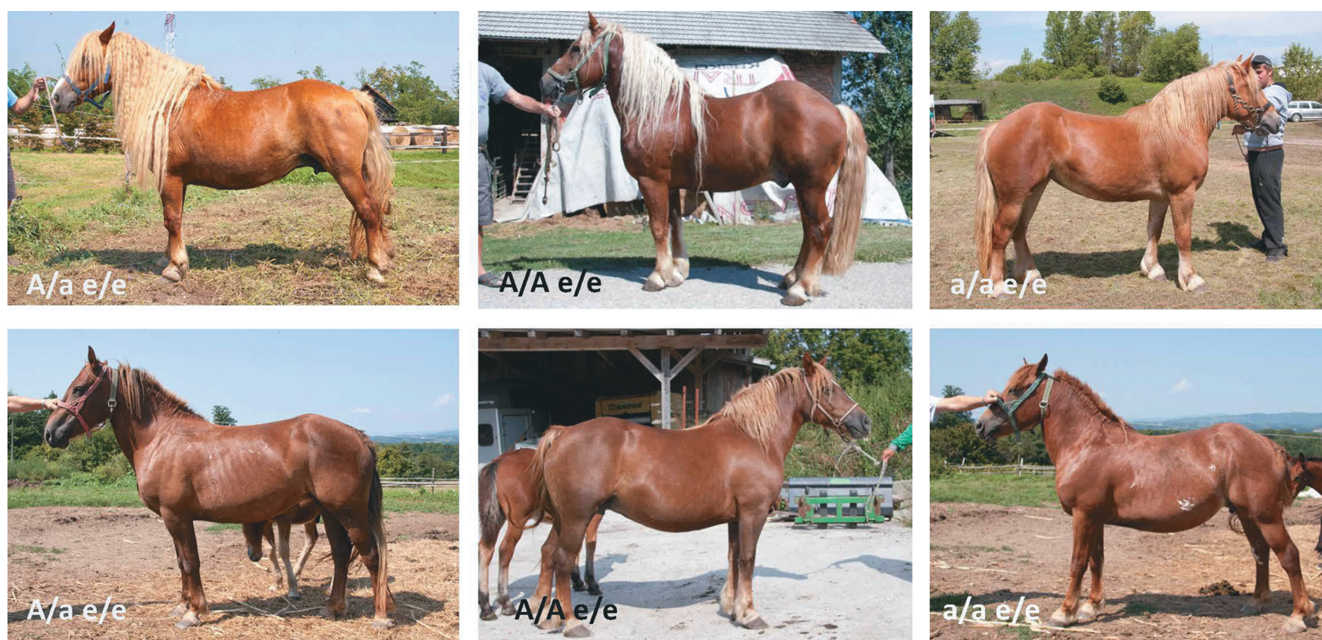



**Figure 2:** Chestnut/sorrel phenotypes and *MC1R/ASIP* genotypes (Fotos Matjaž Mesarič)

**Figure 3:** Bay phenotype classification categories (first line: light bay, bay, brown; second line: red bay, mahogany bay, dark mahogany bay) and genotypes of *MC1R* and *ASIP* (Fotos Matjaž Mesarič)

From 70 genotyped horses, five were black (3 *a/a E/E*, 2 *a/a E/e*), and within the 55 bay Posavje Horses the genotype combination *A/a E/E* was most frequent (52,7%). The genotypes *A/A E/E* and *A/a E/e* were present with 20% each, and 7.3% of the horses had the genotype *A/A e/E* (Figure 3). Within the ten chestnut/sorrel Posavje Horses, all genotype combinations (3 *A/A e/e*, 5 *A/a e/e*, 2 *a/a e/e*) were observed (Figure 2).

Pooling all base-coloured Posavje Horses together, canonical discriminant analysis, which differentiated the spectrophotometric data (L*a*b) according to the *ASIP* and *MC1R* genotype combinations (*a/a e/e, a/a E/E, a/a E/e, A/A E/E, A/a E/E, A/a E/e*), resulted in three main clusters: one cluster containing all black horses (3 *a/a E/E, 2 a/a E/e*), a second wider spread cluster containing mostly darker shaded bay horses (*A/a E/E*), and a third heterogeneous cluster containing lighter shaded horses of all bay genotype combinations and the ten chestnut horses (Figure 4.).

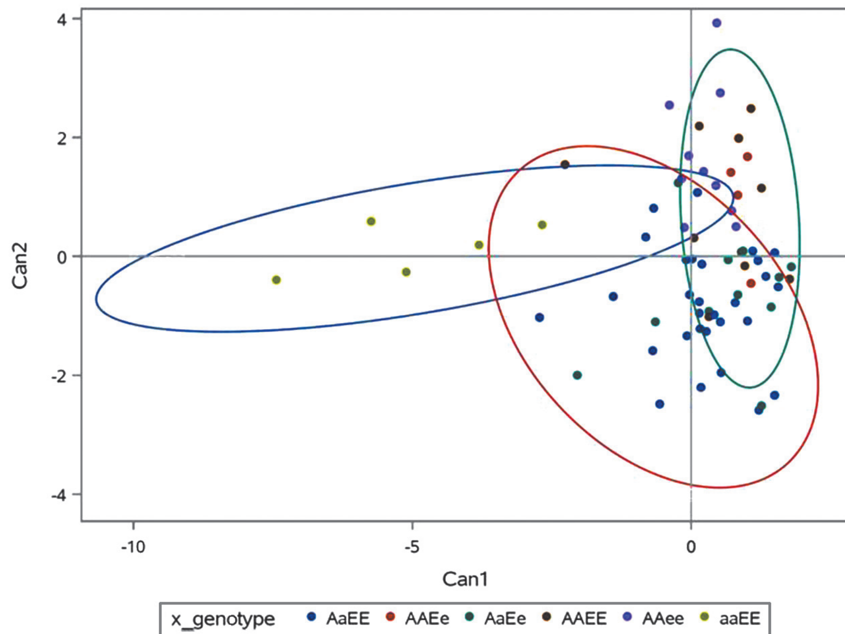



**Figure 4:** Plot of the first two canonical variables (Can1 and Can2) derived from canonical discriminant analysis discriminating individual colour phenotype information (L*a*b) according to genotype information of *MC1R* and *ASIP*. Can1 explained 55.3% of the between group variation and Can2 accounted for 27.2% of the between group variation

*Breed Comparison*

In comparison to the Noriker horse and the Shagya Arabian, Posavje Horses exhibited the highest variability in L* measurements and had a higher proportion of lighter shades of bay. However, Posavje Horses and Shagya Arabians were characterised by higher L*a*b values than those of the Noriker horses (Figure 5).

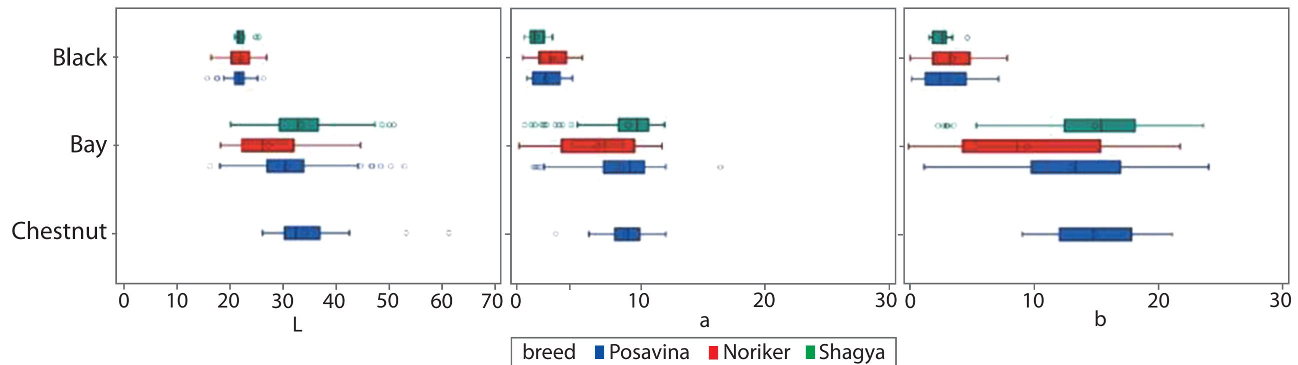



**Figure 5:** Box plots for L*a*b measurements of all body areas in bay, black, and chestnut Posavje Horses, bay and black Noriker and Shagya Arabian horses

Following a MANOVA in order to test the differences of L*a*b measurements between the four genotype combinations in bay horses from the breeds Posavje, Noriker and Shagya Arabian, the generalised model and the Tukey-Kramer test revealed significant differences in all measuring spots between the dark shade associated *A/a E/E* genotype and most of the other genotype combinations (*A/A E/E, A/a E/e, A/A E/e*), except at the axillary area, where the a-value did not differ significantly between the genotype combinations (see Tab. 2.).

**Table 2:** LSmean values for the four genotype groups of *ASIP* and *MC1R* for all five measuring points in 97 bay Noriker, Posavina and Shagya Arabian horses. Superscripts in small letters indicate significant differences between LSmeans at a p-value < 0.05

| | | Rsq | *A/A E/E* | *A/A E/e* | *A/a E/E* | *A/a E/e* |
|---|---|---|---|---|---|---|
| Neck | L* | 0.23 | 27.63$^{b}$ | 30.99$^{b}$ | 25.00$^{a}$ | 27.95$^{b}$ |
| | a* | 0.26 | 8.09$^{b}$ | 9.63$^{b}$ | 5.88$^{a}$ | 8.88$^{b}$ |
| | b* | 0.24 | 11.03$^{b}$ | 14.06$^{b}$ | 7.73$^{a}$ | 11.57$^{b}$ |
| Shoulder[1] | L* | 0.16 | 31.01 | 33.18$^{b}$ | 28.45$^{a}$ | 30.90 |
| | a* | 0.22 | 8.99$^{b}$ | 9.73$^{b}$ | 7.11$^{a}$ | 9.42$^{b}$ |
| | b* | 0.18 | 13.22$^{b}$ | 15.25$^{b}$ | 10.46$^{a}$ | 13.52$^{b}$ |
| Axillary area | L* | 0.10 | 39.35 | 42.87$^{b}$ | 36.17$^{a}$ | 38.02 |
| | a* | 0.10 | 9.68 | 9.65 | 8.39 | 9.60 |
| | b* | 0.13 | 19.26$^{b}$ | 19.89$^{b}$ | 16.37$^{a}$ | 18.57 |
| Belly | L* | 0.15 | 33.53$^{b}$ | 35.09$^{b}$ | 30.02$^{a}$ | 33.49 |
| | a* | 0.14 | 10.09$^{b}$ | 10.53$^{b}$ | 8.27$^{a}$ | 10.14$^{b}$ |
| | b* | 0.14 | 15.74$^{b}$ | 17.06$^{b}$ | 12.64$^{a}$ | 15.88 |
| Croup | L* | 0.09 | 27.52 | 29.18$^{b}$ | 25.71$^{a}$ | 28.27 |
| | a* | 0.16 | 7.85$^{b}$ | 8.57$^{b}$ | 5.71$^{a}$ | 9.10$^{a}$ |
| | b* | 0.13 | 10.76 | 12.09$^{b}$ | 7.88$^{a}$ | 10.88 |

[1]92 animals (5 were missing shoulder measurements)

## Discussion

The three base colours represent the typical coat colour spectrum of the Posavje Horse; the bay coat occurs predominantly and is favoured by the breeders (17). Within the breeding objectives, there is no preference for special shades. As a result, no selection towards distinct chestnut or bay coat colour shades exists. Therefore, in the Posavje breed, a high variation in bay and chestnut is present, whereas in Noriker horses, mahogany bay and brown are preferred, and lighter variants are rare (9). The results of the study showed that the classification categories of Sponenberg (14) can be verified with colour spectrophotometric data. Light bay is characterised by the highest L-values, which are similar across all measuring points, whereas brown is defined by lower and equally dispersed L*a*b values. In mahogany bay, which ranges in the L*a*b spectra between light bay and brown, differences between measuring points occurred. The darkest areas were observed at the neck and croup measuring points, whereas the shoulder and belly were lighter. Lower values of L*a*b in measuring points of the dorsal body area were also observed by Hofmanova et al. (7). In seal brown, which represents the darkest bay category, the typical characteristic is represented by lighter (L-value) and yellowish (b-value) colour measures around the axillary area. Chestnut horses (although the number of animals included in this study were too low for more detailed analysis) could not be differentiated from bay or light bay horses by spectrophotometric data (long hair was not measured).

Breed-specific differences in L*a*b measurements could be shown for Posavje, Noriker and Shagya Arabian horses, whereas in the Noriker sample the darkest phenotypes in the bay and black horses were observed and bay Shagya Arabians exhibited the lightest colour measurements. Toth et al. (13) observed lower L*a*b within the Hungarian Nonius breed, for which the selection allows only dark shades of bay and black. The values

for Nonius (L=24.0; a=4.5; b=4.8) were even lower than in the in Noriker sample.

The phenotypic differences between chestnut and sorrel horses were investigated by Reissmann et al. (18), who were not able to prove a direct relation between genotype and the colour differences of long hair (tail, mane) and body hair. Furthermore, no association between chestnut colour shades and the second identified mutation $e^a$ in *MC1R* (19) was observed. The presence of this mutation was documented in Black Forest Horse, Hungarian Coldblood (20) and in Haflinger breeds (21). Rieder et al. (6) concluded from a small sample of chestnut horses that no association of chestnut coat colour variation with allele status on the *ASIP* locus, whereas Grilz-Seger et al. (22, 23) postulated that the gene *OCA2* may be involved in chestnut coat colour variation and in the mealy (pangare) phenotype. Rieder et al. (6), who analysed associations of *MC1R* and *ASIP* allele status with colour variation in bay horses, concluded an association of *E/E MC1R* genotype with darker shades of bay. In a recent association study, Corbin et al. (24) confirmed the results of Rieder et al. (6). Sakamoto et al. (10), who conducted a pedigree analysis and tested offspring ratios, also observed an association between *A/a* genotype at the Agouti locus and *E/E* genotype at the Extension locus with dark shades of bay. In our study, we were able to confirm these findings and could show that dark shades of bay were associated with the genotype combination *A/a E/E* in bay Posavje Horses, Noriker, and Shagya-Arabians. Interestingly, a comparable gene interaction of *ASIP* and *MC1R* was reported in the red fox (Vulpes Vulpes) by Vage et al. (25). Genotype combination *A/A E/E* resulted in the common phenotype of red foxes, whereas the genotype *A/a E/E* is characterised by dark colour shades in foxes (25).

Conclusions. CIE L*a*b colour spectrophotometry represents an effective tool to characterise and quantify coat colour in horses, which could be used for further analysis of chestnut coat colour, for which the genetic background of coat colour variation remains unknown.

## Acknowledgements

This work was financially supported by the Slovenian Research Agency program P4-0053 to M. Cotman.

Hair samples for Posavje Horses were collected in the context of routine procedures during the studbook registration of horses by the Institute for Breeding and Health Care of Horses of the Veterinary Faculty, Ljubljana.

Author’s contribution: Grilz-Seger, Gertrud: conceptualisation, data analysis, writing - original draft, Mesaric, Matjaz: sampling, data collection; Cotman, Marko: DNA extraction and genotyping, funding acquisition; Brem, Gottfried: funding acquisition; all authors: reading, commenting and reviewing of the final manuscript draft. None of the authors has conflicts of interest.

## References

1. Wutke S, Benecke N, Sandoval-Castellanos E, et al. Spotted phenotypes in horses lost attractiveness in the Middle ages. Sci Rep 2016; 6: e38548. doi: 10.1038/srep38548

2. Pruvost M, Bellone R, Benecke N, et al. Genotypes of predomestic horses match phenotypes painted in Paleolithic works of cave art. Proc Natl Acad Sci U S A 2011; 108: 18626–30.

3. Librado P, Gamba C, Gaunitz C, et al. Ancient genomic changes associated with domestication of the horse. Science 2017; 356: 442–5.

4. Ludwig A, Pruvost M, Reissmann M, et al. Coat colour variation at the beginning of horse domestication. Science 2009; 324(5296): e485. doi: 10.1126/science.1172750.

5. Marklund L, Moller MJ, Sandberg K, Andersson L. A missense mutation in the gene for melanocyte-stimulating hormone receptor (MC1R) is associated with the chestnut coat colour in horses. Mamm Genome 1996; 7: 895–9.

6. Rieder S, Taourit S, Mariat D, Langlois B, Guérin G. Mutations in the agouti (ASIP), the extension (MC1R), and the brown (TYRP1) loci and their association to coat colour phenotypes in horses (*Equus caballus*). Mamm Genome 2001; 12: 450–5.

7. Hofmanová B, Kohoutová P, Vostrý L, Vostrá Vydrová H, Majzlík I. Quantitative aspects of coat colour in old Kladruber Black Horses. Poljoprivreda 2015; 21(Suppl.): 224–7.

8. Stachurska A, Pięta M, Jaworski Z, Ussing AP, Bruśniak A, Florek M. Colour variation in blue dun Polish Konik and Biłgoraj horses. Livest Prod Sci 2004; 90(2): 201–9.

9. Druml T, Grilz-Seger G, Horna M, Brem G. Discriminant analysis of colour measurements

reveals allele dosage effect of ASIP/MC1R in bay horses. Czech J Anim Sci 2018; 63: 347–55.

10. Sakamoto T, Fawcett JA, Innan H. Evaluating the potential roles of the Gray and Extension loci in the coatcoloration of Thoroughbred racing horses. J Equine Sci 2017; 28: 61–5.

11. Curik I, Druml T, Seltenhammer M, et al. Complex inheritance of melanoma and pigmentation of coat and skin in grey horses. PLoS Genetics 2013; 9: e1003248.

12. Lackner C. Quantitative Ansätze der Farbvererbung beim Noriker-Pferd: Dipl. Arbeit. Wien : Universität für Bodenkultur, 2006.

13. Toth Z, Kaps M, Sölkner J, Bodo I, Curik I. Quan¬titative genetic aspects of coat color in horses. J Anim Sci 2006; 84: 2623–8.

14. Sponenberg DP. Equine color genetics. 3rd ed. Hoboken : Wiley-Blackwell, 2009: p. 118.

15. Lauren J, Staiger E, Albright J, Brooks S. The MC1R and ASIP coat colour loci may impact behavior in the horse. J Hered 2016; 107(3): 214–9. doi: 10.1093/jhered/esw007.

16. SAS Institute. Cary : SAS university edition, Cary, USA, 2018.

17. Mesarič M. Die Zucht des Posavina Pferdes in Slowenien. In: Grilz-Seger G, Druml T, ed. Das Posavina Pferd. Graz : Vehling Verlag, 2018.

18. Reissmann M, Brockmann GA. Analyse verschiedener Langhaaraufhellungen beim Pferd. Züchtungskunde 2008; 80: 491–500.

19. Wagner HJ, Reissmann M. New polymorphism detected in the horse MC1R gene. Anim Genet 2000; 31: 289–90.

20. Reissmann M, Wagner M, Gulyaz L, Schuster S. The allele ea – a rare mutation in the MC1R gene in horses (*Equus caballus*). In: 28th International Conference on Animal Genetics. Göttingen : International Society of Animal Genetics, 2002.

21. Reissmann M, Musa L S, Zakizadeh S, Ludwig A. Distribution of coat-colour-associated alleles in the domestic horse population and Przewalskis horse, J.Appl Genetics 2016; 57: 519–525.

22. Grilz-Seger G, Druml T, Neuditschko M, et al. Changes in breeding objectives of the Haflinger horse breed from a genome-wide perspective. Züchtungskunde 2019; 91: 296–311.

23. Grilz-Seger G, Neuditschko M, Ricard A, et al. Genome-wide homozygosity patterns and evidence for selection in a set of European and Near Eastern horse breeds. Genes 2019; 10(7): e491. doi: 10.3390/genes10070491

24. Corbin LJ, Pope J, Sanson J, et al. An independent locus upstream of ASIP controls variation in the shade of the Bay Coat colour in horses. Genes 2020; 11(6): 606. doi: 10.3390/genes11060606.

25. Vage DI, Fuglei E, Snipstad K, Beheim J, Landsem M, Klungland H. Two cysteine substitutions in the MC1R generate the blue variant of the arctic fox (*Alopex lagopus*) and prevent expression of the white winter coat. Peptides 2005; 26(10): 1814–7.

## KARAKTERIZACIJA BARVE DLAKE PRI POSAVSKEM KONJU

G. Grilz-Seger, M. Mesarič, G. Brem, M. Cotman

**Izvleček:** Za opis barv konj se uporabljajo različni pristopi in klasifikacijski sistemi, ki se razlikujejo med posameznimi pasmami in državami. V raziskavi smo najprej opredelili različne barve dlake pri posavskem konju z metodo barvne spektrofotometrije po sistemu CIE L*a*b*. Fenotipsko razdelitev barv dlake po Sponenbergu (light bay/svetli rjavec, bay/rjavec, mahogany bay/kostanjev, brown/temni rjavec and seal brown/črnkast rjavec) smo potrdili s spektrofotometričnimi podatki. Vrednosti L*a*b so pri posavskem konju pokazale primerljivo visoko fenotipsko variabilnost rjave barve, pri tem so bili temnejši odtenki povezani z *ASIP* in *MC1R* kombinacijo genotipa *A/a E/E*. Barvna spektrofotometrija po sistemu CIE L*a*b predstavlja učinkovito orodje za kvalitativno in kvantitativno opredelitev/določanje barv pri konjih, zlasti pri lisjakih, pri katerih še vedno ni znana genska osnova variabilnosti v barvi dlake.

**Ključne besede:** posavski konj; *MC1R*; *ASIP*; barva dlake; spektrofotometrija; CIE L*a*b

Slov Vet Res 2021: 58 (2): 85 –90
DOI 10.26873/SVR-1096-2020
UDC 579.852.11: 616.322-002.3: 598.162.112

**Case Report**

# FIRST REPORT OF *Paenibacillus cineris* FROM A BURMESE PYTHON (*Python molurus bivittatus*) WITH ORAL ABSCESS

Hamid Staji[1]*, Iradj Ashrafi Tamai[2] , Zahra Zeifati Kafi[2]

[1]Department of Pathobiology, Faculty of Veterinary Medicine, Semnan University, Semnan,[2]Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

*Corresponding author, E-mail: hstaji@semnan.ac.ir

**Abstract:** We report a case of oral abscess caused by *Paenibacillus cineris* in a pet Burmese python. Microbiological and nucleic acid sequencing showed high similarity to the reference strain *P. cineris* (113XG30) isolated in China. Although *Paenibacillus* spp. are environmental bacteria, we highlight the importance of careful consideration of such unusual organisms in exotic reptiles.

**Key words:** *Paenibacillus cineris*; python; pharyngeal abscess

## Introduction

Various published investigations are demonstrating the normal microbiata of the oral cavity in snakes (1-3). For instance, it has been stated that gram positive genera such as *Staphylococcus* and *Corynebacterium* are predominant in the oral cavities of healthy snakes while gram negative genera such as *Pseudomonas* and *Providencia* are mainly predominant in snakes with stomatitis (3). Although snake oral health can be affected by such bacteria, there is not enough documented data showing rare bacterial agents isolated from snakes with stomatitis. In the present study for the first time, we identified *Paenibacillus cineris* isolated from the abscess contents of a python received from a vet hospital using phenotypic, biochemical and genotypic taxonomic approaches.

Recent advances in molecular typing methods for bacterial taxonomy have enabled microbiologists to a growing appreciation that a variety of rare organisms may also be recovered from the infectious conditions of exotic species. The present study aimed to describe the recovery of *Paenibacillus cineris* from an abscess in a python with head swelling.

Received: 29 April 2020
Accepted for publication: 24 November 2020

## Case description

A previously healthy python (male, weighting 27.5 kg) presented with respiratory problems and dysphagia for about a week, progressive restlessness and swelling around the oral cavity, was referred to the pet hospital of the faculty of veterinary medicine (University of Tehran, Iran). At the physical examination, an abscess about 2 cm in diameter located in the tissues in the back of the throat behind the posterior pharyngeal wall was observed. To remove the infection and prevent complications it was decided to drain and extract the abscess. For this purpose, the snake was anesthetized by intramuscular (IM) injection with ketamine medicine (KETAMINA MOLTENI, Italy; 20 mg/kg). Then, the surgical site was disinfected with 1.0% chlorhexidine and sterile 0.9% saline (4) and interior fluid and caseous pus contents were extracted for microbiological assessments. Analgesics should be used each time when the animals are subjected to painful sensations. Unfortunately, the knowledge of pain perception in snakes is quite limited. Mitchell et al. (2009) recommended using of pain relieving agents such as butorphanol (0.5 1 mg/kg IM), carprofen (2-4 mg/kg IM, PO) and meloxicam (0.5 mg/kg PO). Thus, for analgesic purposes and less stress induction to the animal, meloxicam (ABURAIHAN, Iran; 0.5 mg/kg PO, once after operation) was administered. After operation, the python was fed every 4 days by mice pinkies soaked in warm chicken broth by using feeding forceps.

### *Microbiological identification of pathogenic agent*

The isolation and identification of the pathogen agent was carried out according to the protocols described by Leão et al. (5) and Sáez-Nieto et al. (6) respectively. Briefly, the aspirated pus sample was plated on Columbia agar with 5% defibrinated sheep's blood and Mac Conkey agar for 24 to 48 hours in the aerobic atmosphere at 37°C. After overnight incubation, yellow colonies with irregular edges and mucoid appearance were observed on enriched Columbia agar and there was no growth on Mac Conkey selective agar. Microscopic examination of colonies showed Gram-negative sporulated bacilli, and some endospores were demonstrated. The isolate was assessed by further biochemical and phenotypic tests as described in Table. 1.

### *Molecular identification of Paenibacillus spp. based on partial sequencing of 16S rRNA*

Genomic DNA extraction was carried out on colonies microbiologically suspected to *Paenibacillus* spp. by using an alkaline lysis method (7). Then, extracted genomes were subjected to PCR for amplification of a 900-1000 bp fragment of 16S rRNA belonging to the genus Paenibacillus. Identification and amplification of 16S rRNA were carried out using universal primer pairs (8).

The nucleotide sequence of the amplified fragment was analyzed with an ABI 3730XL DNA Analyzer according to an automated Sanger dideoxy fluorescent nucleotide method and the obtained sequence was deposited in GenBank with accession number: MT158489. Then, the BLAST software was applied to determine the homology of the amplified fragment to DNA sequences existing in GenBank and the phylogenetic tree was constructed by the neighbor-joining model in MEGA-6.

### *Antimicrobial susceptibility*

According to the previous antimicrobial susceptibility profiles of *Paenibacillus* spp. (5-6), eleven antimicrobials (PADTAN TEB Company, Iran) including imipenem (10 μg), ampicillin (10 μg), penicillin (10 μg), polymixin B (300 μg), trimethoprim sulfamethoxazole (75 μg), cefotaxime (30 μg), enrofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), rifampicin (5 μg), and vancomycin (30 μg) were examined for susceptibility assessments on Mueller-Hinton agar enriched with sheep's defibrinated blood. Disc diffusion (Kirby–Bauer) test was performed on the isolate for antimicrobial susceptibility evaluations. Briefly, a suspension of the isolate (of approximately 1-2 × $10^8$ CFU/mL) was prepared to a 0.5 McFarland standard, then spread evenly onto Mueller-Hinton agar in the Petri dish and the mentioned discs were placed onto the surface of the agar. Then, after overnight incubation zones of growth inhibition around each of the antibiotic discs were measured to the nearest millimeter. The interpretation of results were based on the clinical and laboratory standards institute (CLSI) guidelines.

**Table 1:** Phenotypic and biochemical characteristics of Paenibacillus isolate from a pet python. Adapted from Leão et al. [5] and Logan et al. [9]. ONPG = O-nitrophenyl-β-D-galactopyranoside; PYR = pyrrolidinyl-β-naphthylamide

| Tests | Results | Tests | Results |
|---|---|---|---|
| Gram stain | Gram-negative sporulated bacilli | Esculin | + |
| Oxidase | + | Urea | - |
| Motility | + | ONPG | + |
| Oxidation of Glucose | - | PYR | + |
| Xylose | - | Growth on Mac Conkey | - |
| Maltose | - | 42 °C | + |
| Sucrose | - | Citrate agar | - |
| Lactose 10% | - | NaCl (6.5%) | - |
| Lysine decarboxylase | - | NaCl (0%) | + |
| Ornithine decarboxylase | - | Production of $H_2S$ | - |
| Arginine dihydrolase | - | Indole | - |
| Hydrolysis of Gelatin | - | Nitrate reduction | + |
| DNase activity | - | Gas from nitrate | + |

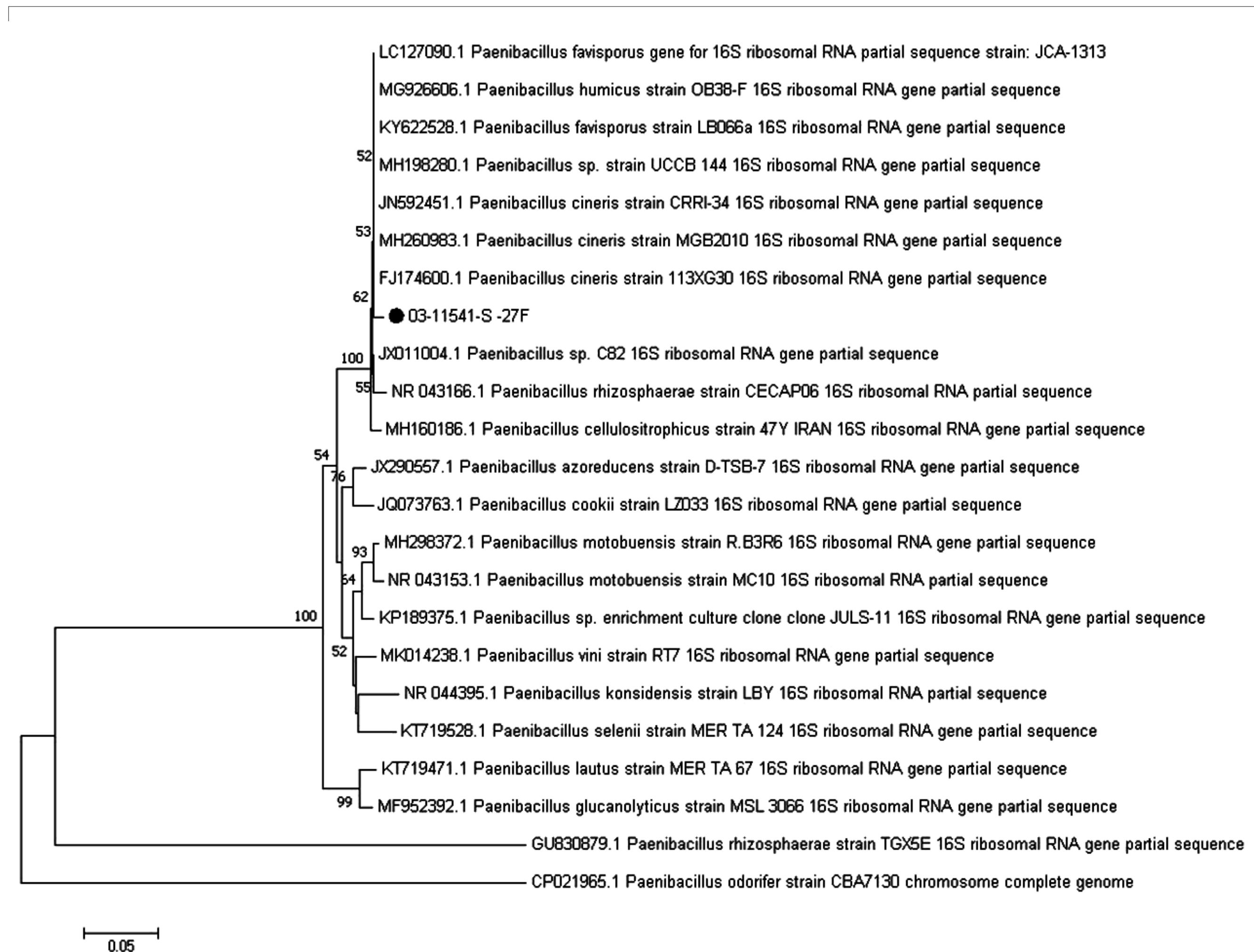



**Figure 1:** Neighbor-Joining phylogenetic tree based on the 16S rRNA gene sequence of *Paenibacillus* spp. *Note:* the marked Latin name (03-11541-S-27F) represents the isolate in this study. The codes before the Latin names are GenBank accession numbers for each type strain. Numbers at nodes are percentages of bootstrap values and scale bar indicates 0.05 nucleotide sequence divergence

## Results

According to the phenotypic and microbiological criteria, the bacterial isolate from snake's abscess secretions was found to be a *Paenibacillus* spp. All the microbiological results of the isolate are presented in the Table. Our findings concerning the microbiological characteristics of the isolate are consistent with the type strains *P. cineris* from previous publications (5, 9).

Sequence analysis of a partial fragment of the 16S rRNA gene amplified from the isolate (MT158489) demonstrated a high similarity level with the *P. cineris* strain 113XG30 deposited in Gene Bank with accession no. FJ174600.1. The neighbor-joining phylogenetic tree of our study indicates the position of our isolate and its identity with the type strains of *P. cineris* and other closely related species (Figure).

Antimicrobial sensitivity testing results belonging to the Paenibacillus isolate in the present study are shown in Table 2. In summary the isolate showed resistance to ampicillin, penicillin, erythromycin, and gentamicin while sensitivity was observed to the rest of tested antimicrobials. Trimethoprim-sulfadimethoxine was administered 20 mg/kg (IM, q24h) for 10 days with the use of a pole syringe (4). Two weeks after the end of the antibiotic therapy, abscess lesions and head swelling signs showed complete treatment and the python recovered completely.

**Table 2:** Zone diameter observed related to the P. cineris isolate and interpretative standards for used antimicrobial disks

| | Zone Diameter Interpretive Criteria | | |
|---|---|---|---|
| | Resistant (R) | Susceptible (S) | Observed zone of inhibition (mm) concerning the *P. cineris* isolate |
| imipenem | ≤ 20 | 28 ≤ | 31 |
| ampicillin | ≤ 12 | 16 ≤ | 8 (R) |
| penicillin | ≤ 27 | 28 ≤ | 14 (R) |
| polymixin B | ≤ 14 | 18 ≤ | 20 |
| Trimethoprim-sulfamethoxazole | ≤ 10 | 16 ≤ | 22 |
| cefotaxime | ≤ 25 | 31 ≤ | 31 |
| enrofloxacin | ≤ 22 | 30 ≤ | 33 |
| erythromycin | ≤ 13 | 23 ≤ | 9 (R) |
| gentamicin | ≤ 12 | 15 ≤ | 11 (R) |
| rifampicin | ≤ 16 | 20 ≤ | 28 |
| vancomycin | ≤ 17 | 21 ≤ | 24 |

## Discussion

More than twenty strains belonging to Paenibacillus genus are recognized as responsible for true infections or transient infections colonizing blood and other human sources, or as a possible contamination occurring during the process of obtaining the sample (6). Since 1989, the number of reports regarding clinical infections in human cases including brain abscesses after contusion, neonatal meningitis, lung diseases, bacteremias, urinary tract infection and endophthalmitis caused by the *Paenibacillus* spp. have increased (5, 10-16). In veterinary medicine, *Paenibacillus* larvae is known as the causal agent of American Foulbrood (AFB) disease in honey bees (17).

Although, *Paenibacillus* spp. can be truly pathogen, but reports concerning the prevalence of these bacteria in clinical samples in medicine and veterinary medicine are limited and this may be related to the fact that such bacteria are considered to be contaminants of clinical specimens (5, 6).

*Paenibacillus cineris* is a part of the inhabitant microflora of a variety of sources such as soil, water, food, plants and sediments (6). To the best of our knowledge, this is the first report of *P. cineris* recovered from a reptile and there is no

other report regarding the infection caused by this species in animals. *Paenibacillus* spp. may cause infections via indigenous route or can enter the host body after trauma (18). The infection by *P. cineris* observed in this study in the oral cavity of a python kept as a pet animal could have been triggered by mechanical injuries that occurred during feeding or as a result of aggressive behaviors. Although, the role of *Paenibacillus* spp. in reptiles diseases is unknown this study highlights the potential pathogenic ability of *P. cineris* in snakes. So, further consideration should be given to the epizootiology of rare microorganisms in reptiles and the implication of *P. cineris* in the evaluation of the snake's infectious conditions.

## Acknowledgments

The authors thank University of Tehran clinical staffs for their help in sampling and recovering the python and microbiology department technicians for their help in bacteriological identification of *P. cineris* isolate.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## References

1. Dehghani R, Sharif MR, Moniri R, Sharif A, Kashani HH. The identification of bacterial flora in oral cavity of snakes. Comp Clin Pathol 2016; 25(2): 279–83. doi:10.1007/s00580-015-2178-9

2. Fonseca MG, Moreira WM, Cunha KC, Ribeiro AC, Almeida MT. Oral microbiota of Brazilian captive snakes. J Venom Anim Toxins Incl Trop Dis 2009; 15(1): 54–60. doi:10.1590/S1678-91992009000100006

3. Jho YS, Park DH, Lee JH, Cha SY, Han JS. Identification of bacteria from the oral cavity and cloaca of snakes imported from Vietnam. Lab Anim Res 2011; 27(3): 213–7. doi:10.5625/lar.2011.27.3.213

4. Mitchell MA. Snake care and husbandry. Vet Clin North Am Exot Anim Pract 2004; 7: 421–6.

5. Leão RS, Pereira RH, Ferreira AG, et al. First report of *Paenibacillus cineris* from a patient with cystic fibrosis. Diagn Microbiol Infect Dis 2010; 66(1): 101–3. doi:10.1016/j.diagmicrobio.2009.06.011

6. Sáez-Nieto JA, Medina-Pascual MJ, Carrasco G, et al. *Paenibacillus* spp. isolated from human and environmental samples in Spain: detection of 11 new species. New Microbes New Infect 2017; 19: 19–27. doi: 10.1016/j.nmni.2017.05.006

7. Staji H, Rassouli M, Jourablou S. Comparative virulotyping and phylogenomics of *Escherichia coli* isolates from urine samples of men and women suffering urinary tract infections. Iran J Basic Med Sci 2019; 22(2): 211–4. doi:10.22038/ijbms.2018.28360.6880

8. Shida O, Takagi H, Kadowaki K, Nakamura LK, Komagata K. Transfer of *Bacillus alginolyticus, Bacillus chondroitinus, Bacillus curdlanolyticus, Bacillus glucanolyticus, Bacillus kobensis,* and *Bacillus thiaminolyticus* to the genus *Paenibacillus* and emended description of the genus *Paenibacillus*. Int J Syst Bacteriol 1997; 47(2): 289–98. doi:10.1099/00207713-47-2-289

9. Logan NA, De Clerck E, Lebbe L, et al. *Paenibacillus cineris* sp. nov. and Paenibacillus cookii sp. nov., from Antarctic volcanic soils and a gelatin-processing plant. Int J Syst Evol Microbiol 2004; 54(4): 1071–6. doi: 10.1099/ijs.0.02967-0

10. Bert F, Ouahes O, Lambert-Zechovsky N. Brain abscess due to *Bacillus macerans* following a penetrating periorbital injury. J Clin Microbiol 1995; 33(7): 1950–3.

11. Lo CI, Sankar SA, Fall B, et al. High-quality genome sequence and description of *Paenibacillus dakarensis* sp. nov. New Microbes New Infect 2016; 10: 132–41. doi: 10.1016/j.nmni.2016.01.011

12. Marchese A, Barbieri R, Pesce M, Franchelli S, De Maria A. Breast implant infection due to *Paenibacillus residui* in a cancer patient. Clin Microbiol Infect 2016; 22(8): 743–4. doi: 10.1016/j.cmi.2016.05.012

13. Ouyang J, Pei Z, Lutwick L, et al. *Paenibacillus thiaminolyticus*: a new cause of human infection, inducing bacteremia in a patient on hemodialysis. Ann Clin Lab Sci 2008; 38(4): 393–400. PMC2955490

14. Rieg S, Bauer TM, Peyerl-Hoffmann G, et al. *Paenibacillus larvae* bacteremia in injection drug users. Emerg Infect Dis 2010; 16(3): 487–90. doi: 10.3201/eid1603.091457

15. Sharma S, Gupta A, Rao D. *Paenibacillus lautus*: a rare cause of bacteremia and review of the literature. Indian J Med Case Rep 2015; 4(2): 56–9.

16. Yoon HJ, Yim HW, Ko KS. A case of *Paenibacillus pasadenensis* bacteremia in a patient with acute respiratory distress syndrome after mi-

crosurgical clipping. J Infect Chemother 2015; 47 (1): 64–7. doi: 10.3947/ic.2015.47.1.64

17. Antúnez K, Anido M, Arredondo D, Evans JD, Zunino P. *Paenibacillus larvae* enolase as a virulence factor in honeybee larvae infection. Vet Microbiol 2011; 147(1/2): 83–9. doi: 10.1016/j.vetmic.2010.06.004

18. Park SN, Lim YK, Shin JH, et al. *Paenibacillus oralis* sp. nov., isolated from human subgingival dental plaque of gingivitis lesion. Curr Microbiol 2019; 12: 1–7. doi: 10.1007/s00284-019-01843-0

## PRVO POROČILO O OSAMITVI *Paenibacillus cineris* IZ BURMANSKEGA PITONA (*Python molurus bivittatus*) Z ORALNIM ABSCESOM

H. Staji, I. A. Tamai , Z. Z. Kafi

**Izvleček:** V članku je opisan primer oralnega abscesa, ki ga je povzročila bakterija *Paenibacillus cineris* pri hišnem burmanskem pitonu. Mikrobiološko in nukleinsko kislinsko zaporedje je pokazalo visoko podobnost z referenčnim sevom *P. cineris* (113XG30), izoliranim na Kitajskem. Čeprav spada *Paenibacillus spp.* med okoljske bakterije, lahko takšni neobičajni mikroorganizmi pri eksotičnih plazilcih povzročajo tudi zdravstvene težave in jim moramo posvečati pozornost.

**Ključne besede:** *Paenibacillus cineris*; piton; žrelni absces

**SLOVENIAN VETERINARY RESEARCH**
**SLOVENSKI VETERINARSKI ZBORNIK**

**Slov Vet Res 2021; 58 (2)**

**Original Research Articles**

Habeeb GA, Durmuşoğlu H, İlhak Oİ. The combined effect of sodium lactate, lactic acid and acetic acid on the survival of *Salmonella* spp. and the microbiota of chicken drumsticks ....47
Sargious MAN, El-Shawarby RM, Abo-Salem ME, EL-Shewy EA, Ahmed HA, Hagag NM, Ramadan SI. Genetic diversity of Egyptian Arabian horses from El-Zahraa Stud based on 14 TKY microsatellite markers .... 55
Aloke C, Igwe ES, Obasi NA, Amu PA, Ogbonnia EC. Anti-diabetic effect of ethanol extract of Copaifera salikounda (Heckel) against alloxan-induced diabetes in rats ....63
Grilz-Seger G, Mesarič M, Brem G, Cotman M. Characterisation of coat colour in the Slovenian Posavje horse ....77

**Case Report**

Staji H, Tamai IA, Kafi ZZ. First report of *Paenibacillus cineris* from a Burmese python (*Python molurus bivittatus*) with oral abscess ....85