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Address
ul. Jana Heweliusza14
10-718 Olsztyn-Kortowo, Poland
tel.: +48 89 523-36-61
fax: +48 89 523-34-38
e-mail: wydawca@uwm.edu.pl

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INHERITANCE OF COAT COLOUR IN FERRETS (*MUSTELA PUTORIUS FURO*) BASED ON PEDIGREE ANALYSIS

***Dominika Grabolus*[1], *Patrycja Wacławik*[2], *Magdalena Zatoń-Dobrowolska*[3]**

[1] ORCID: 0000-0003-0402-0095
[2] ORCID: 0000-0002-0011-0715
[3] ORCID: 0000-0002-3096-4113
[1–3] Department of Genetics
[1] Wrocław University of Environmental and Life Sciences, Wrocław, Poland

Key words: ferret, fur colour, coat pattern, phenotype, heredity.

Abstract

The study aimed to determine the rules governing the inheritance of coat colour in ferrets, including basic colour, colour concentration patterns, and white markings. To reach this aim, pedigree analysis was applied. It covered the pedigrees of 201 pups born between 2009 and 2017 in two household ferretries. In the analysed population, basic colours black and dark brown dominated over brown, beige, and those with copper reflections. The standard colour concentration pattern dominated the other patterns, the point type being the most recessive. The point pattern is phenotypically similar to the Himalayan mutation, which is also recessive to the wild type. In this study, it was impossible to analyse if the lack of white markings dominated over their presence, due to the small differences in the number of animals with and without white markings. The obtained results refer to a specific animal population and are based only on phenotypic classification.

Introduction

The first recorded coat colour variations in ferrets (*Mustela putorius furo*) were sable (so-called standard) and albino (JEŻEWSKA and MACIEJOWSKI 1989, BEDNARZ and FRINDT 1991, BLASZCZYK et al. 2007). The nomenclature used in 1980s in Polish farm breeding distinguished three types of breeding polecats: grey, lemon, and orange, the colour of undercoat hair being their main difference. There were also brown variations, with the varying intensity of colour, from dark brown to light yellow-brown,

Address: Dominika Grabolus, Wrocław University of Environmental and Life Sciences, Kożuchowska st. 7, 51-631 Wrocław, Poland; e-mail: dominika.grabolus@upwr.edu.pl

referred to as fawn (JEŻEWSKA and MACIEJOWSKI 1989, Bednarz and Frindt 1991). The Scandinavians described individuals with silver hair on their tails, hind legs, napes, and hips. In addition to the standard and albino colour variations, they also distinguished pastel ferrets, with light brown fur. Ferrets with a dark brown coat, called chocolate, have been reported in Finland, Norway, and the USA (NES et al. 1988). In 2005, the American Ferret Association distinguished 30 colour variations of ferret coat, 11 of them being basic variations. In addition, the Russians distinguished sable, pearl and goldish variations, differing in undercoat colour. The goldish variation had yellow-orange, pearl had white, and sable had cream-yellow undercoat hair (LEWINGTON 2007). Currently, to correctly characterise the coat colour of a ferret, a three-step analysis should be performed. First, basic colour is determined; then, a concentration pattern is determined; and finally, white markings – if present – are classified (American Ferret Association 2017, Associazione Italiana Furetti 2016). The coat colour of animals depends on the presence of melanin pigments in the hair and skin. In mammals two melanin types responsible for fur colour can be distinguished – namely eumelanin and pheomelanin – they can occur in the hair and skin together or alone. The basic coat colour is determined by the ratio of eumelanin to pheomelanin, which is mainly being controlled by the Agouti signalling protein (*Asip*) and the Melanocortin-1 Receptor (*Mc1r*) genes. The final effects seen in animals can be modified by many other genes, e.g. Tyrosinase-related protein 1 (*Tyrp 1*) can switch the eumelanin colour between black and brown. Some genes may dilute the base colour (e.g. Myosine 5a – *Myo5a*), others may cause white spotting on the fur and skin (e.g. Endothelin receptor type B – *Ednrb*, Dominant white – *Kit*) and the Tyrosinase-related protein gene (*Tyr*) is responsible for the lack of pigment in animals body – albinism (CIESLAK et al. 2011, HOEKSTRA 2006, ATA and MAJEWSKI 2016, RZEPKA et al. 2016). The topic of genetic basics of coat colour inheritance in ferrets has been briefly described by GRABOLUS et al. (2016). The authors were also using pedigrees in their analyses but contrary to this study, they concentrated on the genetics of the coat colour.

This study aimed to determine the inheritance scheme – consisting of basic colours, colour concentration patterns, and white markings – of coat colour in ferrets. To meet this aim, a pedigree analysis was conducted.

Material and Methods

Coat colour classification

The classification of colour variations used in this study is based on the Associazione Italiana Furetti (2016) – Furetomania Onulus – Aif (2016) and the American Ferret Association – AFA (2017). The classification includes six basic colours and two types of uniformly white coat colour, five colour concentration patterns, and five variations of white markings. Unlike AIF, AFA uses neither the self pattern nor milk and striped white markings.

The pedigrees

The research included the pedigrees of 201 ferrets born in 2009–2017. The ferrets came from two household ferretries, namely, Ferretta Passion (Poland; 105 ferrets) and Ferret Vendetta (Italy; 96 ferrets). The pedigrees included 354 specimens (178 females and 176 males) for whom it was possible to determine the basic coat colour, concentration pattern, and white markings. Ferrets from both ferretries were related. In addition, breeding lines were carried out and some matings were repeated, allowing for more accurate analysis and the higher reproducibility of the study. The pedigrees originated from private breeders' resources and "Feritage – Ferret Database System", a ferret pedigree database (2017) supervised by Marit Nybakken.

Pedigree analysis

To determine the rules for the inheritance of coat colour in ferrets, the analysis of pedigrees for the litters born between 2009 and 2017 was carried out. The analysis included all the matings found in the pedigrees, which went back three generations (an offspring, parents, grandparents, and great-grandparents). The analysis was carried out separately for each of the three aspects of determining coat colour, that is, basic colour (including the colour uniformly white), a concentration pattern, and white markings.

Because of the colour uniform white for the albino and DEW (Dark Eyed White) coat colours, it is impossible to determine the concentration pattern and the occurrence of white markings for them. In addition, in ferrets with striped white markings, a colour concentration pattern cannot be determined, which is also due to the predominance of the colour white. Therefore, when analysing concentration patterns, mating with uniformly

white individuals and striped white markings was considered. To analyse white markings, mating with ferrets with the colours albino and DEW was also considered.

Results and Discussion

Basic colour

Twenty-three out of 37 possible mating combinations for basic colour were detected in the population analysed (Table 1). Coats with mostly black and/or dark brown hair (that is, black, black-sable, and sable) clearly dominated over coats with hair in shades of brown (that is, warm shades), beige, and with copper reflections (that is, chocolate, champagne, and cinnamon).

Table 1

The distribution of the eight basic colours for various matings (27 combinations)

Specification	Black	Black-sable	Sable	Chcoco-late	Cinna-mon	Cham-pagne	DEW	Albinos
Black × black	9	2	8	1	–	–	–	–
Black × black-sable	29	34	3	4	–	–	–	–
Black × sable	9	1	3	4	–	–	–	–
Black × chocolate	12	1	2	6	1	–	–	–
Black × cinnamon	1	–	–	4	–	–	1	–
Black × champagne	–	–	–	–	–	2	–	–
Black × DEW	–	–	1	–	–	–	–	–
Black-sable × black-sable	–	11	2	1	–	–	–	–
Black-sable × sable	–	11	16	5	–	–	–	–
Black-sable × chocolate	6	3	1	3	–	–	–	–
Black-sable × champagne	–	1	–	–	–	–	–	–
Sable × sable	–	–	4	–	1	–	–	–
Sable × chocolate	7	4	14	8	4	5	–	–
Sable × cinnamon	–	1	1	–	–	–	–	–
Sable × champagne	–	–	2	1	–	1	–	–
Sable × DEW	–	–	1	–	–	–	–	–
Sable × albino	–	1	1	–	–	–	–	–
Chocolate × chocolate	–	–	–	2	1	–	–	–
Chocolate × cinnamon	–	–	2	2	–	1	–	–
Chocolate × chapmagne	–	–	–	2	1	–	–	–

cont. Table 1

Cinnamon × cinnamon	–	–	–	–	1	–	–	–
Cinnamon × albino	1	–	–	–	–	–	–	–
Champagne × albino	–	–	–	–	–	–	–	1

Eight base coat colours in ferrets can be distinguished, which results in 37 possible mating combinations. The table presents 23 mating combinations for base coat colours that occurred in the study

It was impossible, however, to determine the dominance series for these three most dominating coat colours, because of their similar distributions of mating combinations and offspring colour. Therefore, it is difficult to determine whether the most dominant colour is black, black-sable, or sable: more detailed analyses that would include the genotypes are needed. The small difference between the number of black and black-sable ferrets may also result from mistakes in coat colour determination, a likely reason being that the breeders prefer black ferrets.

The dominance of dark colours over lighter ones is quite common among mammals. In the majority of species, the wild type (so-called agouti) has most hair of the colour black-brown (BENNET and LAMOREUX 2003, HOEKSTRA 2006, CIESLAK et al. 2011). BEDNARZ and FRINDT (1991) and JEŻEWSKA and MACIEJOWSKI (1989) also claimed that brown ferret variations were recessive to standard variations (so-called polecats). Similarly, in American mink, standard variations (black and dark brown) dominate over various pastel variations (i.e., with coat in shades of brown, beige, and those with copper reflections) (SHACKELFORD 1948, NES et al. 1988, KUŹNIEWICZ and FILISTOWICZ 1999). Also in dogs, cats, and mice, the brown coat colour – also known as chocolate or liver – is recessive to the wild type (RUVINSKY and SAMPSON 2001, SCHMIDT-KÜNTZEL et al. 2005). Uniformly white coats (albino and DEW) are the most recessive to other colours. Like in cats, rabbits, cattle, chickens, sheep, American mink, mice, rats, and humans, albinism in ferrets is inherited autosomal recessively (BENNET and LAMOREUX 2003, BLASZCZYK et al. 2007). Such a small number of white ferrets can also be due to diseases associated with this colour – such as deafness in ferrets of the DEW type – and the resulting reluctance of breeders to reproduce animals of this colour (PIAZZA et al. 2014**)**. The obtained pattern of dominance for the basic coat colour in ferrets does not differ from the commonly existing patterns of coat colour dominance in the above-described mammalian species.

Concentration pattern

Seventeen out of 36 possible mating combinations for concentration patterns were detected in the population studied (Table 2).

Table 2

The distribution of eight concentration patterns for various matings (17 combinations)

Specification	Self	Solid	Standard	Roan	Point*	Striped	DEW	Albinos
Self × self	2	–	–	–	–	–	–	–
Self × standard	3	5	7	–	–	–	–	–
Self × roan	–	5	7	10	1	–	–	–
Self × striped	–	6	8	2	–	–	–	–
Self × DEW	–	–	1	–	–	–	–	–
Solid × solid	1	5	2	–	–	–	–	–
Solid × standard	2	3	9	–	–	–	–	–
Solid × roan	3	9	12	12	1	–	–	–
Standard × standard	–	5	56	5	4	–	–	–
Standard × roan	–	1	22	23	3	1	–	–
Standard × point	–	–	1	–	1	–	–	–
Standard × DEW	–	–	1	1	–	–	–	–
Standard × albinos	–	–	2	–	–	–	–	1
Roan × roan	–	–	5	4	–	–	–	–
Roan × point	–	–	5	–	1	–	–	–
Roan × striped	–	–	–	5	–	–	–	–
Point × striped	–	–	–	–	–	–	1	–

* Point – also Siamese

Eight concentration patterns in ferrets can be distinguished, which results in 36 possible mating combinations. The table presents 17 mating combinations for concentration patterns that occurred in the study. The albino and DEW variations were counted in all three categories because it is impossible to determine the concentration pattern or occurrence of white markings in a uniformly white animal

Of the five patterns, the standard one clearly dominated to the others, closely followed by the roan pattern. The solid pattern was placed third in the dominance series, followed by the self pattern. The point pattern was the most recessive. Situations in which the concentration pattern could not be determined were negligible in the population studied.

The wild type coat colour is dominating to the other colour variations. This was confirmed by the results related to the standard concentration pattern in ferrets, which gives a coat similar to that of a European polecat (JEŻEWSKA and MACIEJOWSKI 1989, BEDNARZ and FRINDT 1991). The roan pattern in ferrets is phenotypically similar to the roan pattern in horses,

dogs, and mice. This pattern manifests phenotypically as a mixture of white and coloured hair, in dark-coloured animals giving the salt-and-pepper effect. In addition, like in horses, in ferrets the roan pattern appears for all basic colours, most visible being for dark coat colours (THIRUVENKADAN et al. 2008, WEBB and CULLEN 2010, CIESLAK et al. 2011). The relatively frequent occurrence of the roan pattern – which occurs in neither the wild ferret (polecat) nor polecats bred for fur – may indicate the dominant character of the mutation responsible for the roan pattern. Ferrets in the point (Siamese) pattern are similar in colour to Himalayan mutations in cats (LYONS et al. 2005, SCHMIDT-KÜNTZEL et al. 2005). In mice, rats, guinea pigs, rabbits, and American mink, we can observe a similar phenotype, for which the recessive mutation is responsible (BENKEL et al. 2009, CIESLAK et al. 2011). Due to its recessive nature, the Himalayan mutation is relatively rare if lines of animals of this type are not bred, like in the case of Siamese cats (including Thai, Tonki, and Burmese). In the amateur breeding of ferrets, the point pattern is an unpopular colour variation, additionally decreasing the small number of these animals.

White markings

Twelve out of 35 possible mating combinations of white markings were detected in the population studied (Table 3). The ratio of the number of offspring with no white markings to those having them (two animals with uniformly white coats were classified as having white markings) was 1.5:1.2. In the over half of the mating combinations, at least one of the parents did not have white markings, making it impossible to determine whether in ferrets the presence of white markings is recessive to their lack. In other mammal species, white markings are either recessive (e.g., in mice, rats, and dogs) (WEBB and CULLEN 2010, STRAIN 2011) or – in fewer species – dominant (e.g., in cats and horses) (COOPER et al. 2005, CIESLAK et al. 2011).

Table 3

The distribution of eight white markings for various matings (twelve combinations)

Specification	n.p.*	Milk	Mitt	Blaze	Panda	Striped	DEW	Albinos
N.p. × n.p.	65	2	13	1	–	–	–	–
N.p. × mitt	54	–	64	–	–	–	–	–
N.p. × blaze	1	–	–	–	–	–	–	–
N.p. × panda	2	–	1	1	–	–	–	–
N.p. × striped	13	–	4	–	–	–	1	–
N.p. × DEW	–	–	1	2	–	–	–	–

cont. Table 3

N.p. × albinos	1	–	–	–	–	–	–	1
Mitt × mitt	7	–	22	–	–	–	–	–
Mitt z blaze	1	–	1	–	–	1	–	–
Mitt × panda	–	–	1	1	–	–	–	–
Mitt × striped	–	–	7	–	–	–	–	–
Mitt × albinos	1	–	–	–	–	–	–	–

* N.p. – no pattern (no white markings)

Eight concentration patterns in ferrets can be distinguished, which results in 35 possible mating combinations. The table presents twelve mating combinations for white markings that occurred in the study. The albino and DEW variations were counted in all three categories because it is impossible to determine the concentration pattern or occurrence of white markings in a uniformly white animal

There was a clear dominance of mitts to the other four variations of white markings. The frequencies of ferrets with milk, blaze, panda, and striped white markings were similar, making it impossible to determine which type of white markings followed the mitt variation in the dominance series and which was the most recessive. A low number of ferrets with white markings on their heads (blaze, panda, and striped) is undoubtedly correlated with the large share of deaf ferrets among animals with such white markings. Such a correlation was also found in horses, dogs (for over 90 breeds), cats, pigs, and cattle. In Jack Russell Terriers, this correlation is strong (WEBB and CULLEN 2010, CIESLAK et al. 2011, STRAIN 2011). Similar results were reported in ferrets. Strong correlation between white markings and congenital deafness was observed in the research carried out on 152 ferrets in 2008–2012 at the Veterinary Hospital and Speciality Center of Frégis and Veterinary Clinic ADVETIA in France, regarding the occurrence of congenital deafness undergoing brain-stem auditory evoked response (PIAZZA et al. 2014). Therefore, breeders should avoid pairing two ferrets with white markings on their heads as well as two uniformly white ferrets. Despite deafness that uniformly white ferrets and those with white markings are likely to suffer from, some ferretries breed them because of their attractive appearance and the resulting interest of possible buyers. In addition, unlike in dog and cat breeding, a lack of compulsory hearing tests for individuals in the risk group (blaze, panda, Dark Eyed White, and albino variations) additionally decreases the breeding value of such ferrets.

Summarizing results obtained in our study, darker coat colours in ferrets dominate over the lighter ones. It was impossible to determine which of the three most prevalent coat colours is the most dominant one due to their similar distribution in the database. When analysing the concentra-

tion pattern the standard one proved to be the most frequent one (138 animals), followed by roan (62 animals). It was impossible to determine whether white markings in ferrets are a dominant or recessive trait due to close ratio of ferrets with and without such markings on their body (1.2:1.5) present in the database.

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References

American Ferret Association 2017. *AFA's Breed Standard*. IOP Publishing Physics Web, https://www.ferret.org/read/ferretBreeding.html, access: 1.12.2017.

Associazione Italiana Furetti – Furettomania ONLUS 2016. *Guida Ai Concorsi Italiani*. IOP Publishing Physics Web, https://www.furettomania.it/eventi-e-gallery/guida-ai-concorsi-fm/, access: 1.12 2016.

Ata P., Majewski S. 2013. *Mechanisms of skin pigmentation*. Przegl. Dermatol., 100: 184–188.

Bednarz M., Frindt A. 1991. *Breeding of polecats* (Hodowla tchórzy). PWRiL, Warsaw.

Benkel B.F., Rouvinen-Watt K., Farid H., Anistoroaei R. 2009. *Molecular characterization of the Himalayan mink*. Mamm Genome 20:256–259, https://doi.org/10.1007/s00335-009-9177-6.

Bennet D.C., Lamoreux L.M. 2003. *The color loci of mice. A genetic century*. Pigment Cell Res, 16: 333–344, https://doi.org/10.1034/j.1600-0749.2003.00067.x.

Blaszczyk W.M., Distler C., Dekomien G., Arning L., Hoffmann K.P., Epplen J.T. 2007. *Identification of a tyrosinase (TYR) exon 4 deletion in albino ferrets (Mustela putorius furo)*. Anim. Genet., 38: 421–423, https://doi.org/10.1111/j.1365-2052.2007.01619.x.

Cieslak M., Reissmann M., Hofreiter M., Ludwig A. 2011. *Colours of domestication*. Biol. Rev., 86: 885–899. https://doi.org/10.1111/j.1469-185x.2011.00177.x.

Cooper M.P., Fretwell N., Bailey J.S., Lyons L.A. 2005. *White spotting in the domestic cat (Felis catus) maps near KIT on feline chromosome B1*. Anim Genet 37: 163–165, https://doi.org/10.1111/j.1365-2052.2005.01389.x.

Feritage. 2017. Ferret Database System. IOP Publishing PhysicsWeb, http://www.feritage.no/hoved, access: 1.12 2017.

Grabolus D., Wacławik P., Zatoń-Dobrowolska M. 2019. *Genetic basis of coat colour inheritance in ferrets (Mustela putorius furo). Pedigree analysis*. Acta Sci. Pol. Zootechnica, 18(3): 27–34, https://doi.org/10.21005/asp.2019.18.3.05.

Hoekstra H.E. 2006. *Genetics, development and evolution of adaptive pigmentation in vertebrates*. Heredity, 97: 222–234, https://doi.org/10.1038/sj.hdy.6800861.

Jeżewska G., Maciejowski J. 1989. *Breeding and production of fur animals* (Hodowla i produkcja zwierząt futerkowych). Wyd AR, Lublin.

Kuźniewicz J., Filistowicz A. 1999. *Breeding and husbandry of fur animals* (Chów i hodowla zwierząt futerkowych). Wyd AR, Wroclaw.

Lewington J.H. 2007. *Ferret Husbandry, Medicine and Surgery,* 2nd ed. Elsevier Limited, London.

Lyons L.A., Imes D.L., Rah H.C., Grahn R.A. 2005. *Tyrosinase mutations associated with Siamese and Burmese patterns in the domestic cat (Felis catus)*. Anim Genet, 36(2): 119–126, https://doi.org/10.1111/j.1365-2052.2005.01253.x.

Nes N., Einarsson E.J., Lohi O., Jarosz S.J. 1988. *Beautiful fur animals and their colour genetics*. Scientifur, Glostrup.

Piazza S., Abitbol M., Gnirs K., Huynh M., Cauzinille L. 2014. *Prevalence of deafness and association with coat variations in client-owned ferrets*. J. Am. Vet. Med. Assoc., 9(244):1047–1057, https://doi.org/10.2460/javma.244.9.1047, access: 1.12.2019.

Rzepka Z., Buszman E., Beberok A., Wrześniok D. 2016. *From tyrosine to melanin: Signaling pathways and factors regulating melanogenesis.* Postępy Hig. Med. Dosw., 70: 695–700.

Ruvinsky A., Sampson J. 2001. *The genetics of the dog.* CABI Publ., New York.

Schmidt-Küntzel A., Eizirik E., O'brien S.J., Menotti-Raymond M. 2005. *Tyrosinase and tyrosinase related protein 1 alleles specify domestic cat coat color phenotypes of the albino and brown Loci.* J. Hered., 96(4): 289–301, https://doi.org/10.1093/jhered/esi066.

Shackelford R.M. 1948. *The nature of coat color differences in mink and foxes.* Genetics, 33: 311–336.

Strain G. 2011. *White noise. Pigment-associated deafness,* Vet. J., 188: 247–249, https://doi.org/10.1016/j.tvjl.2010.08.015.

Thiruvenkadan A.K., Kandasamy N., Panneerselvam S. 2008. *Coat colour inheritance in horses.* Livest Sci., 117: 109–129.

Webb A.A., Cullen C.L. 2010. *Coat color and coat color pattern-related neurologic and neuroophthalmic diseases.* Can. Vet. J., 51(6): 653–657.

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GROWTH PARAMETERS, ECONOMIC ANALYSIS AND BLOOD CHARACTERISTICS OF WEANED PIGS FED CASHEW REJECT KERNEL MEAL

***Taiwo Ojediran*[1], *Opeyemi Oyebamiji*[2], *Evelyn Areo*[3], *Isiak Emiola*[4]**

[1] ORCID: 0000-0003-1355-200X
[1–4] Department of Animal Nutrition and Biotechnology
Ladoke Akintola University of Technology, Ogbomoso, Nigeria

Key words: cashew, economy, blood parameters, performance, weaned pigs.

Abstract

Growth parameters, economic analysis and blood characteristics of weaned pigs fed graded levels of cashew reject kernel meal (CRKM) were studied in a 42 day trial. Forty (40) weaned pigs with mean weight of 8.67±0.99 kg were randomly assigned into four experimental diets such that Diets 1, 2, 3 and 4 contained 0%, 5%, 10% and 15% CRKM respectively. The chemical profile of CRKM showed 21.10% crude protein, 35.09% ether extract, 9.20% moisture, 6.83% crude fibre, 4.10% ash, 23.67% nitrogen fee extract, 11.69 MJ/kg metabolizable energy, 0.01% tannin, 0.26% saponin, 0.19% phytate, and 19.22 µg/kg ppm aflatoxin. The results showed that feed intake, economic indices, haematological parameters, and serum metabolites like alanine aminotransferase, asparatate aminotransferase, albumin and glucose were significantly influenced ($p < 0.05$). Conclusively, incorporation of up to 15% CRKM in weaned pigs' diet favour growth performance, lowered feed cost, improved economic advantages and was not deleterious to the haematological and serum metabolites.

Introduction

The declining convectional feedstuff production is aggravated by the rising threat of climate change accompanied with geometric population growth (Li and Kaiser 2011, Coster and Adeoti 2015). Although, crop differs in climatic prerequisite and economic prominence but climate change is affecting productivity. Thus, food/feed security is threatened. For instance, rain starts around March and stabilises in April in south-west, Nigeria but climate has changed and rain does not stabilize until

Address: Taiwo Ojediran, Ladoke Akintola University of Technology, Ogbomoso, Nigeria phone: +234-8034104295, e-mail: tkojediran@lautech.edu.ng

late May. This has affected the production of ephemerals crops like maize to a single cycle per year while other orthodox feedstuffs like soybean and groundnut has been affected resulting in decreased productivity, hike in price coupled with competition between man and livestock. Alternative feedstuffs has to be explored.

Cashew (*Anacardium occidentale*), a tropical and subtropical drought resistant tree crop has received economic attention (KGF 2011), because of the industrial potential of the nut which is now an import-export commodity globally (AKINHANMI et al. 2008) with Cote d'Ivoire being the Africa's leading producer of the nuts (FAO 2015, HEUZE et al. 2017) after Nigeria lost its place in 2010 (ADESANYA et al. 2021). The nut can be separated into the toxic shell and edible kernel (QUIRINO et al. 2014). The nutritional evaluation of the kernel reveals that it contains protein, fat, energy, amino acids, vitamins and minerals.

The cashew nut processing plants are growing in number especially in exporting countries of Africa, Asia and Latin America (AKINHANMI et al. 2008). ALIYU and HAMMED (2008) attributed to the expansion in production to meet local consumption and export. During processing, unsuitable portions of the kernel, unfit for human consumption or export owing to damages are discarded and depending on quality are estimated to be as high as 30% *(*AKANDE et al. 2015) of the total kernel processed. OJEWOLA et al. (2004), FAO (2013) and AKANDE et al. (2015) proved their suitability as an animal feedstuff. These authors have proved its protein efficiency worth (22–38% crude protein), and comparative advantage (lower cost per kg) over soybean meal and groundnut meal in chickens.

The expansion in cashew production, global output and demand from various countries are a proof that the kernels will be available as a feasible livestock feed ingredient (FAO 2013). However, literature on the use of the kernel in swine diet is scanty. Thus, there is research deficit using cashew kernel because deserved awareness has not been given to it. There is therefore need to investigate cashew kernel as another unorthodox feed material for zootechny. This study venture to unveil the potentials of cashew kernel by evaluating the growth parameters, economic analysis, haematological parameters and serum biochemistry of weaned pigs.

Materials and Methods

Location of the experiment

The swine research unit of the Ladoke Akintola University of Technology, Ogbomoso was used for the experiment. Ogbomoso's vegetation is a derived savannah zone which lies on 4º15'E and 8º07'N with an average yearly temperature of around 27ºC.

Animals

Forty (40) weaned Large white-Landrace cross-bred uncastrated male piglets were acclimatized for 7 days before the start of this study. They were eight (8) weeks old with and an average initial weight of 8.67±0.33 kg at the start of the trial. They were randomly divided into four dietary groups of 10 replicates each while each pig served as a replicate. The animals had access to feed and water *ad-libitum*. The trial lasted for 42 days in a open type house with pens. The weaner pigs were farrowed by 6 Large white sows serviced by the same Landrace boar. They were chosen based on sex and weight. The pigs were handled and managed following the NIH Guide for the Care and Use of Laboratory Animals NIH publication No 86-23, revised 1985 and 1991) and the ethical requirements of the United Kingdom for animal experimentation (*Animals Scientific Procedures*, Act 1986).

Preparation of test ingredient and experiment diet

The cashew reject kernel meals were procured from a reputable processing firm. During processing, kernels unsuitable for human utilization or falling below market standards are termed as reject and were procured for this study. After extraneous materials removal, they were milled prior to mixing with other feedstuffs. Four diets were compounded isonitrogeneously (20% CP) and metabolizable energy ranging from 11.44 to 12.15 MJ/kg ME (Table 1). Groundnut meal was replaced with Cashew reject kernel meal in diet 1 (0.00% CRKM) (w/w) at 5.00%, 10.00% and 15.00% in the Diets 2–4 respectively. Other ingredients were varied in an attempt to formulate iso-nitrogenous diets.

Table 1

Formulation of the experimental diets

Ingredients [%]	Diet 1	Diet 2	Diet 3	Diet 4
Maize	21.00	18.00	12.00	2.00
Soya bean meal	1.00	4.00	7.50	10.50
Groundnut cake	15.00	10.00	5.00	0.00
CRKM	0.00	5.00	10.00	15.00
Palm kernel cake	50.00	50.00	50.00	50.00
Corn bran	11.50	11.50	14.00	21.00
Limestone	1.00	1.00	1.00	1.00
#*Premix blend	0.25	0.25	0.25	0.25
Salt	0.25	0.25	0.25	0.25
Total	100.00	100.00	100.00	100.00
Calculated nutrients				
ME [MJ/kg]	11.44	11.84	12.10	12.15
Crude protein	19.58	19.54	19.69	19.72
Ether extract	5.09	6.17	8.42	10.02
Crude fibre	8.26	8.14	8.22	8.59
Calcium	0.49	0.54	0.52	0.64
Lysine	0.70	0.90	1.12	1.33
Methionine	0.33	0.39	0.45	0.51

CRKM – Cashew reject kernel meal, ME – metabolizable energy

supplied the following (per kg feed): vitamin A, 12 500 IU; vitamin D_3, 5 000 IU; vitamin E, 40 mg; vitamin K_3, 2 mg; vitamin B_1, 3 mg; vitamin B_2, 5.5 mg; niacin, 55 mg; calcium pantothenate, 11.5 mg; vitamin B_6, 5 mg; vitamin B_{12}, 25 mg; folic acid, 1 mg; biotin, 50 mg; choline chloride, 500 mg; manganese, 300 mg; iron, 120 mg; zinc, 80 mg; copper, 85 mg; iodine, 1.5 mg; cobalt, 3 mg; selenium, 1.2 mg; anti-oxidant, 120 mg; with (in 1.25 kg/250 kg ration) detoxyzyme, 125 g; superliv, 125 g; prebiotics and probiotics, 62.5 g; herbo-methionine, 125 g and limestone (carrier), 187.5 g

Data collection

Growth parameters and economic analysis

Data taken on growth parameters include feed consumed, weight differences and conversion ratio. Feed left on daily basis was deducted from that given as the intake while weight change was recorded weekly. The relationship between feed intake and weight gain was estimated as feed conversion ratio. Economic analysis was estimated as enumerated by OJEDIRAN et al. (2020a).

Blood parameter

Four pigs per dietary treatment group were randomly picked for blood examination. They were bleed prior to feeding: About 5 ml of blood was each siphoned into pre-labelled tubes for haematological and serum exam-

ination through the jugular vein puncture method using sterilized needles and syringes. Blood portions for haematology determination were emptied into vacutainer tubes having anti-coagulant, ethylene diamine tetra-acetic acid (EDTA) and gently rocked. Haematological parameters like leucocyte (WBC), erythrocyte (RBC), haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and lymphocyte counts were determined.

Serum metabolites determined include the aminotransferases; Alanine (ALT) and Aspartate (AST), Total protein, Albumin, Globuin, creatinine, Urea, Glucose, Triglycerol, Cholesterol including both High density (HDL) and Low density lipoproteins (LDL). Haematocrit and hemoglobin were ascertained by mirco-hematocrit and cyanmethehemoglobn methods correspondingly (MITRUKA and RAWNSLEY 1977). White blood cell and RBC were estimated employing improved neubauer haemacytometer following apt solvent addition (SCHALM et al. 1975). Mean corpuscular volume, MCV and MCHC were estimated according to the procedure of JAIN (1986). LDL was calculated from the Friedewald model (LDL = cholesterol – tryglycerol/5). The cholesterol, triglycerol and HDL were analyzed according to the procedure of ROSCHLAN et al. (1974). The spectrophotometric method of SCHMIDT (1963) was used to evaluate for ALT, AST and Alanine Phosphatase (ALP). Biuret and Bromocresol green methods explained by (PETER et al. 1982) was used to determine serum protein and albumin respectively while globulin was estimated as the difference between the total serum protein and albumin.

Chemical and statistical analysis

The proximate component of the test ingredient (CRKM) was determined using AOAC (2012). The metabolisable energy (ME) content was determined using formula ME:

$$\text{Kcal/kg} = 37 \cdot \%\text{CP} + 81.1 \cdot \%\text{Fat} + 35 \cdot \%\text{NFE}$$ (PAUZENGA 1985)

before being expressed in MJ. The concentration of aflatoxin was determined using OPADOKUN (1999) procedure.

Data were analyzed (Analysis of variance, ANOVA) in a completely randomized design using SPSS (2006) package and means were separated using Duncan multiple range test (at 5% level) of the same package.

Results

The chemical constituents of CRKM is presented in Table 2. The CRKM had 9.20% moisture, 21.10% CP, 35.09% ether extract, 9.20%, 6.83% crude fibre, 4.10% ash and 23.67% nitrogen fee extract. It had 18.96 MJ/kg ME, 0.01% tannin, 0.26% saponin, 0.19% phytate, and 19.22 µg/kg ppm aflatoxin.

Table 2

Nutritional and anti-nutritional composition of cashew reject kernel meal

Chemical composition [%]	Percentage composition [%]
Moisture	9.20
Crude protein	21.10
Ether extract	35.09
Crude fibre	6.83
Ash	4.10
Nitrogen free extract	23.67
Metabolizable energy [MJ/kg]	18.96
Tannin	0.01
Saponin	0.26
Phytate	0.19
Aflatoxin [µg/kg ppm]	19.22

The growth parameters of pigs fed CRKM is shown on Table 3. Only the feed intake parameters were significantly affected ($p < 0.05$). The total feed intake increased in pigs fed diets 1 (32.23 kg), 4 (36.81 kg) 2 (37.84 kg), and 3 (39.68 kg in that pattern). The least feed intake was observed in pigs given diet 1 while those offered diet 3 had the highest. Both total and average daily feed intake had similar pattern. The feed intake was higher in pigs offered CRKM.

Table 3

Growth parameters of pigs fed cashew reject kernel meal

Parameter [kg]	Diet 1	Diet 2	Diet 3	Diet 4	SEM	*P*-value
Initial weight	8.76	8.60	8.68	8.64	0.33	0.99
Final weight	19.90	22.88	24.02	23.84	1.03	0.49
Total weight gain	11.14	14.28	15.34	15.20	0.76	0.18
Av. daily gain	0.27	0.34	0.37	0.36	0.18	0.16
Total feed intake	32.23^{d}	37.84^{b}	39.68^{a}	36.81^{c}	0.63	0.00
ADFI	0.77^{d}	0.90^{b}	0.94^{a}	0.88^{c}	0.01	0.00
FCR	3.24	2.70	2.63	2.54	0.16	0.44

ab – means with varying superscripts along the same row are significant ($p < 0.05$); SEM – standard error of the mean; Av. – Average; ADFI – daily feed intake; FCR – feed conversion ratio

Economic indices of pig fed cashew reject kernel meal is presented in Table 4. All parameters including feed cost per kg (FC/kg), FC/kg weight gain (WG), income per kg WG, profit per kg WG and economic efficiency of gains were influenced ($p < 0.05$). A linear decrease was observed in the feed cost from diet 1 (₦74.28) – diet 4 (₦57.85). The FC/kg WG was highest in those given diet 1 and least for those on diet 4. No significant difference was observed in those offered diets 3 and 4 ($p > 0.05$) unlike those fed the control ($p < 0.05$) while those on diet 2 were comparable. The income/kg WG observed in pigs fed diets 2–4 were similar ($p > 0.05$) but differ significantly from those fed diet 1. The income per kg weight gain followed the same trend as observed in the income per kg weight gain. A linear increase was observed in the economic efficiency of gain as the CRKM increased with those fed diet 5 having the highest value.

Table 4

Economic indices of pig fed cashew reject kernel meal

Parameter (₦)	Diet 1	Diet 2	Diet 3	Diet 4	SEM	*P*-value
Feed cost per kg	74.28^{a}	69.19^{b}	64.34^{c}	57.85^{d}	1.55	0.00
Feed cost per kg WG	238.02^{a}	186.82^{ab}	168.65^{b}	146.20^{b}	12.46	0.04
Income per kg WG	1100.8^{a}	962.76^{b}	939.66^{b}	942.63^{b}	18.95	0.00
Profit per kg WG	862.77^{a}	775.93^{b}	771.01^{b}	796.44^{b}	11.87	0.01
Economic efficiency of gain	395.05^{b}	423.94^{b}	462.75^{ab}	570.52^{a}	26.05	0.01

ab – means with varying superscripts along the same row are significant ($p < 0.05$); ₦ – Nigerian naira; WG – weight gain

Table 5 shows the hematological parameters of pigs fed cashew reject kernel meal. All parameters differ significantly ($p < 0.05$) except white blood cell count. RBC of pigs given diets 2 and 3 were similar ($p > 0.05$) but were differ from those offered diet 4 ($p < 0.05$) and were comparable to those fed diet 1. Hemoglobin values obtained ranged from 9.70 (diet 3) – 11.45g/dl (diet 2) ($p < 0.05$). Haematocrit values for pigs offered diets 2 and 4 were higher and not different ($p > 0.05$) but differs significantly ($p < 0.05$) compared with those offered diet 3. However, those offered control diet was comparable. Mean corpuscular volume were 56.00, 60.20, 54.75 and 53.55 fl for pigs fed diets 1–4 respectively such that pigs fed diets 2 and 4 were significantly different ($p < 0.05$) while those fed diet 3 were comparable to with those fed diets 1 and 4. Mean Corpuscular Haemoglobin values of pigs fed with diet 3 and 4 were similar ($p > 0.05$) while others varied significantly ($p < 0.05$). Mean Corpuscular Haemoglobin Concentration of pigs fed with diet 1 and 2 did not differ significantly ($p > 0.05$) as with pigs fed diet 3 and 4. However, both pairs differ significantly ($p < 0.05$). The lymphocyte counts showed that pigs fed diets 1 and 3 differ significantly ($p < 0.05$) while others were comparable.

Table 5

Haematological parameters of pigs fed cashew reject kernel meal

Parameters	Diet 1	Diet 2	Diet 3	Diet 4	SEM	*P*-value
White blood cell [·10^3/μL]	12.85	13.10	15.25	13.30	0.41	0.13
Red blood cell [·10^3/μL]	7.66^{ab}	7.31^{b}	7.41^{b}	8.16^{a}	0.13	0.03
Haemoglobin [g/dL]	10.90^{ab}	11.45^{a}	9.70^{c}	10.55^{b}	0.21	0.00
Haematocrit [%]	42.85^{ab}	44.00^{a}	40.50^{b}	43.65^{a}	0.54	0.05
Mean corpuscular volume [fL]	56.00^{b}	60.20^{a}	54.75^{bc}	53.55^{c}	0.79	0.00
MCH [pg]	14.25^{b}	15.70^{a}	13.10^{c}	12.90^{c}	0.35	0.00
MCHC [g/dL]	25.45^{a}	26.05^{a}	23.95^{b}	24.15^{b}	0.31	0.01
Lymphocytes [g/L]	7.05^{b}	7.65^{ab}	9.45^{a}	8.40^{ab}	0.40	0.16

ab – means with varying superscripts along the same row are significant ($p < 0.05$); MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular haemoglobin concentration

Table 6 showed the Serum metabolites of pigs fed cashew reject kernel meal. Values obtained for alanine aminotransferase, Asparatate aminotransferase, Albumin and glucose differs significantly ($p < 0.05$).

Table 6

Serum metabolites of pigs fed cashew reject kernel meal

Parameters	Diet 1	Diet 2	Diet 3	Diet 4	SEM	*P*-value
ALT [U/L]	59.60^{b}	83.28^{a}	94.23^{a}	80.36^{a}	4.53	0.02
AST [U/L]	121.32^{ab}	131.18^{a}	92.34^{b}	109.21^{ab}	6.13	0.11
ALP [U/L]	59.10^{a}	48.32^{b}	45.82^{b}	54.36^{ab}	1.94	0.03
Total protein [g/l]	5.25	6.25	5.59	5.15	0.20	0.21
Albumin [g/l]	2.38^{b}	3.08^{a}	2.61^{b}	2.49^{I}	0.10	0.02
Globulin [g/l]	2.87	3.17	2.98	2.67	0.14	0.68
Creatinine [mmol/L]	1.51	1.51	1.03	1.23	0.09	0.13
Urea [mmol/L]	3.74	4.19	3.35	5.85	0.44	0.21
Glucose [mg/dl]	99.84^{a}	121.70^{a}	111.58^{a}	87.39^{b}	11.59	0.02
Cholesterol [mg/dl]	166.99	154.37	156.96	150.49	3.85	0.53
Triglycerides [mg/dl]	42.11	50.20	38.46	36.84	3.07	0.47
HDL [mg/dl]	37.80	27.52	35.06	31.55	1.77	0.19
LDL [mg/dl]	120.76	116.81	114.20	111.56	2.79	0.74

ab – means with varying superscripts along the same row are significant ($p < 0.05$);
ALT – alanine aminotransferase; AST – aspartate aminotransferase; ALP – alkaline phosphatase; HDL – high density lipoprotein; LDL – low density lipoprotein

Values recorded for alanine aminotransferase were higher, 80.36–94.23 U/L compared with those offered the control diet (59.60 U/L) ($p < 0.05$). Asparatate aminotransferase in pigs given diet 2 (131.18 U/L) and 3 (92.34 U/L) differ significantly ($p < 0.05$) from each other although, others compared favourably. Albumin in the serum of pigs fed diet 2 were significantly different ($p < 0.05$) from those fed other diets. Alkaline phosphate in pigs offered diet 1 (59.10 U/L) differ significantly ($p < 0.05$) compared to those on 2 (48.32 U/L) and 3 (45.82 U/L). Significant low glucose value was obtained for pigs given diet 4 ($p < 0.05$).

Discussion

The chemical composition of CRKM affirms it moderate protein content (AKANDE et al. 2015). The ether extract content showed it can be classified as an oil seed like *Jatropha curcas* kernel (OJEDIRAN et al. 2014). ODDOYE et al. (2012) observed a high fibre content of 27.50% than reported in this study. This can be linked to processing efficiency. The ash content and nitrogen free extract recorded is like that observed by AKANDE et al. (2015). The metabolizable energy in CRKM is similar to 19.89 MJ/kg for groundnut cake as observed by AKANDE et al. (2015). Tannin interfere with protein digestion, saponnin interfers with taste while phytate reduces availability of mineral bioavailablity in monogastric animals. However, the level of these anti nutrients were tolerable not to elicit negative response (FDA 2011, AQUILINA et al. 2014). The processing methods employed in the processing of CRKM since determined for human consumption may have played a role in the low tannin, saponin, phytate and the aflatoxin content. However, AKANDE et al. (2015) cautioned that long storage may raise the content of aflatoxin.

Observed growth results are similar to that of OJEDIRAN et al. (2020a) where supplemented palm kernel diet was assessed. LI and PATIENCE (2017) attributed such changes in intake to feed factors like energy concentration amidst other non-dietary factors. HENRY (1985) had earlier asserted that energy density determines feed intake. This was corroborated by ORESANYA et al. (2007, 2008) and BLACK et al. (2009) established that apart from reduced intestinal motility caused by dietary fat content, increased fatty acyl-CoA in the brain hypothalamus results in changes in hormone thus decreased feed consumption. The voluntary feed intake peaked at 10% CRKM inclusion in the feed. The ether extract content of the feed may have played a role rather than to the metabolizable energy. OJEDIRAN et al. (2020b) reported a non-significant weight changes and feed conversion similar to this study and affirmed that enzyme supplementation in a PKC based diet may not be necessary. This established that weaned pigs may well tolerate PKC based (50–55%) diets with adequate energy and protein.

Economic indicators studied showed the advantages in the use of CRKM over the control diet. Consensually, inclusion of non-conventional feedstuff or ingredients in diet formulation lowered feed cost (DONKOH et al. 2004, OJEWOLA et al. 2004). CHOI et al. (2015) established that for increased profitability, unconventional feedstuffs are needed to lower the production cost which accounts for 64.8–75.2% in a pig enterprise. The cost of convectional feed ingredients like maize, a food crop and biofuel

feedstock (De Gorter et al. 2013) is hiked because of low production in the face of population growth. The feed cost in relation to weight increment and reduced income and profit in pigs fed diets 2–4 is attributable to the feed consumed and conversion ratio. Satisfactory performance from lowered feed cost in economic terms is of utmost importance (Adesehinwa 2009) as demonstrated by the economic efficiency of gain as the use of CRKM increased. This revealed that incorporating cashew reject kernel meal in the diets of pig up to 15% bears a relevant and practical use for a commercial pig production.

Hematological parameters associates blood and its producing organs (Waugh and Cirant 2001), thus signals the functional condition and health state of the livestock (Eze et al. 2000). Blood haematological constituents are responsive to the quantity and quality of feed or level of anti-nutrients inherent in the feed (Ani et al. 2013, Nse Abasi et al. 2014). The results on haematological parameters showed that the values reported were of range for healthy pigs (Gianotti et al. 2010, Coronado 2014). Pigs with normal blood parameter values are believed to display good performance (Buzzard et al. 2013). But, Dlamini et al. (2017) said lower haematological values could be due to malnutrition resulting in to anaemia. In this case the animals were not anaemic. White blood cells are critical in the defensive mechanism of pigs (Nse Abasi et al. 2014, Ojediran et al. 2020). However, CRKM exert no adverse effect on the immune system of the pigs. Recorded numerical quantities for RBC, Hb, PCV, and the corpusculars indicated that the pigs were not anaemic as reported by Ojediran et al. (2020b) who fed pigs with biscuit dough and attributed such to the fact that the dough since meant for human consumption had no or tolerable anti-nutrients. For heametocrit values above 30% indicate adequate blood ion status (Perri et al. 2016). Therefore, since erythropoiesis of the RBC was not impaired, thus oxygen carrying capacity in the animals showed that the pigs were in good health. Lymphocyte values were within normal range for pigs and such indicative of antibody function (Dlamini et al. 2017).

Pig blood metabolites can be affected by nutrition (Etim et al. 2014). Transaminases, ALT and AST are liver serum markers and when they are elevated, they suggest liver impairment (Unigwe et al. 2018). Elevated ALT was observed in pigs fed CRKM. Other organs found to produce ALT are the heart, kidney and muscles but Unigwe et al. 2018) proved that it is more liver specific while AST is a direct indicator of other cell damages including the liver (Rochling 2001). Alanine amino transaminase and AST has been found to be elevated in biliary duct obstruction, medications, diseaseas and fatty liver (steatohepatitis). However, increased mus-

cular activity has been reported to cause increased AST in blood serum and UNIGWE et al. (2018) attributed such in feed with more energy and protein availability. Rise in serum ALP was attributed to cholestatic disease, a situation HYDER et al. (2013) believed that ALP is made mostly in the liver and osteoblasts in hepatic bile duct obstruction. The pattern in the AST and ALP rules out a liver damage because AST falls within normal range for the class of pigs and ALP were not high as in pigs offered the control.

DVORÀK (1986), proved that weight gain and albumin synthesis are interdependent in piglets as influenced by nutritional status. The link between nutrition and albumen level was demonstrated by FUHRMAN et al. (2004) as albumen levels lowers during malnutrition. ELBERS et al. (1992) and CAPRARULO et al. (2020) revealed that in pigs, albumin level is a predictor of growth response. Albumin and insulin synthesis are directly correlated (CHEN et al. 2016) because insulin is connected with muscle protein synthesis (Davis et al. 2010). The observation by CAPRARULO et al. (2020) buttressed the findings among the CRKM fed pigs. CAPRARULO et al. (2020) attributed such to values within physiological range (ELBERS et al. 1992)) thus positively affected the weight gain. Reduced glucose with increased CRKM level could be associated with fibre level in the feed as noted by ADESEHINWA (2007). However, ether extract and nitrogen free extract may have played a role. MANELL et al. (2016), DAVIE (2003), CHRISTENSEN et al. (2011) and KAWADA et al. (2017) supposed that butyrate level triggers histone deacetylase inhibition activity in glucose homeostasis or release via pancreatic β-cells.

Observed urea and creatinine levels in this study showed that CRKM did not affected the kidney functions. This is similar to the report of ADESEHINWA (2007). It implied that there were no muscular wastage as the feed was well utilized. The cholesterol, triglycerides, HDL and LDL levels also showed that CRKM was well tolerated by the pigs akin to the result of Hellwing et al. (2007). AKANDE et al. (2015) observed that high ether extract may not translate to high cholesterol or fractions because it is crucial in homeostasis and in series of metabolic cycle. HDL has been identified by PODREZ (2010) and NAVAB et al. (2011) to exhibit and promote cholesterol efflux which causes reverse cholesterol movement by affecting foam cell formation owing to it anti-atherogenic effects. Triglycerides being fatty acids with high energy chains important for fueling cell functions are at high levels believed to cause hearth diseases (YUE et al. 2015). RAUW et al. (2004) proved that a positive relationship between HDL and triglyceride exist in pigs. These relationship may have been responsible for the observation in this study.

Conclusion and recommendation

It could be concluded that weaned pigs could tolerate up to 15% cashew reject kernel meal inclusion because of the performance in terms of growth and economics indices. Also the blood parameter were not deleteriously affected. Further research could look into the use of cashew reject kernel meal in other phases of growth.

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References

ADESANYA K.A., AGBOLA-ADEDOJA M.O., ADELUSI A.A., OGUNWOLU Q.A., UGWU C.A., ALLI M.A., AKINPELU A.O. 2021. *Opportunities in Nigerian cashew nut value chain.* World J. Adv. Res. Rev., 9(1): 168–174.

ADESEHINWA A.O.K. 2007. *Utilization of palm kernel cake as a replacement for maize in diet of growing pigs. Effects on performance, serum metabolites, nutrient digestibility and cost of feed conversion.* Bulg. J. Agric. Sci., 13(5): 593–600.

ADESEHINWA A.O. 2009. *Palm kernel cake supplemented with cassava flour waste as an energy source for pigs.* Rev. Bras. Ciênc. Agrár., 4(4): 479–484.

AKANDE T.O., AKINWUMI A.O., ABEGUNDE T.O. 2015. *Cashew reject meal in diets of laying chickens: nutritional and economic suitability*. J. Anim. Sci. Technol., 57: 1–7.

AKINHANMI T.F., ATASIE V.N., AKINTOKUN P.O. 2008. *Chemical composition and physicochemical properties of cashew nut (Anacardium occidentale) oil and cashew nut shell liquid.* J. Agric. Food. Environ., 2(1): 1–10.

ALIYU O.M., HAMMED L.A. (2008). *Nigerian cashew economy. A review of nut production sector*. Paper Presented at the International Academy of African Business and Development (IAABD) Conference. University of Florida, Gainesville, USA.

ANI A.O., EZEMAGU I.E., UGWUOWO L.C. 2013. *Response of weaner pigs to diets containing graded levels of soybean hull (SBH).* Online J. Anim. Feed Res., 3(3):137–142.

AOAC. 2012. *Official methods of analysis (19th ed). Association of Official Analytical Chemists*, Arlington, VA.

AQUILINA G., BAMPIDIS V., BASTOS M., COSTA L.G., FLACHOWSKY G., GRALAK M.A. 2014. *Scientific opinion on the safety and efficacy of tannic acid when used as feed flavouring for all animal species.* EFSA J., 12(10): 3828.

BLACK J.L., WILLIAMS B.A. GIDLEY M.J. 2009. *Metabolic regulation of feed intake in monogastric mammals.* In: *Voluntary feed intake in pigs*. Eds. D. Torrallardona, E. Roura. Wageningen Academic, Wageningen, The Netherlands, pp. 189–213.

BUZZARD B.L., EDWARDS-CALLAWAY L., ENGLE T., ROZELL T., DRITZ S. 2013. *Evaluation of blood parameters as an early assessment of health status in nursery pigs.* J. Swine Health Prod., 21: 148–151.

CAPRARULO V., HEJNA M., GIROMINI C., LIU Y., DELL'ANNO M., SOTIRA S., REGGI, SGOIFO-ROSSI C., CALLEGARI M., ROSSI L. 2020. *Evaluation of dietary administration of chestnut and quebracho tannins on growth, serum metabolites and fecal parameters of weaned piglets*. Animals, 10(11): 1945.-

CHEN Q., LU M., MONKS B.R., BIRNBAUM M.J. 2016. *Insulin is required to maintain albumin expression by inhibiting forkhead box O1 protein.* J. Biol. Chem., 291: 2371–2378.

CHOI H.B., JEONG J.H., KIM D.H., LEE Y., KWON H., KIM Y.Y. 2015. *Influence of rapeseed meal on growth performance, blood profiles, nutrient digestibility and economic benefit of growing-finishing pigs.* Asian-Australa J. Anim. Sci., 28: 1345–1353.

Christensen D.P., Dahllöf M., Lundh M., Rasmussen D.N. Nielsen M.D., Billestrup N., Mandrup-Poulsen T. 2011. *Histone deacetylase (HDAC) inhibition as a novel treatment for diabetes mellitus*. Mol. Med., 17: 378.

Coronado K. 2014. *Haematological values, pathological services*, www.uac.arizona.edu.pathology.

Coster A.S., Adeoti A.I. 2015. *Economic effects of climate change on maize production and farmers adaptation strategies in Nigeria: A Ricardian approach.* J. Agric. Sci. Arch.*,* 7(5): 67.

Dlamini Z.C., Langa R.L.S., Aiyegoro O.A., Okoh A.I. 2017. *Effects of probiotics on growth performance, blood parameters, and antibody stimulation in piglets.* South African J. Anim. Sci., 47(6): 766–775.

Davie J.R. 2003. *Inhibition of histone deacetylase activity by butyrate.* J. Nutr., 133: 2485S–2493S.

Davis T.A., Suryawan A., Orellana R.A., Fiorotto M.L., Burrin D.G. 2010. *Amino acids and insulin are regulators of muscle protein synthesis in neonatal pigs.* Animal, 4: 1790–1796.

De Gorter H., Drabik D., Just D.R. 2013. *Biofuel policies and food grain commodity prices 2006–2012: All boom and no bust?* AgBioForum, 16(1): 1–13.

Donkoh A., Attoh-Kotoku V., Kwame R.O., Gascar R. 2012. *Evaluation of Nutritional quality of Dried Cashew Nut Testa using Laboratory Rat as a Model for Pigs.* Sci. World J., 984249.

Dvorák M. 1986. *Changes in blood protein levels in piglets during development and during stress.* Vet. Med. Czech, 31: 403–414.

Elbers A.R.W., Counotte G.H.M., Tielen M.J.M. 1992. *Haematological and clinicochemical blood profiles in slaughter pigs: Reference values, between and within herd variability and the relationship with herd factors and growth performance.* Vet. Q., 14: 57–62.

Etim N.N., Offiong E.E., Williams M.E., Asuquo L.E. 2014. *Influence of nutrition on blood parameters of pigs.* Am. J. Biol. Life Sci., 2: 46–52.

Eze J.I., Onunkwo J.I., Shoyinka S.V.O., Chah F.K., Ngene A.A., Okolinta N., Nwanta J.A., Onyenwe I.W. 2010. *Haematological profiles of pigs raised under intensive management system in South-Eastern Nigeria.* Niger. Vet. J., 31 (2):115–123.

Food and Agriculture Organization (FAO) 2013. Food and Agriculture Organization of the United Nations Statistics division, http://faostat3.fao.org/browse/Q/QC/E.

Food and Agriculture Organization (FAO) 2015. *Food and Agriculture Organization of the United Nations*, Rome, Italy, pp: 68–70.

FDA 2011. *Mycotoxin Regulatory Guidance. A guide for grain elevators, feed manufacturers, grain processors and exporters.* 1250 Eye St, N., Suite 1003, Washington, DC: National Grain and Feed Association, https://www.ngfa.org/wp-content/uploads/NGFACompliance Guide-FDARegulatoryGuidanceforMycotoxins8-2011.pdf

Fuhrman M.P., Charney P., Mueller C.M. 2004. *Hepatic proteins and nutrition assessment.* J. Am. Diet. Assoc., 104: 1258–1264.

Gianotti G.C., Beheregaray W.K., Bianchi S.P., Mombach V.S., Carregaro A.B., Contesini E.A. 2010. *Swine in biomedical research: normal physiological values.* Acta Sci. Vet., 38(2): 133–137.

Hellwing A.L.F., Tauson A.H. Skrede A. 2007. *Blood parameters in growing pigs fed increasing levels of bacterial protein meal.* Acta Vet. Scand., 49: 33.

Henry Y. 1985. *Dietary factors involved in feed intake regulation in growing pigs: a review.* Livest. Prod. Sci., 12: 339–354.

Heuzé V., Tran G., Hassoun P., Bastianelli D., Lebas F. 2017. *Cashew (Anacardium occidentale) nuts and by-products.* Feedipedia, a programme by INRA, CIRAD, AFZ and FAO, http://www.feedipedia.org/node/56.

Hyder M.A., Hasan M., Mohieldein A.H. 2013. *Comparative level of ALT, AST, ALP and GGT in liver associated disease.* Eur. J. Exp. Biol., 3(2): 280–284.

Jain N.C. 1986. *Schalms Veterinary Haematology*. 4th Edition. Philadephia, USA. Lea and Febier.

Kawada Y., Asahara S.I., Sugiura Y., Sato A., Furubayashi A., Kawamura M., Koyanagi-K., imura M. 2017. *Histone deacetylase regulates insulin signaling via two pathways in pancreatic β cells.* PLoS ONE, 12, e0184435.

KFG 2011. *Export potentials for high value crops from Bangladesh; An assessment based on global market analysis*, www.kfg.org.bd.../10-project%20code%20.

Li X., Kaiser H. M. 2011. *The impact of climate change on maize yield in the United States and China.* Agric. Syst., 104(4): 348–353.

Li Q., Patience J. F. 2017. *Factors involved in the regulation of feed and energy intake of pigs.* Anim. Feed Sci. Technol., 233: 22–33.

Manell E., Hedenqvist P., Svensson A., Jensen-Waern M. 2016. *Establishment of a refined oral glucose tolerance test in pigs, and assessment of insulin, glucagon and glucagon-like peptide-1 responses.* PLoS ONE, 11: e0148896.

Mitruka B.M., Rawnsley H.M. 1977. *Chemical biochemical and haematological reference values in normal experimental animals.* Masson, New York. Masson publishing, pp. 287–380.

Navab M., Reddy S.T., Van Lenten B.J., Fogelman A.M. 2011. *HDL and cardiovascular disease. Atherogenic and atheroprotective mechanisms.* Nat. Rev. Cardiol., 8: 222.

Nse Abasi N.E., Edem E.A., Offiong M.E., Williams L., Asuquo E. 2014. *Influence of nutrition on blood parameters of pigs.* Am. J. Biol. Life Sci., 2(2): 46–52.

Oddoye E .O. K., Agyente-Badu K., Johnson V. 2012. *Broiler chicks performance on finisher diets containing different levels of cashew kernels.* Agric. Sci. Res. J., 2(4): 154–158.

Ojediran T.K., Adisa Y.A., Yusuf S.A. Emiola I.A. (2014). *Nutritional evaluation of processed jatropha curcas kernel meals. Effect on growth performance of broiler chicks.* J. Anim. Sci. Adv., 4(11): 1110–1121.

Ojediran T.K., Olayiwola S., Adeyeye M., Ajayi A. F., Emiola I.A. (2020a). *Effects of palm kernel meal-based diet with or without enzyme supplementation on growth performance, economic benefits and villi morphometry of weaned pigs.* Pol. J. Nat. Sci., 35(2): 129–139.

Ojediran T.K., Fawamide T., Adeyeye O., Babajide N., Ajayi A.F., Shittu M.D., Emiola I.A. (2020b). *Haematological parameters, organ weight and villi morphometrics of weaner pigs fed biscuit dough.* Pol. J. Nat. Sci., 35(2): 141–150.

Ojewola G.S., Okoye F.C. Agbakuru I. 2004. *Replacement of cashew nut meal for soybean meal in finishing broiler chickens.* Int. J. Poult. Sci., 3: 513–516.

Opadokun T.A. 1999. *The aflatoxin content of locally consume food stuff. Part II. Sorghum*. Ann. Rep., 99: 101.

Oresanya T.F., Beaulieu A.D., Beltranena E., Patience J.F. 2007. *The effect of dietary energy concentration and total lysine/digestible energy ratio on the growth performance of weaned pigs.* Can. J. Anim. Sci., 87: 45–55.

Oresanya T.F., Beaulieu A.D., Patience J.F. 2008. *Investigations of energy metabolism in weanling barrows. The interaction of dietary energy concentration and daily feed (energy) intake.* J. Anim. Sci., 86: 348–363.

Pauzenga U. 1985. *Feeding parent stock.* Zootenica International, pp. 22–24.

Perri A.M., Friendship R.M., Harding J.S.C, O'Sullivan T.L. 2016. *An investigation of iron deficiency and anemia in piglets and the effect of iron status at weaning on post-weaning performance.* J. Swine Health Prod., 24(1): 10–20.

Peters T., Biomont C.T. Doumas B.T. 1982. *Protein (total protein) in serum, urine and cerebrospinal fluid, albumin in serum: In selected methods of clinical chemistry*, volume 9. W.R.

Podrez E.A. 2010. *Anti-oxidant properties of high-density lipoprotein and atherosclerosis.* Clin. Exp. Pharmacol. Physiol., 37: 719–725.

Quirino R.L., Garrison T.F. Kessler M.R. 2014. *Matrices from vegetable oils, cashew nut shell liquid, and other relevant systems for biocomposite applications.* Green Chem., 16: 1700–1715.

Rauw M., Portoles O., Soler J., Reixach J., Prat Cuffi J. M., Diaz I., Gomez-Raya L. 2004. *Preliminary results on the relationship between cholesterol and triglyceride serm levels and bogy weight around weaning in pigs.* Conference. XII Reunion Nacional de Majora Genetica Animal at Arucas, Las Palmas, Gran Canaria. ITEA, 100(3): 284–288.

Rochling F.A. 2001. *Evaluation of abnormal liver tests*. Clinical cornerstone, 3: 1–12.

Roschlan P., Bernet E., Gruber W. 1974. *Enzymaticsche best immugoles gesamt cholesterius in serum.* J. Clin. Chem. Biochem., 12: 403–407.

SCHALM O.W., JANE N.C., CAROL E.J. 1975. *Veterinary heamatology*. 3rd Edition. Lea and Febiger, Philadephia.

SCHMIDT E., SCHMIDT F.W. 1963. *Determine of serum glutamic oxaloacetic and glutamic pyruvic transaminase*. Biological Clinic., 3: 1.

SPSS 2016. *Statistical Package for Social Sciences*. PC version15. SPSS Inc. 444N. Michigan Avenue Chicago, United State of America 2006.

UNIGWE C.R., BALOGUN F.A., ODEYEMI T.A. 2018. *Liver enzymes an histo-morphology of pigs fed fermented and enzyme-supplemented cassava peel meal based diets*. Niger. J. Anim. Sci., 20(3): 106–116.

WAUGH A., GRANT A. 2001. *Ross and Wilson. Anatomy and Physiology in Health and Illness* (9th edn). Churchill Livingstone, London.

YUE L., HAO Z., LI Y., LILI Z., TIAN W. 2015. *Effect of medium-chain triglycerides on growth performance, nutrient digestibility, plasma metabolites and antioxidant capacity in weanling pigs*. Anim. Nutr., 1(1): 12–18.

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OPTIMIZATION OF LACCASE ACTIVITY MEASUREMENT IN A CRUDE OIL POLLUTED SOIL

***Lawrence Edemhanria*[1], *Ruth O. Shafe*[2], *Christopher C. Osubor*[3]**

[1] ORCID: 0000-0002-6034-3780
[1,2] Chemical Sciences Department, Biochemistry Unit
Samuel Adegboyega University, Ogwa, Nigeria
[3] Department of Biochemistry
University of Benin, Benin City, Nigeria

Key words: bioremediation, optimization, polluted soil, microbial oxidative enzymes, laccase activity.

Abstract

The effect of substrate concentration, soil incubation buffer volume and substrate incubation duration on laccase activity in a crude oil polluted soil were evaluated in this study. These parameters were studied over a specified range of values; substrate concentrations: 1, 5, 10, 15, 20 and 25 mM; soil incubation buffer volume: 25, 50, 75, 100 and 125 mL; and incubation duration: 30, 60, 90, 120 and 150 min. Laccase activity was evaluated by spectrophotometric method using pyrogallol as substrate. The optimum values of laccase activity in micromole per hour per gram soil [µmol/h/g] obtained were 2.80, 2.16 and 2.12 for the corresponding optimum substrate concentration (5 mM), soil incubation buffer volume (50 mL) and incubation duration (60 min) respectively. The use of these optimum values in laccase assay will provide a very useful indication of microbial metabolic activities during the process of bioremediation of the polluted soil.

Introduction

Crude oil exploration and other related activities globally over the years have greatly impacted on the environment with its attendant negative effect on the ecosystem (ITE et al. 2013). The exposure of microbial communities in the environment to petroleum hydrocarbon pollution leads to selective enrichment and genetic changes resulting in increased proportion of hydrocarbon degrading microbes including bacteria and fungi (primary degraders) encoding hydrocarbon catabolic genes (LEAHY and

Address: Lawrence Edemhanria, Samuel Adegboyega University, PMB 0001, Ogwa, Nigeria, e-mail: ledemhanria@gmail.com

COLWELL 1990, SAFDARI et al. 2018). These catabolic genes mediate the degradation of petroleum hydrocarbon pollutants in the soil using their respective enzyme systems (SILES and MARGESIN 2018). Most organic pollutants including crude oil are transformed aerobically (ROJO 2009) through several metabolic processes catalysed by various microbial enzymes including oxygenases, reductases, hydroxylases and dehydrogenases (VARJANI and UPASANI 2017, POLYAK et al. 2018). The various hydrocarbon fractions of crude oil have different and diverse degradation pathways controlled by different catabolic genes (WANG and SHAO 2013). The initial attack incorporates oxygen into the organic pollutant followed by stepwise peripheral degradation pathways that convert the petroleum hydrocarbon to tricarboxylic acid cycle (TCA) intermediates (WANG and SHAO 2013). The metabolites formed such as acetyl – CoA, succinate and pyruvate are then used by the microbes to synthesize cell biomass (CHANDRA et al. 2015).

The oxidative enzymes expressed by soil microorganisms have been broadly classified based on the electron acceptor involved in their oxidative process (SINSABAUGH 2010, BACH et al. 2013). The first group utilizes oxygen as electron acceptor while the second uses hydrogen peroxide. In assays of environmental samples, SINSABAUGH (2010) used the generic term phenol oxidase to refer to the activity of several enzymes that require oxygen as electron acceptor including monooxygenases, dioxygenases, tyrosinase, catechol oxidase, laccase etc. Similarly, all hydrogen peroxide requiring oxidative enzymes are assayed as peroxidase. Laccases (benzediol: oxygen oxidoreductase EC 1.10.3.2) are the largest class of phenol oxidase present in the soil (BALDRIAN 2006, SINSABAUGH 2010). They are a group of multicopper enzyme family involved in phenolic compounds oxidation with oxygen as electron acceptor (STREK and TELESINSKI 2015). Extracellular laccases are produced by microorganisms in response to organic pollutants in the soil; plants also produce both intracellular and extracellular laccases used in the synthesis of lignin and other secondary compounds (BALDRIAN 2006, SINSABAUGH 2010). Although, laccases are primarily taken to be fungal enzymes, some bacteria are known to also secrete laccase – like enzymes (BALDRIAN 2006, SINSABAUGH 2010). These bacterial laccases exist as components of larger protein complexes and they exhibit much greater activity in the soil than fungal laccases (BACH et al. 2013). Laccases are more active than peroxidases at beginning of organic pollutant transformation due to their low redox potential (MARTINEZ et al. 2005).

The estimation of the potential oxidative enzymes activity in the soil is done by measuring oxidation rate of a model substrate (BACH et al. 2013,

BURNS et al. 2013). Phenol oxidase (laccase) activity is assayed with the substrate alone in contrast to the addition of hydrogen peroxide for peroxidase activity (BACH et al. 2013). Pyrogallol, L-3,4-dihydroxyphenylalanine (L-DOPA) and 2,2'-azinobis-3-ethylbenzothiazoline-6-sofonic acid (ABTS) are some commonly used model substrates for oxidative enzymes assay in the soil (SINSABAUGH 2010, BACH et al. 2013). Pyrogallol is the most easily oxidized and is also less susceptible to interference by soil minerals (HEWINS et al. 2016). Soil enzyme activities and microbial indices are useful indicators of the recovery of the soil during bioremediation (ALRUMMAN et al. 2015). These indicators provide evidence of the metabolic activities and viability of the microorganisms in the soil (MARGESIN et al. 2000). Laccase was chosen as an indicator of soil microbial activity in this study due to its involvement in the degradation of complex molecules including recalcitrant soil organic matter and carbon cycling (BALDRIAN 2006).

The development of appropriate protocol for the estimation of laccase activity in a crude oil polluted soil is integral to the deployment of an effective bioremediation strategy as it provides a basis to monitor the soil attenuation process. Such protocol must take into consideration factors including substrate concentration, soil incubation buffer volume and duration of substrate incubation that are involved in the enzyme activity measurement. Hence, this work was designed to determine optimum levels of these experimental factors in the estimation of laccase activity in a crude oil contaminated soil.

Materials and Methods

Reagents and equipment

Pyrogallol was purchased from Kermel Chemicals, China. All other chemicals and reagents used were of analytical grade. The following equipment were used in the study: analytical balance (BL – 200S, Setra Systems, USA); centrifuge (800D, Phoenix, USA); constant temperature magnetic stirrer (78 HW – 1, China); pH meter (PHS – 3C, Phillips Scientific, USA); top – loading precision balance (PA512, Ohaus – Pioneer, USA); spectrophotometer (Model 6305, Jenway, England).

Sample collection and preparation

Soil sample was collected from a fallow area without any history of petroleum hydrocarbon pollution in Samuel Adegboyega University, Ogwa, Edo State, Nigeria. The sample was artificially contaminated in the laboratory with crude oil at a concentration of 1% (w/v) in an open container and kept for two weeks. During this period, the contaminated soil was replenished with water and mixed once a week. Thereafter, the soil samples were air-dried for five days and sieved with a 2 mm sieve. Baseline properties of both the contaminated and uncontaminated soil samples were determined using standard analytical procedures (data not included).

Laccase activity assay

Laccase activity was estimated using pyrogallol as substrate (ALLISON and JASTROW 2006). One gram (1 g) crude oil contaminated soil was weighed into a 250 mL conical flask and 50 mL acetate buffer (50 mM, pH 5) was added. The flask was incubated at room temperature for 1 h with vigorous shaking every 20 min. Thereafter the volume of the buffer was increased to 125 mL and the flask shaken vigorously. An aliquot of the soil suspension (10 mL) was centrifuged at 4000 rpm for 10 min to obtain the supernatant used for the enzyme assay. The test experiment contained 2 mL of the supernatant (soil suspension) and l mL substrate (25 mM). This was incubated in the dark at room temperature for 1 h. A sample control containing 2 mL supernatant and 1 mL buffer and also substrate control containing 1 mL substrate and 2 mL buffer were treated as the test experiment. The absorbance was measured at 460 nm using a spectrophotometer. Buffer solution was used as blank. Laccase activity was calculated using Eq. (1).

$$\text{Laccase activity [µmol/h/g]} = \frac{A \cdot \text{V1}}{E \cdot \text{V2} \cdot T \cdot W}$$

where:
A – net absorbance [test – sample control – substrate control]
V1 – volume of buffer used [mL]
E – molar extinction coefficient for pyrogallol – 4.2 per µmol
V2 – volume of soil suspension [mL]
T – substrate incubation time [h]
W – mass of soil sample [g]

Effect of buffer incubation volume on laccase activity

One gram (1 g) contaminated soil sample was weighed into five different 250 mL conical flasks and 25, 50, 75, 100 and 125 mL of 50 mM acetate buffer pH 5 was added to each flask respectively. The flasks were incubated at room temperature for 1 h with vigorous shaking every 20 min. Thereafter the volume of the buffer in each flask was increased to 125 mL by the addition of 100, 75, 50, 25 and 0 mL of 50 mM acetate buffer pH 5 respectively. Laccase activity was then determined after centrifugation of the soil suspension as already described above.

Effect of substrate incubation time on lacccase activity

One gram (1 g) contaminated soil sample was weighed into 250 mL conical flasks and 50 mL of 50 mM acetate buffer (pH 5) was added. The flask was incubated at room temperature for 1 h with vigorous shaking every 20 min. Thereafter the volume of the buffer was increased to 125 mL by the addition of 75 mL of 50 mM acetate buffer (pH 5). Aliquot (10 mL) of the soil suspension was centrifuged at 4000 rpm for 10 minutes. The supernatant was used for laccase activity assay as already described using different substrate incubation duration of 30, 60, 90, 120 and 150 min respectively.

Effect of substrate concentration on laccase activity

One gram (1 g) contaminated soil sample was weighed into 250 mL conical flasks and 50 mL of 50 mM acetate buffer (pH 5) was added. The flask was incubated at room temperature for 1 h with vigorous shaking every 20 min. Thereafter the volume of the buffer was increased to 125 mL by the addition of 75 mL of 50 mM acetate buffer (pH 5). An aliquot (10 mL) of the soil suspension was centrifuged at 4000 rpm for 10 minutes. The supernatant was used for laccase activity assay as already described using different substrate concentrations of 1, 5, 10, 15 and 25 mM respectively.

Data analysis and presentation

All experiments were performed in triplicates. The values were presented as means ± standard error (SE).

Results and Discusion

Laccase catalyse the oxidation of pyrogallol (a phenolic substrate) to quinone. This is autooxidised to dark brown pigment whose absorbance was measured with a spectrophotometer at 460 nm. The colour intensity is proportional to the enzyme activity. Experimental conditions including soil incubation buffer volume, substrate incubation duration and substrate concentration in the enzyme activity assay were studied. The result obtained for the effect of soil incubation buffer volume is presented in Figure 1. The optimum buffer incubation volume was 50 mL with laccase activity of 2.16 µmol/h/g. Microbial oxidative enzymes in soil are usually measured in extracts using appropriate buffer (BALDRIAN 2009). The buffer controls pH and dilute sample (GERMAN et al. 2011). Acetate buffer (50 mM) used in this study is reported in literature to support high recovery of lignolytic enzymes from the soil (BALDRIAN 2009). The use of high buffer volume before sample incubation in the substrate is to reduce the possibility of interference from organic matter present in the soil (BALDRIAN 2009). ALLISON and JASTROW (2006), BACH et al. (2013) and SAIYA-CORK et al. (2002) all reported use of 125 mL acetate buffer (50 mM, pH 5) in assay for laccase in forest soil.

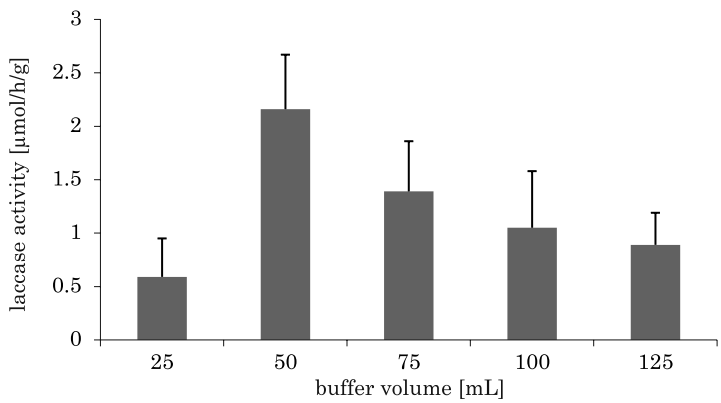

Fig. 1. Effect of buffer incubation volume on laccase activity in crude oil contaminated soil

In the determination of the effect of substrate incubation duration, the various enzyme activities obtained in the study were linear up to 60 min incubation (Figure 2). This time was therefore taken as the optimum incubation duration. Since soil enzyme activity is usually calculated as a rate with assumption of zero order kinetics (GERMAN et al. 2011), it is important to use substrate incubation duration that will produce linear response with time (SAIYA-CORK et al. 2002). ALLISON and JASTROW (2006) used

incubation time of 1 h to improve detection limit; this is in agreement with the optimum substrate incubation time obtained in this study. In contrast, BACH et al. (2013) reported that reaction rates for pyrogallol remained linear over 3–4 h, they however noted that longer incubation times yielded inconsistent results.

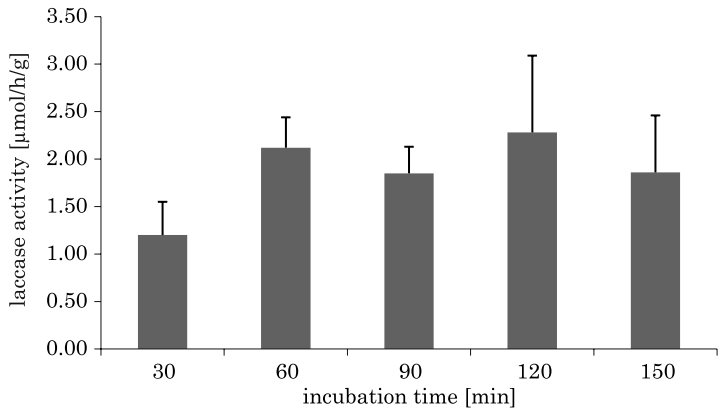

Fig. 2. Effect of substrate incubation time on laccase activity in a crude oil contaminated soil

In this study, the enzyme activity was assayed by monitoring the rate of oxidation of the model substrate pyrogallol over a range of concentrations. Substrate concentration is an important consideration in soil enzyme assays (BALDRIAN 2009, SINSABAUGH 2010).

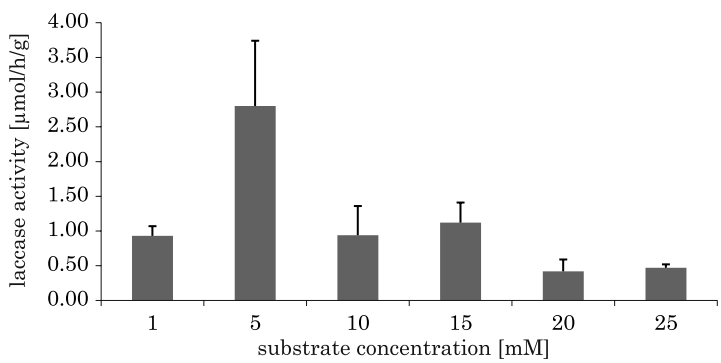

Fig. 3. Effect of substrate concentration on laccase activity in a crude oil contaminated soil

Assays for oxidases that may not follow Michaelis-Menten kinetics may require excess concentration of substrate in the reaction system to allow the enzymatic reaction to proceed at a maximal rate (BALDRIAN 2009). This explains the substrate concentration (25 mM) used in this study. However, the results obtained revealed that the optimum substrate

concentration for maximum enzyme activity during this study was 5 mM (Figure 3). This result is in agreement with the 5 mM Pyrogallol reported by BACH et al. (2013).

Conclusion

Laccase activity was measured in a crude oil contaminated soil using pyrogallol as a model substrate. Experimental conditions including incubation buffer volume, substrate incubation duration and substrate concentration were optimized. The use of these optimum values of the various parameters studied in laccase assay will ensure optimal assay conditions for efficient evaluation of laccase activity in the soil. This will provide a very useful indication of microbial metabolic activities and the viability of the microorganism during the process of bioremediation of crude oil polluted soil.

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References

ALLISON S.D., JASTROW J.D. 2006. *Activities of extracellular enzymes in physically isolated fractions of restored grassland soils*. Soil Biol. Biochem., 38: 3245–3256.

ALRUMMAN S.A., STANDING D.B., PATON G.I. 2015. *Effects of hydrocarbon contamination on soil microbial community and enzyme activity*. J. King Saud Univ. Sci., 27: 31–41.

BACH C.E., WARNOCK D.D., VAN HORN D.J., WEINTRAUB M.N., SINSABAUGH R.L., ALLISON S.D., GERMAN D.P. 2013. *Measuring phenol oxidase and peroxidase activities with pyrogallol, L-DOPA and ABTS: Effect of assay conditions and soil types*. Soil Biol. Biochem., 67: 183–191.

BALDRIAN P. 2009. *Microbial enzyme-catalyzed processes in soils and their analysis*. Plant Soil Environ., 55: 370–378.

BURNS R.G., DEFOREST L., MARXSEN J., SINSABAUGH R.L., STROMBERGER M.E., WALLENSTEIN M., ZOPPINI A. 2013. *Soil enzymes in a changing environment: current knowledge and future directions*. Soil Biol. Biochem., 58: 216–234.

CHANDRA S., SHARMA R., SINGH K., SHARMA A. 2013. *Application of bioremediation technology in the environment contaminated with petroleum hydrocarbon*. Ann. Microbiol., 63: 417–431.

German D.P., Weintraub M.N., Grandy A.S., Lauber C.L., Rinkes Z.L., Allison S.D. 2011. *Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies*. Soil Biol. Biochem., 43: 1387–1397.

Hewins D.B., Chuan X., Bork E.W., Caryle C.N. 2016. *Measuring the effect of freezing on hydrolytic and oxidative extracellular enzyme activities associated with plant litter decomposition*. Pedobiologia, 59: 253–256.

Ite A.E., Ibok U.J., Ite M.U., Peters S.W. 2013. *Petroleum exploration and production. Past and present environmental issues in Nigeria's Niger Delta*. Am. J. Environ. Prot., 1: 78–90.

Leahy J.G., Colwell R.R. 1990. *Microbial degradation of hydrocarbons in the environment*. Microbiol. Mol. Biol. Rev., 54: 305–315.

Margesin R., Zimmerbauer A., Schinner F. 2000. *Monitoring of bioremediation by soil biological activities*. Chemosphere, 40: 339–346.

Martinez A.T., Speranza M., Ruiz-Duenas F.J., Ferreira P., Camarero S., Guillen F., Martinez M.J., Gutierrez A., Del Rio J.C. 2005. *Biodegradation of lignocellulosics. Microbial, chemical and enzymatic aspects of the fungal attack of lignin*. Int. Microbiol., 8: 195–204.

Polyak Y.M., Bakina L.G., Chugunova M.V., Mayachkina N.V., Gerasimov A.O., Bure V.M. 2018. *Effect of remediation strategies on biological activity of oil-contaminated soil. A field study*. Int. Biodeterior. Biodegradation, 126: 57–68.

Rojo F. 2009. *Degradation of alkane by bacteria*. J. Environ. Microbiol., 11: 2477–2490.

Safdari M.S., Kariminia H.R., Rahmati M., Fazlollahi F., Polasko A., Mahendra S., Wilding W.V., Fletcher T.H. 2018. *Development of bioreactors for comparative study of natural attenuation, biostimulation, and bioaugmentation of petroleum-hydrocarbon contaminated soil*. J. Hazard. Mater., 342: 270–278.

Saiya-Cork K.R., Sinsabaugh R.L., Zak D.R. 2002. *The effects of long term nitrogen deposition on extracellular enzyme activity in an Acer Saccharum I forest soil*. Soil Biol. Biochem., 34: 1309–1315.

Siles J.A., Margesin R. 2018. *Insights into microbial communities mediating the bioremediation of hydrocarbon-contaminated soil from an alpine former military site*. Appl. Microbiol. Biotechnol., 102: 4409–4421.

Sinsabaugh R.L. 2010. *Phenol oxidase, peroxidase and organic matter dynamics of soil*. Soil Biol. Biochem., 42: 391–404.

Strek M., Telesinski A. 2015. *Assessment of selenium compounds use in limitation of petroleum impact on antioxidant capacity in sandy soil*. Environ. Prot. Nat. Res., 26: 6–11.

Varjani S.J., Upasani V.N. 2017. *A new look on factors affecting microbial degradation of petroleum hydrocarbon pollutants*. Int. Biodeterior. Biodegradation, 120: 71–83.

Wang W., Shao Z. 2013. *Enzymes and genes involved in aerobic alkane degradation*. Front. Microbiol., 4: 1–7.

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OPTIMIZATION OF GROWTH AND TOLUENE DEGRADATION BY FREE AND IMMOBILIZED *BACILLUS CEREUS* ATHH39

***Fatemeh Heydarnezhad*[1], *Mehran Hoodaji*[2], *Mahdi Shahriarinour*[3], *Arezoo Tahmourespour*[4]**

[1] ORCID: 0000-0002-1188-7175
[2] ORCID: 0000-0001-8993-6152
[3] ORCID: 0000-0002-4596-591X
[4] ORCID: 0000-0002-5300-3251
[1,2] Soil Science Department, Isfahan (Khorasgan) Branch
Islamic Azad University, Isfahan, Iran
[3] Department of Microbiology
Rasht Branch, Islamic Azad University, Rasht, Iran
[4] Basic Medical Sciences Department, Isfahan (Khorasgan) Branch
Islamic Azad University, Isfahan, Iran

Key words: *Bacillus cereus* ATHH39, biodegradation, immobilized bacterium, toluene.

Abstract

A toluene-degrading bacterium with high tolerance of toluene was isolated from oil-contaminated soils. DNA sequencing and homologous analysis identified it as *Bacillus cereus* sp. Toluene degradation was optimized in respect to pH, temperature and toluene concentration using response surface methodology with central composite design. At optimal pH (6.7), temperature (33°C) and toluene concentration (825 mg/L) predicted degradation was 65.5%. Carbon nanotubes were used to immobilize the *Bacillus*; immobilized cells degraded toluene by 87.5% tolerated a higher toluene levels and protected the bacteria against changes in temperature and pH. These results indicate that immobilized *Bacillus cereus* strain ATHH39 possesses a good application potential in the treatment of toluene-containing soils.

Introduction

Monoaromatic hydrocarbons (BTEX) are a group of hazardous pollutants which originate from sources such as drilling, storage, transport, refineries, gas, and oil extraction fields, petrochemicals and paint and glue industries (SEIFI et al. 2011). This oily waste poses a crucial environmen-

Address: Fatemeh Heydarnezhad, Guilan Agricultural and Natural Resources Research and Education Center, Rasht, Iran, phone: +989112389488, e-mail: fatemehheydarnezhad@yahoo.com

tal pollutant and creates the problem of ground water contamination due to improper disposal/discharge on nearby lands of refineries (HU et al. 2013). They exert harmful and toxic effect on the environment and have been classified as hazardous chemicals due to carcinogenic and mutagenic nature. Degradation of such compounds is on top priority work, needs safer, and environmentally friendly treatment method. During the last decade, much research has been carried out on treatment technology for oily sludge management, which includes land farming, incineration, solidification/stabilization, solvent extraction, ultrasonic treatment, pyrolysis, photocatalysis, chemical treatment, and biodegradation (HU et al. 2013). But no single method is capable of removing/degrading total components of oily sludge. One of the most effective of these methods is adsorption because processes based on this concept are simple, highly efficient, and easy to operate; therefore, adsorption processes are widely used (JIA et al. 2013, LIU et al. 2013). Various adsorbents have been developed for the removal of organic pollutants from water (ATUL et al. 2013). Recently, a great deal of attention has been focused on the application of nano-structured materials as adsorbents to remove toxic and harmful organic substances from wastewater (ADITYA et al. 2011, MOHMOOD et al. 2013). Carbon nanotubes (CNTs), are one of the most widely studied carbon nanomaterials and can serve as excellent adsorbents (LATORRE et al. 2012) because of their hollow and layered structure and large specific surface area, which is why CNTs are the most commonly used nano-materials for adsorbing toxic material (SUI et al. 2012b, SWEETMAN et al. 2012). The use of immobilized cells or microorganisms in reactors offers many advantages over suspended cell systems, such as easier separation, greater operational flexibility, and higher cell density, resulting in higher rates of biodegradation per reactor unit volume (PARAMESWARAPPA et al. 2008). It has also been reported that immobilized cells are protected from harsh environmental conditions, becoming more tolerant to high concentrations of toxic compounds (CHEN et al. 2007). In this sense, there is a continuous search for more efficient, easier to handle, and lower cost supports. However, the degradation efficiency is decided by the environment, key factors like contaminant concentration, bioavailability, and catabolic strength of microflora, nutrient requirement, moisture level and geographical situations. Therefore, it is essential to study the simultaneous effects of different environmental conditions. Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for the modeling and problem analysis in which a response of interest is influenced by several variables and the objective is to optimize the response (MYERS et al. 2016). The central composite design (CCD) is the standard RSM and allows

estimating the second-degree polynomial of the relationships between the independent variables and the dependent variable and gives information about the interaction between variables in relation to the dependent variable (ZHAO et al. 2017).

In this study, a bacterial strain that can degrade elevated concentration of toluene, *Bacillus cereus* strain ATHH39, was isolated and characterized. Moreover, multi-walled carbon nanotubes (MWCNTs) used to immobilize the strain ATHH39. Factors affecting a toluene degradation process and operational and store stability of immobilized cells investigated.

Materials and Methods

Medium and culture conditions

The toluene-degrading *Bacillus cereus* strain ATHH39 used in this study isolated by enrichment for growth on toluene as the sole carbon source from the oil contaminated soils of Caspian Sea (Bandar-Anzali Guilan, Iran). This strain was grown aerobically at 30°C mineral salt medium (MSM) containing (per liter) 4 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g Na_2HPO_4, 0.2 g $MgSO_4$, $7H_2O$, 0.0011 g $FeSO_4$, H_2O, 0.01 g $CaCl_2$ (Merck, Darmstadt, Germany) (ZHANG al. 2013). The medium was adjusted to pH 7.0 and autoclaved to sterilize at 121°C for 15 min. Bacterial isolate was grown 24 h at 30°C, 150 rpm, in MSM broth supplemented with 1% (v/v) toluene (99.5% purity; Merck, Darmstadt, Germany) as the sole carbon and energy sources. Cells were harvested by centrifugation (10,000 × g for 10 min), washed twice in sterile MSM broth and resuspended in one-tenth volume of medium. Bacterial suspension with the density equal to 0.5 McFarland used as inoculum to predict the optimal medium composition for toluene degradation. The growth rate of the isolate was determined turbidometrically at 600 nm (MALATOVA 2005). The toluene degradation done by dissolving residual toluene of the medium in 3 ml n-hexane and reading the optical density against a blank at 200–400 nm wavelengths (WANG et al. 2008). All the experiments were carried out independently and the results were the average of three replicate experiments.

Identification of strain ATHH39 by 16S rDNA sequence

16S rDNA was amplified with the primers 27F-AGAGTTTGATCMTGGCTCAG and 1492R- GGTTACCTTGTTACGACTT. PCR reaction was done as originally described by MADUENO et al. (2011). 16S rDNA sequenced

by the Iranian Biological Resource Center. The final sequence of 1500 bp submitted to the GeneBank under accession number KX344721. The sequence submitted to a BLAST search of the NCBI GeneBank database to identify the organism.

Optimization of the degradation process

Optimization of *Bacillus cereus* ATHH39 growth and toluene degradation carried out in MSM medium. RSM was employed to optimize pH (X_1), temperature [°C] (X_2), and toluene concentration [mg/L] (X_3). RSM with a three-factor, three-level CCD design used to optimize the response, Y (toluene degradation) of three variables:

$$Y=\beta_0+\sum_{i=1}^{3}\beta_i X_i+\sum_{i=1}^{3}\beta_{ii}X_i^2+\sum_{i=1}^{2}\sum_{j=1}^{3}\beta_{ij}X_iX_j \quad (1)$$

Where β_0, β_i, β_{ii} and β_{ij} are intercept, linear, quadratic and cross product regression terms, respectively. X_i and X_j are coded independent variables, linearly related to X_1, X_2, and X_3. The actual factor level corresponding to the coded factor levels are shown in Table 1. The ranges of factor levels for the experimental design selected based on the original medium.

Table 1

Levels and codes of variables for central composite design

Variables	Level code				
	-1.68	-1	0	1	+1.68
X_1	5.32	6	7	8	8.68
X_2	21.59	25	30	35	38.41
X_3	195.46	400	700	1000	1204.54

Explanation: X_1 – pH; X_2 – temperature [°C]; X_3 – toluene concentration [mg/L]

The optimal culture conditions for maximum toluene degradation estimated by statistical analysis using the Design Expert Software (version 7.1). The coefficients in the second-order polynomial (Eq. 1) calculated by multiple regression analysis on the experimentally obtained data.

Bacterial immobilization

MWCNTs with 5–10 nm inner and 20–30 nm outer diameter, surface area of > 110 m^2/g and purity above 98% were purchased from US Research Nanomaterials. The amount of 1 g of MWCNTs treated with 60 mL of a 3:1 mixture of nitric (15.6 mol/L) to sulfuric (14.8 M) acid and were dispersed using probe sonicator for 3 h (ZHANG et al. 2011, PETERSEN et al. 2010).

To prepare a stable stock solution, MWCNTs dispersed in deionized water by ultrasonication (200 W, Cole-Parmer CV33) for 30 min, and then the mixture left at room temperature. Then the solution was filtered through a 0.45 μm membrane filter and then washed with deionized water until the neutral pH. The treated MWCNTs dried for 12 h at 60°C and stored for further use (PAN et al. 2007).

10 μl of freshly adapted bacteria with the density equal to 0.5 McFarland were transferred in to 10-ml of MSM containing 100 μl of 0.05 g/L treated MWCNT and incubated at different cultural conditions. The quantities of toluene in liquid medium degraded by free and immobilized bacteria estimated by measuring the absorbance of the culture solution at 200–400 nm.

Free and Immobilized *Bacillus cereus* ATHH39 were analysed using Scanning Electron Microscopy (SEM). For SEM analysis, Free and Immobilized strain were rinsed three times with sterile distilled water.

Results and Discussion

Isolation and characterization of Bacillus cereus strain ATHH39

By enrichment culture, the strain ATHH39 was selected for detailed studies because of its high toluene-degrading rate and capable of growth on toluene as the sole carbon and energy source. Microorganisms, in addition to being able to metabolize pollutants, are used as an analytical technology to accelerate the decomposition of soils and contaminated sediments. This strain tested for their ability to utilize toluene at concentrations from 100 mg/L to 1200 mg/L and was capable of removing 100 mg toluene per liter in the liquid MSM by 47% in 24 h (Figure 1) and metabolizing toluene up to 59% in 48 h. By PCR amplification, a 1500-bp 16S rDNA gene fragment of strain ATHH39 obtained and submitted for sequencing. The blast search of this sequence indicated that ATHH39 matched at 99.9% to *Bacillus cereus* SATCC 14579 (T). Thus, ATHH39 was identified to be a *Bacillus cereus* sp.

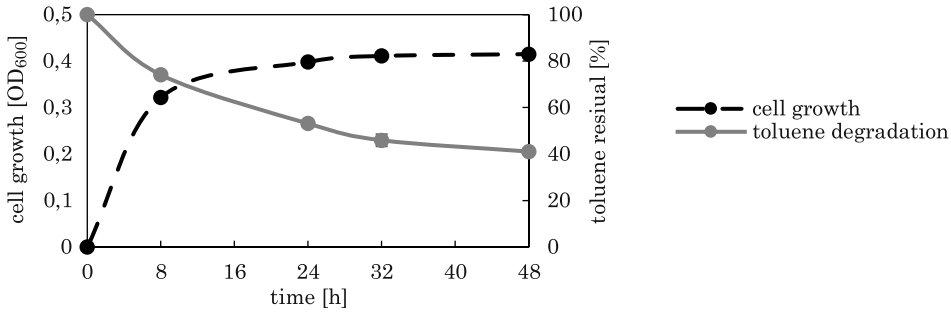

Fig. 1. Toluene-degrading and growth curves of strain ATHH39

RSM model development

Instead of optimizing medium composition by a one factor at a time approach, the statistical RSM design provides the opportunity to determine the optimal conditions, in any given parameters by establishing the relationship between factors and predicted responses. The RSM design was applied to obtain the precise factor values which results in the higher toluene degradation. The results are summarized in Table 2.

Table 2

RSM design for the three factors and their experimental results

Run order	Factors			Toluene degradation [%]	
	X_1	X_2	X_3	experimental	predicted
1	7	30	195.46	47.44	47.02
2	6	25	400	46.32	47.47
3	8	25	400	53.06	52.29
4	6	35	400	56.63	57.25
5	8	35	400	53.24	54.26
6	7	21.59	700	54.57	54.17
7	5.32	30	700	59.90	57.83
8	7	30	700	65.46	64.80
9	7	30	700	65.53	64.80
10	7	30	700	65.54	64.80
11	7	30	700	65.33	64.80
12	7	30	700	63.22	64.80
13	7	30	700	63.31	64.80
14	8.68	30	700	64.10	63.86
15	7	38.41	700	63.44	61.53

cont. Table 2

16	6	25	1000	54.77	55.37
17	8	25	1000	64.53	65.53
18	6	35	1000	59.75	62.15
19	8	35	1000	64.03	64.51
20	7	30	1204.54	64.16	62.27

Explanation: X_1 – pH; X_2 – temperature [ºC]; X_3 – toluene concentration (mg/L)

Toluene degradation

By applying multiple regression analysis on the experimentally determined data in Eq. (1), the regression coefficients were estimated and the following second-order polynomial equations was obtained using Design Expert software for optimum toluene degradation:

$$Y = 64.80 + 1.79X_1 + 2.19X_2 + 4.54X_3 - 1.40X_1^2 - 2.46X_2^2 - 3.59X_3^2 - 1.95X_1X_2 + 1.34X_1X_3 - 0.75X_2X_3 \quad (2)$$

The predicted optimum levels of X_1, X_2, and X_3 were obtained by applying regression analysis of Eq. (2), using Design Expert software, and they were 6.72 of pH, 33.16ºC of temperature and 824.15 mg/L of toluene concentration, respectively. The predicted value of toluene degradation was 65.85%. The coefficient of determination (R^2) value of the regression for the response related to significant effects on the model was 0.9601, which means that the sample variation of 96.01% of biomass production was attributable to the factors. The adequacy of the full quadratic model of toluene degradation also evaluated by ANOVA. Model summary statistics in Table 3 indicated the adequacy of the models including linear, 2-factor interactions, and quadratic terms. Linear and interaction models were significant at the 1% level.

Table 3

Analysis of variance for response surface quadratic model obtained from experimental design

Source	Sum of squares	DF	Mean square	*F*-Value	Prob. > *F*
Model	699.84	9	77.76	26.75	$< 0.0001^{***}$
X_1	43.86	1	43.86	15.09	0.0030^{**}
X_2	65.43	1	65.43	22.51	$< 0.0008^{***}$
X_3	280.93	1	280.93	96.65	$< 0.0001^{***}$
X_1^2	28.13	1	28.13	9.68	$< 0.0110^{***}$

cont. Table 3

X_2^2	86.88	1	86.88	29.89	$< 0.0003^{***}$
X_3^2	185.61	1	185.61	63.85	$< 0.0001^{***}$
X_1X_2	30.48	1	30.48	10.49	$< 0.0089^{***}$
X_1X_3	14.27	1	14.27	4.91	$< 0.0511^{ns}$
X_2X_3	4.50	1	4.50	1.55	$< 0.2419^{ns}$
Residual	29.07	10	2.91	–	–
Lack of fit	22.57	5	4.51	3.48	0.0989^{ns}
Pure error	6.49	5	1.30	–	–
Cor total	728.90	19	–	–	–
Std. dev. = 1.70 Mean = 59.72 C.V.= 2.86 PRESS = 184.41		R-squared = 0.9601 Adj. R-squared = 0.9242 Pred. R-squared = 0.7470 Adeq. precision = 15.356			

Explanation: X_1 – pH; X_2 – temperature [°C]; X_3 – toluene concentration [mg/L] *values of "probability > *F*-Value" less than 0.05 indicates model terms are significant

SEM observations analysis

Free *Bacillus cereus* ATHH39 and Immobilized *Bacillus cereus* ATHH39 adhesion on the surface of MWCNTs in the presence of 100 mg/L toluene were observed using SEM (Figure 2). Figure 2(b) demonstrate that bacteria cells are trapped among the MWCNTs arrays bundles. It can be due to the interactions of bacteria cells with the external surfaces of MWCNTs arrays. Also, Figure 2 indicates that there are no major changes in the morphology of the bacteria cells after incubating with MWCNTs arrays. These SEM images reveal that MWCNTs clusters only capture the bacteria cells due to sieve mechanisms without any damage of the cell wall.

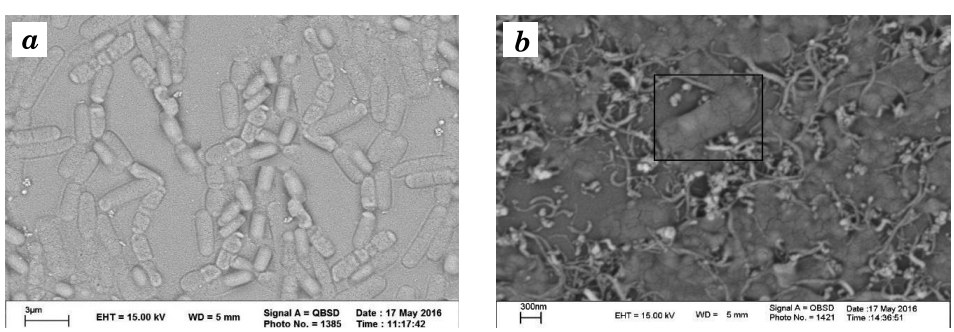

Fig. 2. Scanning electron microscopy image of (*a*) *Bacillus cereus* ATHH39, (*b*) immobilized *Bacillus cereus* ATHH39 by MWCNTs

No changes in the structures of the carbon nanotubes after bacterial immobilizing, which is the benefits of the method has been observed in other studies (KOLANGIKHAH et al. 2010). This observation differs from other studies (KANG et al. 2008a, KANG et al. 2008b). Using non-array CNTs have shown that CNTs rupture cell wall-membrane due to toxicity mechanisms such as oxidative stress (NEL et al. 2006) and physical damage (KANG et al. 2008a, KANG et al. 2008b) while this observation has not been observed here.

Effect of pH, temperature and toluene concentration

Cell immobilization has been used for biodegradation of several toxic chemicals. Immobilization facilitates the separation and retrieval of cells and leads to reuse and cost reductions (BAYOUMI 2009). MWCNTs used to immobilize *Bacillus cereus* strain ATHH39. Factors affecting toluene degradation of immobilized cells, such as pH, temperature and toluene concentration investigated. DÍAZ et al. (2002) reported that the immobilization of bacterial cells in comparison with free-living cells increased the rate of biodegradation of crude oil in a wider range of salinity. The pH optimum experiments for toluene degradation by free and immobilized cells performed at 30ºC with toluene concentration of 700 mg/L and the results are shown in Figure 3. The optimal pH was 7 for free and immobilized cells, the degradation rates were 65.5% to 95.2% for free and immobilized cells, respectively.

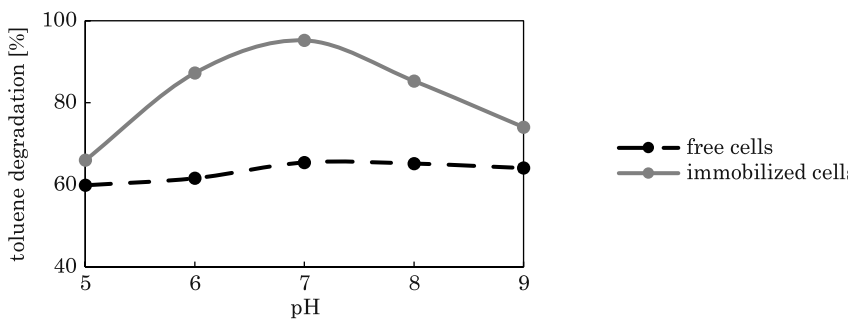

Fig. 3. Effect of pH on toluene degradation of free and immobilized cells

The immobilized cells have higher degradation rate constant and wider pH range than that of free cells. The changes of the toluene degradation of free cells were not as sharp as the immobilized cells. When pH was at about 7, toluene-degrading of free cells was completely inhibited. However, immobilized cells still maintained an acceptable degradation rate at the same pH. When pH increase or decrease the degradation rate of the immobilized and free cells were lower. To confirm that immobilized cells can also

tolerate a wider temperature change, toluene degradation experiments performed at a temperature range from 20 to 40ºC at pH 7 with toluene concentration of 700 mg/L. Figure 4 shows the effect of temperature on toluene degradation of free and immobilized cells. The optimal temperature was (30ºC) and (35ºC) for free and immobilized cells, the degradation rates were 65.5% to 95.1% for free and immobilized cells, respectively.

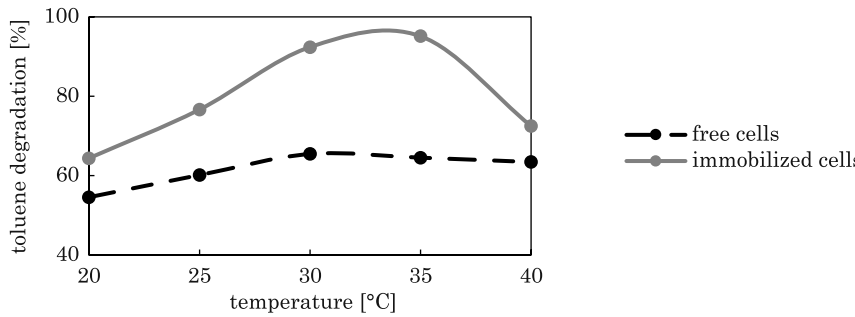

Fig. 4. Effect of temperature on degradation of free and immobilized cells

In the range of 30 to 40ºC, the immobilized cells showed a higher value of degradation rate than free cells. Especially when the temperature reached 35ºC, with the increase of the temperature, the curve of free cells declined slowly, but the curve of immobilized cells fell sharply. Figure 5 shows the effect of toluene concentration on toluene degradation of free and immobilized cells. The optimal toluene concentration was (700 mg/L) for free and immobilized cells, the degradation rates were 65.5% to 87.5% for free and immobilized cells, respectively. The immobilized cells have higher degradation rate constant. Especially when the toluene concentration reached 700 mg/L, with the increase of the toluene concentration, the curve of free cells and immobilized cells declined slowly.

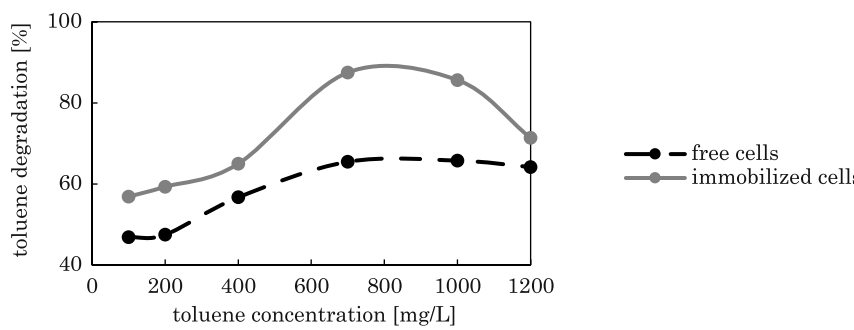

Fig. 5. Effect of initial toluene concentration on toluene degradation of free and immobilized cells

Conclusion

The results of this study showed the efficiency of 65.5% of toluene adsorption by free cells and the efficiency of 87.5% of toluene adsorption, by carbon nanotubes coated with *Bacillus cereus*. This is due to increase in biodegradation rate and active sites available for toluene adsorption through exopolysaccharides secreted from bacteria. The use of bacteria in the form of biofilm coating can play an effective and meaningful role in improving the absorption of toluene by carbon nanotubes.

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References

ADITYA D., ROHAN P., SURESH G. 2011. *Nano-adsorbents for wastewater treatment: a review*. Research Journal of Chemistry and Environment, 15(2): 1033–1040.

ATUL W.V., GAIKWAD G.S., DHONDE M.G., KHATY N.T., THAKARE S.R. 2013. *Removal of organic pollutant from water by heterogenous photocatalysis: A review*. Research Journal of Chemistry and Environment, 17: 84–94.

BAYOUMI R.A. 2009. *Bacterial bioremediation of polycyclic aromatic hydrocarbons in heavy oil contaminated soil*. Journal of Applied Sciences Research, 5: 197–201.

CHEN Y.M., LIN T.F., HUANG C., LIN J.C., HSIEH F.M. 2007. *Degradation of phenol and TCE using suspended and chitosan-bead immobilized Pseudomonas putida*. Journal of Hazardous Materials, 148(3): 660–670.

DÍAZ M.P., BOYD K.G., GRIGSON S.J., BURGESS J.G. 2002. *Biodegradation of crude oil across a wide range of salinities by an extremely halotolerant bacterial consortium MPD-M, immobilized onto polypropylene fibers*. Biotechnology and Bioengineering, 79(2): 145–153.

HU G., LI J., ZENG G. 2013. *Recent development in the treatment of oily sludge from petroleum industry: a review*. Journal of Hazardous Materials, 261: 470–490.

JIA Y., LUO T., YU X.Y., SUN B., LIU J.H., HUANG X.J. 2013. *A facile template free solution approach for the synthesis of dypingite nanowires and subsequent decomposition to nanoporous MgO nanowires with excellent arsenate adsorption properties*. RSC advances, 3(16): 5430–5437.

KANG S., HERZBERG M., RODRIGUES D.F., ELIMELECH M. 2008a. *Antibacterial effects of carbon nanotubes: size does matter!* Langmuir, 24(13): 6409–6413.

KANG S., MAUTER M.S., ELIMELECH M. 2008b. *Physicochemical determinants of multiwalled carbon nanotube bacterial cytotoxicity*. Environmental Science & Technology, 42(19): 7528–7534.

KOLANGIKHAH M., MAGHREBI M., GHAZVINI K., FARHADIAN N. 2012. *Separation of salmonella typhimurium bacteria from water using MWCNTs arrays*. International Journal of Nanoscience and Nanotechnology, 8(1): 3–10.

LATORRE C.H., MÉNDEZ J.Á., GARCÍA J.B., MARTÍN S.G., CRECENTE R.P. 2012. *Carbon nanotubes as solid-phase extraction sorbents prior to atomic spectrometric determination of metal species: A review*. Analytica Chimica Acta, 749: 16–35.

LIU F., JIN Y., LIAO H., CAI L., TONG M., HOU Y. 2013. *Facile self-assembly synthesis of titanate/Fe_3O_4 nanocomposites for the efficient removal of Pb^{2+} from aqueous systems*. Journal of Materials Chemistry A, 1(3): 805–813.

MADUEÑO L., COPPOTELLI B.M., ALVAREZ H.M., MORELLI I.S. 2011. *Isolation and characterization of indigenous soil bacteria for bioaugmentation of PAH contaminated soil of semiarid Patagonia, Argentina*. International Biodeterioration & Biodegradation, 65(2): 345–351.

MALATOVA K. 2005. *Isolation and characterization of hydrocarbon degrading bacteria from environmental habitats in Western New York State*, Master of Science in Chemistry, Rochester Institute of Technology, Rochester, New York.

MOHMOOD I., LOPES C.B., LOPES I., AHMAD I., DUARTE A.C., PEREIRA E. 2013. *Nanoscale materials and their use in water contaminants removal-a review*. Environmental Science and Pollution Research, 20(3): 1239–1260.

MYERS R.H., MONTGOMERY D.C., ANDERSON-COOK C.M. 2016. *Response surface methodology: process and product optimization using designed experiments*. John Wiley & Sons.

NEL A., XIA T., MÄDLER L., LI N. 2006. *Toxic potential of materials at the nanolevel*. Science, 311(5761): 622–627.

PAN X., FAN Z., CHEN W., DING Y., LUO H., BAO X. 2007. *Enhanced ethanol production inside carbon-nanotube reactors containing catalytic particles*. Nature Materials, 6(7): 507–511.

PARAMESWARAPPA S., KARIGAR C., NAGENAHALLI M. 2008. *Degradation of ethylbenzene by free and immobilized Pseudomonas fluorescens-CS2*. Biodegradation, 19(1): 137–144.

PETERSEN E.J., HUANG Q., WEBER JR W.J. 2010. *Relevance of octanol-water distribution measurements to the potential ecological uptake of multi-walled carbon nanotubes*. Environmental Toxicology and Chemistry, 29(5): 1106–1112.

SEIFI L., TORABIAN A., KAZEMIAN H., BIDHENDI G.N., AZIMI A.A., NAZMARA S., ALIMOHAMMADI M. 2011. *Adsorption of BTEX on surfactant modified granulated natural zeolite nanoparticles: parameters optimizing by applying taguchi experimental design method*. CLEAN–Soil, Air, Water, 39(10): 939–948.

SUI Z., MENG Q., ZHANG X., MA R., CAO B. 2012. *Green synthesis of carbon nanotube-graphene hybrid aerogels and their use as versatile agents for water purification*. Journal of Materials Chemistry, 22(18): 8767–8771.

SWEETMAN L.J., NGHIEM L., CHIRONI I., TRIANI G., RALPH S.F. 2012. *Synthesis, properties and water permeability of SWNT buckypapers*. Journal of Materials Chemistry, 22(27): 13800–13810.

WANG L., QIAO N., SUN F., SHAO Z. 2008. *Isolation, gene detection and solvent tolerance of benzene, toluene and xylene degrading bacteria from nearshore surface water and Pacific Ocean sediment*. Extremophiles, 12(3): 335–342.

ZHANG L., PETERSEN E.J., HUANG Q. 2011. *Phase distribution of 14C-labeled multiwalled carbon nanotubes in aqueous systems containing model solids: Peat*. Environmental Science & Technology, 45(4): 1356–1362.

ZHANG L., ZHANG C., CHENG Z., YAO Y., CHEN J. 2013. *Biodegradation of benzene, toluene, ethylbenzene, and o-xylene by the bacterium Mycobacterium cosmeticum byf-4*. Chemosphere, 90(4): 1340–1347.

ZHAO X., WANG L., MA F., BAI S., YANG J., QI S. 2017. *Pseudomonas* sp. ZXY-1, *a newly isolated and highly efficient atrazine-degrading bacterium, and optimization of biodegradation using response surface methodology*. Journal of Environmental Sciences, 54: 152–159.

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PHYSICOCHEMICAL PROPERTIES AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OIL OF *LITSEA CUBEBA* PERS. FRUIT

Le Pham Tan Quoc

ORCID: 0000-0002-2309-5423
Institute of Biotechnology and Food Technology
Industrial University of Ho Chi Minh City, Ho Chi Minh City, Vietnam

Key words: *Litsea cubeba* Pers., fruit essential oil, physicochemical properties, antimicrobial activity.

Abstract

The physicochemical properties of the essential oil (EO) extracted from the fruit of *Litsea cubeba* Pers. were determined, such as pH (5.33±0.15), acid value (8.92±1.02 mg KOH/g EO), saponification value (20.99±2.83 mg KOH/g EO), relative density (0.8836±0.0031), absolute density (0.8820±0.0023 g/mL), and freezing point (-12.67±0.58ºC). The antioxidant capacity was quite low: IC_{50} was not found in this study. In addition, some chemical components were identified using gas chromatography-mass spectrometry (GC/MS) method with some major compounds such as 4-methyl-1,5-heptadiene (26.02%), 1-methoxy-2-butyne (20.05%), and cyclobutane, 1,3-diisopropenyl-, trans (18.06%). The EO was tested for its antibacterial activities against gram-positive (*Staphylococcus aureus* – ATCC 25923, *Bacillus subtilis* – ATCC 11774), and gram-negative (*Escherichia coli* – ATCC 25922), *Salmonella* Enteritidis – ATCC 13076) bacteria. The results showed the highest activity against *B. subtilis* and the lowest activity against both *S.* Enteritidis and *S. aureus.*

Introduction

Litsea cubeba Pers. is a shrub or small deciduous tree, distributed widely in Asian regions including China, Nepal, northeastern India, Indonesia, Laos, Myanmar, Thailand, and Vietnam (SAIKIA et al. 2013) with berry-like spherical fruit. The leaves are staggered, the lanceolate leaflets are 10 cm long, 1.5–2.5 cm wide, thick, the face is green, the gray underside turns black, the edges are intact, and the stems of leaf and veins are clear. In Vietnam, the local citizens exploit the essential oil (EO) from the fruit and leaves of this plant. However, EOs from the leaf are lower in

Address: Le Pham Tan Quoc, Industrial University of Ho Chi Minh City, Ho Chi Minh City, Vietnam, e-mail: lephamtanquoc@iuh.edu.vn

quality. Yield of EO from the fruit is 3–5% and contains about 60–75% citral (a mixture of geranial and neral). This component is used for soft drinks and fragrances (SELL 2010).

In addition, *L. cubeba* has been used in traditional Chinese medicine for many centuries. The roots, stems, leaves, and fruits of this plant have been used to treat diseases such as fatigue, headache, muscle aches, and depression. The extract of bark from this tree contains some antioxidants (HWANG et al. 2005) with anti-inflammatory properties (CHOI and HWANG 2004). The EO from *L. cubeba* also has antifungal and antibacterial activity similar to the EOs from other plants (WANG and LIU 2010). Alkaloids, litseacubebic acid, and monoterpene lactones isolated from *L. cubeba* have good antibiotic activity against many bacterial pathogens (FENG et al. 2009). Besides, TAYLOR et al. (2013) pointed out that the EO from the leaves and fruits of *L. cubeba* harvested in northeast India is able to resist *Staphylococcus aureus*, *Listeria monocytogenes, Escherichia coli,* and *Aspergillus niger*. In fact, this EO has many applications in food, medicine, and the cosmetic industry, according to JIANG et al. (2009), *L. cubeba* EO is used as a food additive. It has also been used as a raw material for the manufacture of vitamin A, E, K, ionine, methylionone, and perfumes.

Currently, this plant is widely cultivated in North Vietnam for the extraction of EO. The quality of the EO depends on climate, season, soil, and extraction methods. Although there are many reports on the chemical components of *L. cubeba* EO, until now there are no reports on the chemical components and physicochemical properties of this EO in Vietnam. Hence, the main aim of this study was to clarify some of the issues mentioned above and, in addition, to evaluate its antibacterial activity and antioxidant capacity, and give an overview on the general quality of *L. cubeba* EO.

Materials and Methods

Plant material and EO extraction

Fresh fruit of *L. cubeba* was collected and harvested from Hanoi province (Vietnam). The fresh fruit was distilled by Clevenger-type apparatus. The total mass of sample (fruits), in round-bottom flasks, was about 100 g per extraction time. Firstly, the fruit was completely soaked in water and heated to boiling (fruit/water ratio of 1/10, w/v). Then, the EO was evaporated together with water vapor and finally collected after decantation. The yield of EO obtained was approximately 3% (v/w). The resulting EO was stored at 4ºC until analysis.

Bacteria strains

Antibacterial activity was determined against two gram-positive bacteria such as *B. subtilis* (ATCC 11774), *S. aureus* (ATCC 25923), and two gram-negative bacteria such as *S.* Enteritidis (ATCC 13076), *E. coli* (ATCC 25922). All bacteria strains were purchased from Microbiologics (USA).

Determination of the relative and absolute density

The relative density was evaluated by the ratio between the mass of a given volume of EO at 20°C to the mass of an equal volume of distilled water at 20°C, while the absolute density was determined by ratio of the mass of a given volume of the EO at 20°C to the same volume (*Essential oils…* ISO 279:1998).

Determination of the freezing temperature

According to *Essential oils…* ISO 1041:1973, 5 mL of EO was added to the test-tube. Then, the test-tube was put into the freezing container. The temperature in the freezing container was decreased slowly until the EO appeared to crystallize and the freezing temperature recorded.

Determination of acid value (AV)

The acid value was determined by the titration method. The EO (1 g) was dissolved in 5 mL of 96 % ethanol and about three drops of 1% phenolphthalein solution. The mixture was titrated with 0.1 N KOH until the solution turned pink.

$$\mathrm{AV}=\frac{V_{\mathrm{KOH}} \cdot 0.1 \cdot 56.1}{\text{mass of essential oil}}$$

Determination of saponification value (SV)

The EO (1.5 g) was put into a glass flask (250 mL) and 25 mL of 0.5 M ethanolic KOH added. The mixture was heated for 1 h under reflux and then 25 mL of deionized water and five drops of 1% phenolphthalein solution added. The solution was titrated with 0.5 M HCl until the solution turned colorless.

$$\mathrm{SV}=\frac{\left(V_{\mathrm{blank}}-V_{\mathrm{sample}}\right) \cdot 56.1 \cdot 0.5}{\text{mass of essential oil}}$$

Determination of antioxidant activity

Determination of the antioxidant activity of the EO to scavenge DPPH free radicals was as described by KIRBY and SCHMIDT (1997) with some small modifications. The EO was dissolved in ethanol to achieve the various concentrations (1, 2, 4, 8, 16, 32, and 64 mg/mL). Then, 50 µL of the solution was mixed with 1950 µL of an ethanolic solution of DPPH ($6 \cdot 10^{-5}$ M). The solution was kept in the dark for 30 min at room temperature (OUIS and HARIRI 2018). Antioxidant activity was recorded by monitoring the decrease in absorbance at 517 nm against a control consisting of DPPH in ethanolic solution ($6 \cdot 10^{-5}$ M) and the antioxidant activity was described using the following expression:

$$\% \text{ inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \cdot 100$$

Determination of antibacterial activity

Antibacterial activity was determined by the paper disk diffusion method for antibiotic susceptibility testing (BAUER et al. 1966) with some slight modifications. At first, 100 µL of bacterial suspension (0.5 McFarland standard, approximately $1.5 \cdot 10^8$ cfu/mL) was spread on MHA medium (Mueller-Hinton agar) by a sterile hockey stick. Then, the sterile paper disks of 6 mm diameter were impregnated with the selected EO (10 µL), while gentamicin (10 µg/disk) were used as positive controls to compare the antibacterial activity of the EO, and 20% dimethylsulfoxide (DMSO) solution (10 µL/disk) was used as negative control. These dishes were incubated for 24 h at 37ºC and the diameter of the inhibition zones were expressed in mm including the disk diameter of 6 mm.

Analysis of EO by GC–MS

A volume of 1 µl of EO was injected into a gas chromatograph (Agilent HP 6890 N, USA) equipped with a quadrupole mass analyzer (Agilent HP 5972, USA) in the electron impact ionization (EI) mode (70 eV), split/splitless injector. A capillary column (HP-5 ms, 30 m × 0.25 mm × 0.25 µm, Agilent Technologies, USA) was used with helium as a carrier gas at a constant flow of 1 mL/min. Temperatures: injector = 280ºC, column = 60ºC (5 min), then 15ºC/min to 300ºC (5 min).

Data analysis

Experimental results were analyzed by one-way analysis of variance (ANOVA), and significant differences among the means from triplicate analyses at ($p < 0.05$) were determined by Fisher's least significant difference (LSD) procedure using Statgraphics software (Centurion XV). The values obtained were expressed in the form of a mean±standard deviation (SD).

Results and Discussion

Determination of physicochemical properties of EO

Table 1 shows that the magnitude of the pH of *L. cubeba* fruit EO was recorded in the acidic range (pH = 5.33). This result is in agreement with the EO of *Ceratonia siliqua* seeds (pH = 5.2) and higher than EO of *C. siliqua* pulp (pH = 4.3) (OUIS and HARIRI 2018). This can be explained by the pH value depending on the various main components of the EO.

Table 1

Physicochemical properties of *L. cubeba* EO

Physicochemical properties	Value
pH	5.33±0.15
Relative density	0.8836±0.0031
Absolute density [g/mL]	0.8820±0.0023
Freezing point [ºC]	–12.67±0.58
Acid value [mg KOH/g EO]	8.92±1.02
Saponification value [mg KOH/g EO]	20.99±2.83

The absolute density of *L. cubeba* fruit EO is also higher than that of coriander EO (0.8737 g/mL) (PORTER and LAMMERINK 1994), *C. siliqua* pulp EO (0.833 g/mL), and lower than that of *C. siliqua* seeds EO (0.910 g/mL) (OUIS and HARIRI 2018). The different temperatures and the components of initial material caused the differences in the relative and absolute density. PORTER and LAMMERINK (1994) believed that the absolute density of all EOs decreased as temperatures increased, whereas PORNPUNYAPAT et al. (2011) noticed that an increase in the distilling temperatures can lead to an increase in the relative density for EO of *Aquilaria crassna* wood. The freezing point of the *L. cubeba* fruit EO is higher than that of the cocoa beans EO (–16ºC) (BAINBRIDGE and DAVIES 1912) and lower than that of *Eucalyptus camaldulensis* leaves EO (0ºC) (ABDUL-MAJEED et al. 2013). In fact, all EOs have a freezing point; some EOs need quite low tem-

peratures to freeze, some EOs will freeze in a refrigerator and others can even be solid or crystalline at room temperature. This strongly depends on the main component of the EO.

The acid and saponification values of *L. cubeba* fruit EO are 8.92 mg KOH/g EO and 20.99 mg KOH/g EO, respectively. The acid value of this study is higher than that of the EO of *C. siliqua* seeds EO (2.2 mg KOH/g EO), and lower than that of *Plectranthus amboinicus* Lour. leaves (10.35 mg KOH/g EO), while the saponification value is lower than that of *P. amboinicus* Lour. leaves EO (54.72 mg KOH/g EO) and *C. siliqua* seeds EO (37.2 mg KOH/g EO) (THUYEN et al. 2012; OUIS and HARIRI 2018). The acid value depends on the extraction method and the freshness of the initial material and increases with increasing time preservation because EOs are oxidized and the esters are hydrolyzed to release the free acid.

Determination of antioxidant activity of EO

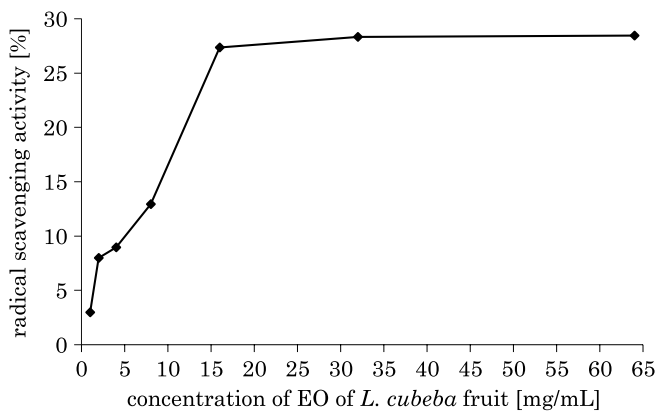

Fig. 1. Radical scavenging activity of *L. cubeba* fruit EO

The appearance of yellow color due to bleaching the purple color of the DPPH attested to the positive antioxidant activity of EO. The percentage inhibition increased with increasing concentration from 1 to 64 mg/mL. Figure 1 shows the percentage inhibition of the EO, but the IC_{50} values could not be determined in this study because the antioxidant activity of *L. cubeba* fruit EO is quite low (< 30%). The antioxidant activity of different EOs depends directly on the concentrations used. However, it is lower than that of studies from the different materials; for instance, the EOs of *Piper betle* L. (IC_{50} = 3.6 µg/mL) (PRAKASH et al. 2010) or of *C. siliqua* pulp and seeds (IC_{50} = 7.8 and 31.25 µg/mL, respectively) (OUIS and HARIRI

2018). Compared with the similar material, *L. cubeba* fruit EO collected from Nepal and China possessed the high antioxidant activity (IC_{50}=1629 and 850 μg/mL, respectively) (BAJRACHARYA and PRATIGYA 2019; BI et al. 2017). This finding revealed that the antioxidant activity of the EO was affected by the initial material, production region, and chemical compositions.

Determination of the chemical compositions of EO

GC–MS analysis of the *L. cubeba* fruit EO revealed 17 different components, which made up approximately 100% of the EO (Table 2). The major components included 4-methyl-1,5-heptadiene (26.02%); 1-methoxy-2-butyne (20.05%); cyclobutane, 1,3-diisopropenyl-, trans (18.06%); 2,7-dimethyl-2,6-octadien-1-ol (9.98%); cyclobutanone, 2-methyl-2-oxiranyl (7.11%) and 2-methyl-6-methylene-2-octene (5.76%). Some components belonged to the terpene group and its derivatives such as 2-methyl-6-methylene-2-octene; 2,7-dimethyl-2,6-octadien-1-ol, and *α*-terpineol, which made up a significant proportion of the EO and other small groups (Table 2). These bioactive compounds have an important role in the antibacterial and antioxidant activity of the EO.

Table 2

Chemical composition of *L. cubeba* fruit EO

Compound	Rt. [min.]	[%]
4-Methyl-1,5-heptadiene	7.851	26.02
1-Methoxy-2-butyne	7.584	20.05
Cyclobutane, 1,3-diisopropenyl-, trans	5.540	18.06
2,7-Dimethyl-2,6-octadien-1-ol	7.484	9.98
Cyclobutanone, 2-methyl-2-oxiranyl	6.750	7.11
2-Methyl-6-methylene-2-octene	7.732	5.76
5-Methoxy-1-pentene	5.004	3.99
2-Propenamide	6.244	3.52
2-Heptyne	6.978	1.42
α-terpineol	4.280	0.76
Butanedinitrile	4.856	0.67
2,5-Dimethyl-2-hexanol	7.088	0.63
6-Methylene bicyclo [3.1.0] hexane	4.806	0.61
2-Furanmethanamine	8.476	0.44
Methyl isocyanoacetate	8.238	0.4
2,5-Octadiene	6.820	0.39
Butanedinitrile	8.843	0.20

However, some previous studies have reported that the prime components of *L. cubeba* EO in Tipet (China) were limonol (44.2%); *β*-linalool (8.8%); and 1,8-cineole (5.4%) (YANG et al. 2010), while the EO of this material from India consisted of sabinene (58.52%); *α*-pinene (12.59%); and 1.8-cineole (12.66%) (SAIKIA et al. 2013). These findings differ from our results. However, some chemical components of this study are similar to those of *L. cubeba* EO in the study of WANG and LIU (2010), such as terpenoid groups. Changes in chemical composition have been recorded in most EOs due to the age of the plant, genes, time of harvesting, extraction method, and geographical and ecological conditions. Therefore, the percentage of the main constituents of *L. cubeba* fruit EO should be mentioned if it is applied as a food additive.

Determination of antimicrobial activity of EO

According to the results in Table 3, it can be observed that the *L. cubeba* fruit EO shows positive antibacterial activity for four strains of bacteria including two gram-positive bacteria (*S. aureus, B. subtilis*) and two gram-negative (*E. coli, S.* Enteritidis). The inhibitor zones of positive control are in order of susceptibility: *S. aureus* < *S.* Enteritidis < *E. coli* < *B. subtilis*. The EO shows the highest antimicrobial activity against *B. subtilis* (inhibition zone 44.33 mm) and the lowest antibacterial activity against *S. aureus* (inhibition zone 14.33 mm). The sensitivity of EOs is classified by the diameter of the inhibition zone. Those of *S.* Enteritidis, *S. aureus*, and *E. coli* were considered "very sensitive" with diameters of 15–19 mm, while that of *B. subtilis* was considered "extremely sensitive" with a diameter of > 20 mm (PONCE et al. 2003). The presence of terpenoids in an EO can strongly inhibit bacteria because their site of action appears to be at the phospholipid bilayer, caused by biochemical mechanisms catalyzed by the phospholipid bilayers of the cell. These processes include the inhibition of phosphorylation steps, electron transport, protein translocation, other enzyme-dependent reactions and microbial oxygen uptake (KNOBLOCH et al. 1986). In addition, the bacterial inhibition also depends on the structure of the terpenoids. However, until now the actual structure-activity relationships of terpenoids are not well understood (GRIFFIN et al. 1999). Compared with other previous studies on disk diffusion assay, *L. cubeba* fruit EO from northeast India also inhibited the grown of *B. cereus* and *S. aureus*, whereas it did not show any appreciable inhibition toward *B. subtilis* (GOGOI et al. 2018). *L. cubeba* fruit were collected from southern regions of China and the EO revealed antibacterial activity against all six bacteria tested: *B. subtilis, E. coli, E. faecalis,*

M. albicans, P. aeruginosa, and S. aureus (WANG and LIU 2010). This proved that the antibacterial activity strongly depends on the chemical composition of the EO.

Table 3

Inhibition zones of *L. cubeba* fruit EO for some bacteria

Microorganism	Diameter of the inhibition zones [mm]
S. Enteritidis	14.67 ± 0.58^a
E. coli	17.67 ± 0.58^b
B. subtilis	44.33 ± 0.58^c
S. aureus	14.33 ± 0.58^a

Different lowercase letters in the same column denote significant difference ($p < 0.05$) with respect to the type of microorganism.

Conclusion

The 17 main chemical components in the *L. cubeba* fruit EO were identified by GC-MS. Further, the *L. cubeba* fruit EO showed marked in vitro antimicrobial activities against *S.* Enteritidis, *E. coli*, *B. subtilis* and *S. aureus*. In addition, the data demonstrated antioxidant activity and determined some physicochemical properties of the *L. cubeba* fruit EO. This is a cheap source of bioactive compounds with high potential as green and natural additives in the food and cosmetics industries.

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References

ABDUL-MAJEED B.A., HASSAN A.A., KURJI B.M. 2013. *Extraction of oil from Eucalyptus camaldulensis using water distillation method*. Iraqi J. Chem. Pet. Eng., 14(2): 7–12.

BAINDRIDGE J.S., DAVIES S.H. 1912. *The essential oil of cocoa*. J. Chem. Soc. Trans., 101: 2209–2221.

BAJRACHARYA G.B., PRATIGYA K.C. 2019. *A high antibacterial efficacy of fruits of Litsea cubeba (Lour.) Pers. from Nepal. GC-MS and antioxidative capacity analyses*. Pharmacogn. J., 11(5): 889–893.

BAUER A.W., KIRBY W.M.M., SHERRIS J.C., TURCK M. 1966. *Antibiotic susceptibility testing by a standardized single disk method*. Am. J. Clin. Pathol., 45(4): 493–496.

BI Y., ZHANG Y., KONG F.S. 2017. *Experimental study on scavenging DPPH free radical activity of Litsea cubeba oil*. Adv. Comput. Sci. Res., 75: 939–942.

CHOI E.M., HWANG J.K. 2004. *Effects of methanol extract and fractions from Litsea cubeba bark on the production of inflammatory mediators in RAW 264.7 cells*. Fitoterapia, 75(2): 141–148.

Essential oils. Determination of freezing point. ISO 1041:1973. International Organization for Standarization, Geneva, Switzerland.

Essential oils. Determination of relative density at 20°C. ISO 279:1998. International Organization for Standarization, Geneva, Switzerland.

FENG T., XU Y., CAI X.H., DU Z.Z., LUO, X.D. 2009. *Antimicrobially active isoquinoline alkaloids from Litsea cubeba*. Planta Med., 75: 76–79.

GOGOI R., LOYING R., SARMA N., MUNDA S., PANDEY S.K., LAL M. 2018. *A comparative study on antioxidant, anti-inflammatory, genotoxicity, antimicrobial activities and chemical composition of fruit and leaf essential oils of Litsea cubeba Pers. from North-east India*. Ind. Crops Prod., 125: 131–139.

GRIFFIN S.G., WYLLIE S.G., MAKRHAM J.L., LEACH D.N. 1999. *The role of structure and molecular properties of terpenoids in determining their antimicrobial activity*. Flavours Fragrances, 14: 322–332.

HWANG J.K., CHOI E.M., LEE J.H. 2005. *Antioxidant activity of Litsea cubeba*. Fitoterapia, 76: 648–686.

JIANG Z., AKHTAR Y., BRADBURY R., ZHANG X., ISMAN M.B. 2009. *Comparative toxicity of essential oils of Litsea pungens and Litsea cubeba and blends of their major constituents against the cabbage looper, Trichoplusia ni*. J. Agric. Food Chem., 57(11): 4833–4837.

KIRBY A.J., SCHMIDT R.J. 1997. *The antioxidant activity of Chinese herbs for eczema and of placebo herbs – I*. J. Ethnopharmacol., 56: 103–108.

KNOBLOCH K., WEIGAND N., WEIS H.M., VIGENSCHOW H. 1986. *Action of terpenoids on energy metabolism*. In: *Progress in Essential Oil Research*. Eds. E.J Brunke, Walter de Gruyther, Berlin, pp. 429–445.

OUIS N., HARIRI A. 2018. *Antioxidant and antibacterial activities of the essential oils of Ceratonia siliqua*. Banat's J. Biotechnol., IX(17): 13–23.

PANDEY R.R., DUBEY R.C., SAINI S. 2010. *Phytochemical and antimicrobial studies on essential oils of some aromatic plant*. Afr. J. Biotechnol., 9(28): 4364–4368.

PONCE A.G., FRITZ R., DEL-VALLE C., ROURA S.I. 2003. *Antimicrobial activity of essential oils on the native microflora of organic Swiss chard*. LWT – Food Sci. Technol., 36: 679–684.

PORNPUNYAPAT J., CHETPATTANANONDH P., TONGURAI C. 2011. *Mathematical modeling for extraction of essential oil from Aquilaria crassna by hydrodistillation and quality of agarwood oil*. Bangladesh J. Pharmacol., 6: 18–24.

PORTER N.G., LAMMMERINK J.P. 1994. *Effect of temperature on the relative densities of essential oils and water*. J. Essent. Oil Res., 6: 269–277.

PRAKASH B., SHUKLA R., SINGH P., KUMAR A., MISHRA P.K., DUBEY N.K. 2010. *Efficacy of chemically characterized Piper betle L. essential oil against fungal and aflatoxin contamination of some edible commodities and its antioxidant activity*. Int. J. Food Microbiol., 142: 114–119.

SAIKIA A.K., CHETIA D., ARRIGO M.D., SMERIGLIO A., RUBERTO G. 2013. *Screening of fruit and leaf essential oils of Litsea cubeba Pers. from north-east India – chemical composition and antimicrobial activity*. J. Essent. Oil Res., 25(4): 330–338.

SELL C. 2010. *Chemistry of essential oils*. In: *Handbook of essential oil. Science, technology and application*. Eds. K.H.C. Baser, G. Buchbauer, CRC Press, New York, pp. 121–150.

TAYLOR P., CHEN Y., WANG Y., HAN X., SI L., WU Q., LIN L. 2013. *Biology and chemistry of Litsea cubeba, a promising industrial tree in China*. J. Essent. Oil Res., 25(2): 37–41.

THUYEN N.T.B., THUY, N.T.D., HANG, C.T.T. 2012. *Study on chemical composition and some anti-microorganism activity of Plectranthus amboinicus L. essential oil*. Can Tho univ. J. Sci., 21: 144–147.

WANG H., LIU Y. 2010. *Chemical composition and antibacterial activity of essential oils from different parts of Litsea cubeba*. Chem. Biodiversity, 7: 229–235.

YANG Y., JIANG J., QIMEI L., YAN X., ZHAO J., YUAN H., QIN Z., WANG M. 2010. *The fungicidal terpenoids and essential oil from Litsea cubeba in Tibet*. Molecules, 15: 7075–7082.

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TOURISM IMPACT ON THE SHORE ZONE OF LAKES: A CASE STUDY OF FOUR LAKES OF MRĄGOWO LAKELAND*

Grażyna Furgała-Selezniow[1], *Małgorzata Jankun-Woźnicka*[2]

[1] ORCID: 0000-0003-0324-1726
[2] ORCID: 0000-0001-6785-3591
Department of Tourism, Recreation and Ecology
University of Warmia and Mazury in Olsztyn

Key words: lake tourism, tourism load monitoring, LU/LC, human pressure, Mrągowo Lakeland (Poland).

Abstract

Lakes are an essential element of human well-being and lake tourism is becoming an increasingly important branch of the tourism industry worldwide. Numerous investments in tourism infrastructure, located in the shore zone of lakes, attract more and more tourists. This phenomenon has a negative impact on the natural environment of the lakes. Their shore zone, which is an ecotone zone, is particularly vulnerable to human pressure. Four lakes were selected for the study. They differed substantially in their use and land cover in the shore zone. GIS datasets were used for the analysis. A set of indices was proposed to monitoring the lake shore zone tourism load. The shore zone of the lakes with a high share of the residential buildings and the forest was the most affected by tourism. The shore zone of reservoirs with a high share of agricultural land and non-forest semi-natural areas was less loaded.

Introduction

Tourism is a complex phenomenon and its new forms are still appearing. Many of them are based on the tourist resources of the water environment and its nearest surroundings. These include sea, river and lake tourism (JENNINGS 2003, *Tourism and global…* 2006, *Lake tourism…* 2006, VENOHR et al. 2018). In the mid-twentieth and early 2000s there was a significant increase in interest in water tourism, leisure, recreation and sport (JENNINGS 2007). Water tourism is developing in two directions. The

Address: Grażyna Furgała-Selezniow, University of Warmia and Mazury in Olsztyn, ul. M. Oczapowskiego 5, 10-719 Olsztyn, Poland, e-mail: graszka@uwm.edu.pl

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first one is the so-called nature-based tourism. It is defined as tourism "associated primarily with the direct use of an almost untouched natural environment" (KUENZI and MCNEELY 2008, WOLF et al. 2019). Tourists undertake activities such as bird watching, canoeing and nature hiking (JENNINGS 2007, ROCHELLE et al. 2015). Nature-based tourism often supports the development of rural areas (LANFRANCHI et al. 2014, LIU et al. 2018). In Canada, lake tourism is often seen as a synonym for rural tourism as a result of its numerous lakeside cottages (SMITH 2003, TUOHINO 2015). The second direction is tourism related to destinations (especially cities) located by water bodies, based on the infrastructure offered by them. Tourism is one of the important functions of cities (BIAGI et al. 2019). The importance of this function is growing, which results from the fact that they are more and more frequently chosen for tourist destinations (ŁAPKO and PANASIUK 2019). Although lakes have long been an important area of recreational and tourism activity, they were not the subject of international tourism research until the beginning of the 21st century (HADWEN et al. 2005, *Lake tourism*… 2006, TUOHINO 2015).

In recent decades, demand for tourism and leisure services has steadily increased worldwide. The development of tourism, in addition to its contribution to the national economy is a major force affecting negatively basic environmental resources (air, water, biodiversity, soil and land) both in tourist destinations (locally) (DORNIER and MAURI 2018, TRANCOSO GONZÁLEZ 2018, among others) and globally (BUCKLEY 2011). The increase in some areas of tourism (e.g. cruise tourism) and the increased frequency and seasonality of holiday trips have a severe impact on the environment at regional and local level (BRIDA and ZAPATA 2009, CARIC and MACKELWORTH 2014, BARNETT et al. 2018, MACNEILL and WOZNIAK 2018). Major tourist destinations face challenges in terms of water supply, waste management and waste water treatment. In addition, changes in land use/land cover (LU/LC), air and noise pollution from the means of transport and landscape disturbances caused by the constantly increasing surface area of built-up land are also quite common consequences of tourism development (ASHA 2013, ATZORI et al. 2018, TRANCOSO GONZÁLEZ 2018). According to GÖSSLING (2002), there are five main aspects of environmental change related to tourism: change in land use and land cover, excessive energy consumption, reduction of biodiversity and introduction of invasive species, spread of diseases and changes in the perception and understanding of the natural environment.

The shore zone is an ecologically and economically important component of lake ecosystems. The natural shores are a habitat for aquatic and terrestrial organisms, affect the cycling of nutrients and organic matter

between land and water and reduce soil erosion (SCHMIEDER 2004). The recreational and aesthetic potential of the lake shores makes them attractive for human settlement, hence the development of settlement is often concentrated around the lakes (SCHNAIBERG et al. 2002, WALSH et al. 2003). The problem, concerning protected areas, was described in relation to conflicts resulting from excessive pressure from the growing number of tourists (WHITELAW et al. 2014, SPENCELEY 2017).

Most forms of tourist activity are not environmentally friendly (BUCKLEY 2011, ASHA 2013). Research on lakes and other water landscapes in the context of tourism development was relatively rare at the beginning of the 21st century, especially in the field of tourism geography (TUOHINO 2015). Most previous studies in this field have focused on the direct impact of tourism on water bodies (BURGIN and HARDIMAN 2011, DOKULIL 2014, TANDYRAK et al. 2016). RAMAZANOVA et al. (2019) made a number of important observations on the indirect effects of lake tourism, with particular emphasis on the accommodation sector in the vicinity. The impact of tourism development on the shore zone of the lake is an important research topic, but publications in this field are scarce (FOLGADO-FERNÁNDEZ et al. 2019). The aim of our study was to assess the load of tourism on the shore zone of the selected lakes of the Mrągowo Lakeland. The lakes selected for the study differed in the type of land use/cover around the reservoir. Moreover, the aim of the study was to propose a complex method of assessing the impact of tourism on the shore zone of lakes on the basis of a set of indices, in connection with the LU/LC type around lakes.

Material and Methods

Study area

The study focused on four lakes located in the Mrągowo Lakeland (Masurian Lakeland, North-Eastern Poland, Central Europe), in the district of Mrągowo. Czos Lake (area 279.1 ha) is situated within the administrative boundaries of Mrągowo city (Figure 1). The western and northern part of its shore zone (more than two thirds) is a typical urban area. The southern edge of the lake is protected under the Natura 2000 network (PLB280008 Puszcza Piska). Probarskie Lake (201.4 ha) is separated from the southern edge of Lake Juksty by about 300 meters wide strip of land. The national road No. 16 and railway tracks run in this strip. The lake is located in the buffer zone of the Mazurian Landscape Park. It is a typical lake located in rural areas (more than 20% of the

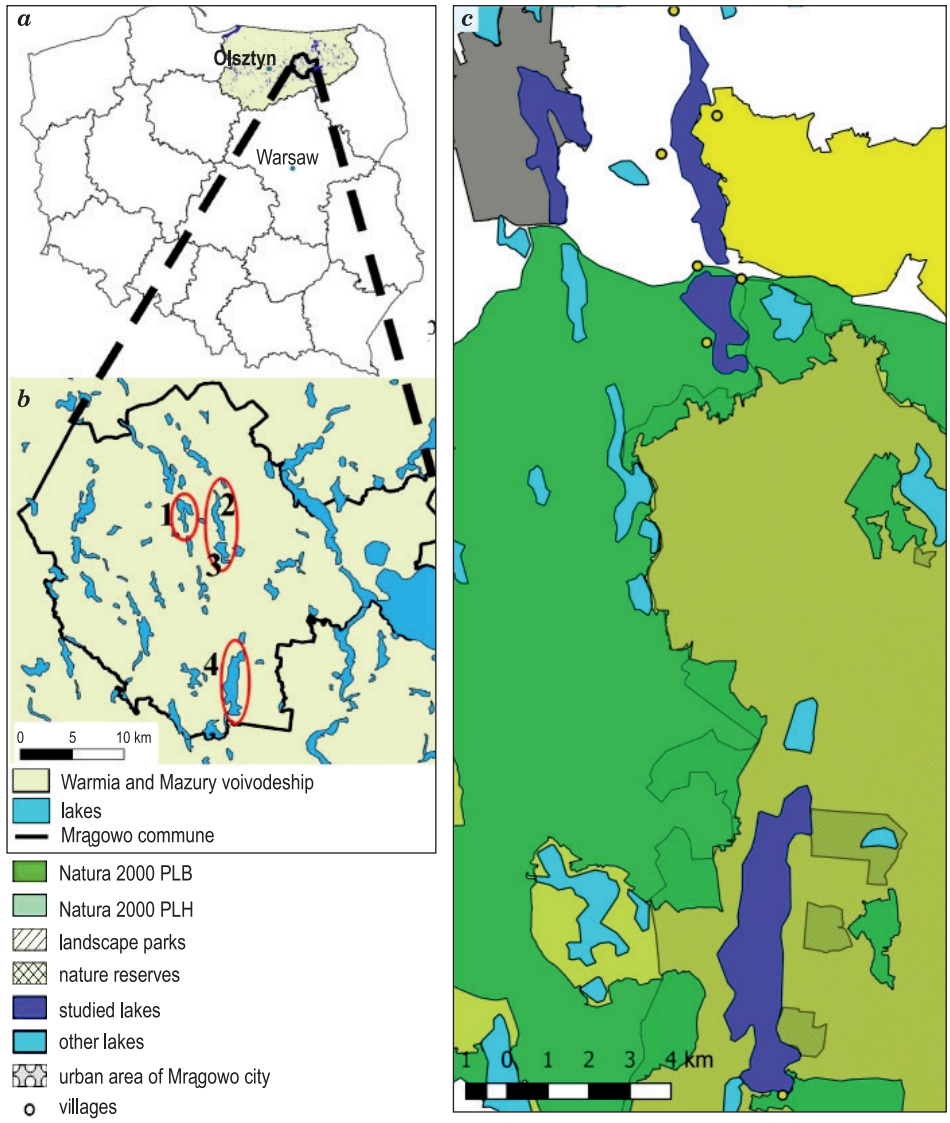

Fig. 1. Mrągowo District with studied lakes; *a* – warmińsko-mazurskie voivodship; *b* – Mrągowo District; *c* – study area

shore zone is covered by rural housing). In the close vicinity of the shoreline of the lake there are four villages: Kosewo, Probark, Nowy Probark and Jakubowo. Lake Juksty (330 ha), located about 3 km east of Lake Czos, is protected within the Protected Landscape Area of the Legińsko-Mrągowskie Lakes and its eastern edge belongs to Natura 2000 network (Masurian Tortoise Refuge Baranowo PLH 280055). Its shore zone is

mainly covered by semi-natural and agricultural land. The built-up areas cover less than 1.5% of the shore zone of the lake. The largest of the studied lakes, Mokre (814 ha), is located about 10 km south of Lake Probarskie and is situated entirely in the Mazurian Landscape Park. In addition, a fragment of the shore zone (along a 1.7 km long shoreline) in the south-eastern part of the lake is protected as a nature reserve (the Reserve Royal Pine). The north-east edge of the lake belongs to nature reserve Krutynia.

Fig. 2. The different landscape types of the lake shore zone: *a* – urban (Lake Czos); *b* – rural (Lake Probarskie), *c* – semi-natural (Lake Juksty), *d* – forest (Lake Mokre)

Mokre Lake is also protected under the Natura 2000 network (PLH280048 Ostoja Piska and PLB280008 Puszcza Piska). Approximately 85% of the shore zone is covered by forest and less than 5% is occupied by built-up areas (mainly belonged to the Zgon village). Figure 2 shows the different landscape types in the lakeshore zone.

Methodology

In the assessment of the tourist attractiveness of studied lakes, the scoring method was applied, which qualifies water reservoirs to appropriate classes of tourist and recreational attractiveness on the basis of the number of points awarded for the value of selected morphometric parameters (Deja 2001). Seven morphometric features of the lake were taken into account: surface area, mean depth, shoreline development index, elongation index, index of the shore zone cover by vegetation [%], index of the lake

surface area cover by water vegetation [%], index of the shore zone cover by forest [%]. The point values for the individual indices were in the range from 0 to 6. Table 1 illustrates the classification into attractiveness classes.

Table 1

Classes of lakes attractiveness depending on the evaluation score (DEJA 2001)

Attractiveness level (in points)	Class of attractiveness
Unattractive ≤ 10	IV
Moderately attractive 10.1–16	III
Attractive 16.1–22	II
Very attractive > 22	I

In order to assess the impact of tourism on the lake environment, an index defining the impact of tourism development on lake shores was created (MIKA 2004). While constructing this index, it was necessary to identify various types of areas used by tourism in the lake shore zone. In order to differentiate the character and directions of tourism impact on the shore zone of lakes, an evaluation system was created (Table 2).

Table 2

Forms of tourist use of the natural environment of lakes shore zone and evaluation of their effect (according to MIKA 2004, FURGAŁA-SELEZNIOW et al. 2010)

Forms of tourist use	Type of area	Area symbol (Pi)	Type of effect	Bi valuation score
Tourist settlement	technogenic areas under permanent tourist use	P1	permanent transformation of land use, denaturalization of environment, permanent changes of landscape, noise, litter, vehicles pollutions, wastewater and sewage	5
Active recreation areas	beaches, marinas, water equipment rentals, piers, sport grounds, playgrounds, car parks, catering facilities	P2	trampling and mechanical damage of plants, erosion of shores, litter, pollution of lakeshores, water turbidity and pollution, noise	4
	tent fields, camp sites, bicycle trails	P3	destruction of plants and soil cover, noise, litter, vehicles pollutions, wastewater and sewage	3
	hiking trails, angling piers and sites, paths	P4	trampling and mechanical damage of plants, pollution, soil erosion, water turbidity and pollution	2
Other recreational areas	recreational plots, green areas around tourist facilities, green areas around villages	P5	change to the type of use of green areas, noise, litter, wastewater and sewage	1

The index of the impact of tourism infrastructure on the environment of the lake shore zone was applied in accordance with the proposal of MIKA (2004) and modified by FURGAŁA-SELEZNIOW et al. (2010). The index was calculated according to the formula (MIKA 2004):

$$K = ((\sum Pi \cdot Bi))/Po$$

where:
K – index of the impact of tourism on the shore zone of lakes (tourism impact index)
Pi – type of area under tourist use [ha]
Po – reference unit area – total area of a delimitated field [ha]
Bi – valuation score

The K index ranged from 0 to 5. In order to group areas in the shore zone according to the degree of tourism load, appropriate classes were created depending on the value of the K index (Table 3).

Table 3

Classes due to the level of tourism impact on the environment according to the tourism impact index (K)

Class	Impacts on environment	Range of K value
I	very high	$K \geq 1.0$
II	high	$0.1 \leq K < 1.0$
III	moderate	$0.01 \leq K < 0.1$
IV	small	$0 < K < 0.01$
V	none	$K = 0$

The study was provided using current topographic maps scaled 1:10 000. All raster maps were obtained from Provincial Centre for Geodetic and Cartographic Documentation in Olsztyn. Vector polygons (territories and area objects) and lines (linear objects) were made over the raster topographic maps using QGIS 2.18 software. A 200 m wide strip of land was determined around the shoreline of the lakes. In the water part of shore zone the 100 m wide strip was determined. The designated area was divided into basic fields covering about 500-metre sections along the shoreline. In order to calculate the K index, data on the current use of the shore zone for tourism and recreation were collected. The total area of land under particular forms of tourism and recreation was determined according to the criteria given in Table 2. The total area of the basic fields was also calculated. A vector polygon layer was created to compare the LU/LC status of the shore zone of studied lakes. The status of land use and land cover at the studied area was additionally verified by direct observations in summer seasons of 2017–2018.

Morphometric data of the studied lakes were assumed according to JAŃCZAK (*Atlas jezior*… 1999) and CHOIŃSKI (2006). Topographic maps of the area were used as well as bathymetric plans of the lakes which were amended on the basis of data gathered during the field studies. Tourist information brochures and available literature were used for additional information.

An analysis of the density of accommodation and other tourist facilities was also carried out, as well as an index of the development of other tourist facilities in the shore zone of the studied lakes. In the analysis of the density of accommodation facilities, all the accommodation facilities located in the 200-metre strip around the lakes were taken into account. For the analysis of the other tourist facilities, water equipment and/or bicycle rentals, gastronomy facilities, car parks, beaches, access to reservoirs and other facilities used by tourists (e.g. sports fields, recreation areas, amphitheatre) were selected.

The index of density of accommodation facilities (P_A) was calculated according to the following formula:

$$P_A = N/S$$

In the formula, N is the number of beds, S is the area of the delimited strip [km^2].

The density index of the other tourist facilities (P_O) was calculated according to the following formula (FURGAŁA-SELEZNIOW et al. 2019):

$$P_O = (N_O \cdot 100)/S$$

In the formula, S is the area of the delimited strip [km^2], N_O is the number of other tourist facility items.

Calculation of the other tourist facilities development index (B_O) was performed according to the formula created for the present study:

$$B_O = N_O \cdot 100/N$$

In the formula, N is the number of beds, N_O is the number of other tourist facility items.

In addition, the dock density index (D_d) was calculated as a measure of the impact of docks and related activities on the water shore zone of the lake. The actual state of development was determined by manual identification of artificial water structures such as marinas, harbors, bathing sites with piers, large recreational platforms and small fishing piers on the orthophotograph maps. According to BECK et al. (2013) and DUSTIN and JACOBSON (2015), these structures are referred to in this study as "docks". Smaller docks (small angling piers) have been designated as single objects,

while large docks (with several branches) have been designated as complex objects. Each branch within a complex dock has been treated as a separate object so that the total number of docks better reflects the actual impact of the individual docks on the development status of the water shore zone of the lake. According to DUSTIN and JACOBSON (2015) lakes with D_d index over 5 has been defined as highly developed:

$$D_d = D/M$$

In the formula, D means the number of docks and M is the shoreline length [km].

Information on the number of accommodation facilities, the number of beds at these facilities and the number of other tourist facility items was obtained from the Tourist Information Office in Mrągowo, direct interviews with accommodation facility managers and a website with the function of browsing and searching for accommodation offers under the domain e-turysta.pl and Google Maps.

Results

Two lakes (Juksty and Czos) are classified as the first class of tourist attractiveness (Table 4). Both lakes were characterized by a low index of the lake surface area covered by water vegetation and the highest shoreline development index. The Probarskie and Mokre lakes were classified

Table 4

Assessment of the degree of tourist attractiveness of selected lakes in Mrągowo Lakeland

Parameter	Juksty		Czos		Probarskie		Mokre	
	value	score	value	score	value	score	value	score
Surface area [ha]	330	6	279.1	5	201.4	5	814	6
Mean depth [m]	8	2	11.1	2	9.2	2	12.7	2
Shoreline development index	3.11	5	2.53	4	1.88	2	2.52	4
Elongation index	5.95	2	5.31	2	1.7	2	2.08	2
Index of the shore zone cover by vegetation [%]	13	4	32	2	50	2	8	4
Index of the lake surface area cover by water vegetation (%)	5	4	9	4	15	2	10	2
Index of the shore zone cover by forest [%]	5	1	23	3	35	3	85	1
Total	24		22		18		21	
Class	I		I		II		II	

as the second class of tourist attractiveness. The shores of Mokre Lake were the most forested. Probarskie Lake had the lowest shoreline development index. Table 5 presents the number of accommodation facilities in

Table 5

Accommodation objects in studied localities

Lake	Type of accomodation object	Number of accomodation objects	Number of beds in tourists objects		Range of beds
			permanent	seasonal	
Juksty	guesthouse	6	98	0	6–30
	summer house	3	10	18	6–12
	guest rooms	2	16	12	12–16
	agritourism farm	1	15	0	15
	total	**12**	**139**	**30**	**6–30**
Czos	hotel	8	869	49	9–182
	guesthouse	2	132	0	32–100
	summer house	1	6	0	6
	guest rooms	9	145	0	5–40
	agritourism farm	1	8	0	8
	camping	1	0	300	300
	total	**22**	**1160**	**349**	**5–300**
Probarskie	hotel	1	50	0	50
	guesthouse	2	48	0	18–30
	resort	1	0	150	150
	guest rooms	8	82	34	4–30
	summer house	4	9	40	4–24
	agritourism farm	3	41	15	15–24
	camping	2	0	120	60
	total	**21**	**230**	**359**	**4-150**
Mokre	guesthouse	2	16	12	12–16
	resort	2	36	474	210-300
	camping	3	0	240	30-130
	total	**7**	**52**	**726**	**12-00**
Total number of objects and beds		**62**	**1581**	**1464**	**4-474**

the analyzed zone divided into particular types, the number of accommodation places and the range of the number of accommodation places in particular types of facilities. It was found that the most numerous group of objects were guest rooms (20). The largest number of beds was offered by hotels (968) and camping sites (420). More than half of all accommodation

places and more than one third of all accommodation facilities (including eight out of nine hotels) were located on Lake Czos (Table 5). More than half of the seasonal accommodation facilities and almost half of the seasonal beds were located on Lake Probarskie. Apart from accommodation facilities, there were other tourist facilities at the lakes under study (Table 6).

Table 6

A comparison of other tourist facilities in the shore zone of the studied lakes

Lake	Gastronomy facilities	Car parks	Access to reservoirs	Beaches	Water equipment and/or bicycle rentals	Remaining objects	Total
Juksty	4	0	0	0	0	0	4
Czos	10	12	2	2	4	9	39
Probarskie	4	1	2	4	1	1	13
Mokre	6	5	2	1	4	2	20
Total	**24**	**18**	**6**	**7**	**9**	**12**	**76**

The largest number of other tourist facilities was located on Lake Czos. Remaining objects included: a promenade, an eco-marina, an amphitheatre, a playground, an outdoor gym and five sports fields for Lake Czos, while for Lake Probarskie the bicycle trail and for Lake Mokre the sculpture gallery in Zgon and the bicycle trail around the lake. The most numerous group of objects were angling piers and walking platforms. The largest number of other tourist objects were located on Czos and Probarskie lakes.

The area of the distinguished shore zone of the examined lakes ranged from 237.7 ha for Probarskie Lake to 401.1 ha for Mokre Lake, and the water part of the shore zone area from 67.6 ha to 258 ha respectively. The number of distinguished basic fields ranged from 13 (Probarskie) to 38 (Juksty). The K index for the whole shore zone ranged from 0.140 (Juksty) to 1.892 (Czos) – Table 7. The value of the accommodation facility density index (P_A) and other tourist facility density index (P_O) was definitely the highest for Lake Czos and the lowest for Lake Juksty. The values of the other tourist facility development index (B_O) were very similar for all the lakes under study. Dock density index (D_d) reached values above 5 for Probarskie and Czos lakes, whose water part of the shore zone was defined as highly developed.

More than 50% of the shore zone of Lake Juksty and about 25% of the shore zone of Lake Czos were the areas without any tourist facilities. Basic fields without any tourist facilities did not occur in the shore zone of Probarskie and Mokre lakes (Figure 3). The most uniform pressure of

tourism on the shore zone was noted for Lake Probarskie (*K* ranged from 0.011 to 0.85). The maximum range in *K* index was noted for the lake Czos (0–4.97).

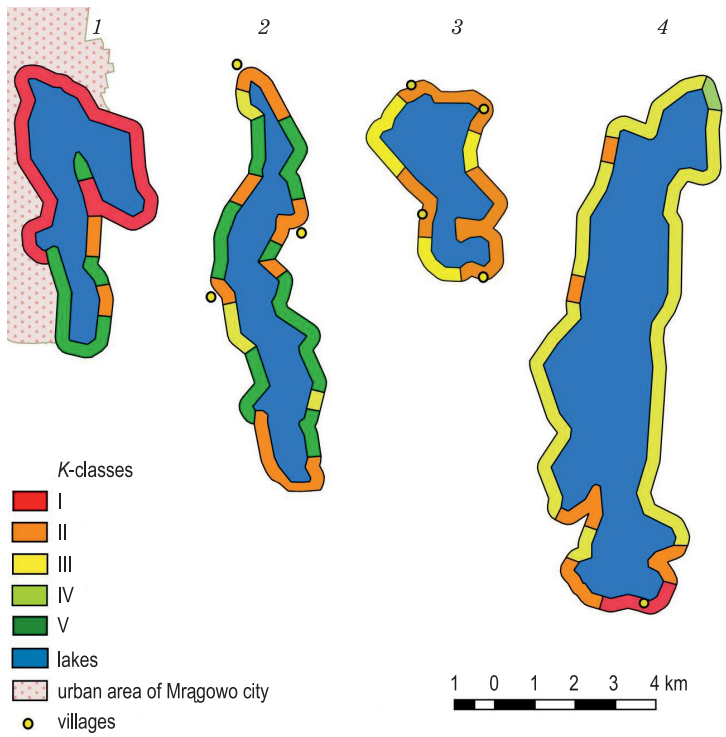

Fig. 3. Spatial pattern of tourism impact in shore zone of studied lakes: *1* – Czos; *2* – Juksty; *3* – Probarskie; *4* – Mokre

Discussion and conclusions

All lakes selected for the study were attractive or very attractive in terms of tourism. According to the authors of this study, the method of evaluation of the shore zone of lakes, taking into account their natural conditions (DEJA 2001), was inadequate in the case of the forest cover share. The lakes Juksty (5% of the forest) and Mokre (85% of the forest) received one point each. For this reason Mokre Lake was classified lower (attractiveness class II) than Juksty Lake (attractiveness class I), whereas P_A, P_O and *K* indices assumed higher values for Mokre Lake, which means that it was more often visited by tourists than Juksty Lake. According to the authors, the lake which shore zone is covered with forest in more than 40% should receive the maximum number of points (5). The authors believe

that reducing the number of points for the "excess" of forests in the shore zone of the lake is unjustified, if only because resting in forest areas positively affects the well-being of tourists (BIELINIS et al. 2018, 2019, TAKAYAMA et al. 2019).

The shore zone of the lakes, on which there are towns and villages, is the most loaded with tourism. The tourism load level is related to the share of residential buildings in the shore zone of the lake. The location of Czos Lake within the administrative borders of Mrągowo city makes the development level of the shore zone approximately 60%. This lake was characterized by the highest values of tourism load indices in the shore zone. The presence of four villages in the shore zone of Lake Probarskie caused that 20% of the zone was covered by residential buildings. Lake Probarskie was characterized by relatively high values of tourism load indices in the shore zone. Probarskie and Czos lakes were also characterized by the highest values of dock density index (D_d above 5). According to the criteria defined by DUSTIN and JACOBSON (2015), the water part of the shore zone of these lakes was highly developed. According to BURAK et al. (2004), small coastal settlements have become tourist destinations in Turkey as a result of legal and institutional incentives to invest in tourism. This has had a negative impact on landscape aesthetics and the loss of fertile land. However, the greatest pressure on coastal areas was exerted by urbanization. Masurian lakes are in a similar situation (FURGAŁA-SELEZNIOW et al. 2020). This results from the development strategy of the warmińsko-mazurskie voivodeship. One of the most important components of this strategy is the development of tourism as one of the main economic sectors in the region (*Strategia rozwoju…* 2013). Lake Mokre, whose shore zone is covered mainly by forest and which is located in the Mazurian Landscape Park, had a moderately developed water part of the shore zone. Its land shore zone was developed very unevenly, mainly by large seasonal resorts and campsites located on the south side of the lake, near the village of Zgon. Mokre Lake is located on the canoeing trail (116 km long) of the Krutynia River. These route is considered to be of international importance (LIJEWSKI et al. 1998). The eastern shore of the lake is undeveloped, there is only a bicycle path that runs around the whole lake.

The development of tourism can stimulate economic growth and play an important role in promoting the social development of backward regions. However, the development of lake tourism should also take into account the protection of lakes. Benefits for the natural environment should be transformed into economic and social benefits (DAVID et al. 2012). According to DAVID et al. (2012), the main principles of lake tourism development are environmental protection, reasonable use of resources,

coherent management and sustainable use. The planning of lake tourism development should be based on scientific principles and include monitoring of tourism development plans and prevention of damage resulting from inappropriate development. Despite very high tourist attractiveness (class I) and short distance (3 km) from Mrągowo city, Lake Juksty was characterised by significantly lower tourism load indices than other lakes. This was probably due to the particular type of land cover in the shore zone, in which the share of forest and residential buildings was negligible (ca. 5% and 1.5% respectively). The level of attractiveness plays an important role in the assessment of the place as a tourist resource. For planners and tourism managers it is of key importance. However, from the point of view of tourists, it is not enough to ensure sufficient investment in this area. Tourists expect the appropriate infrastructure to be in place before they visit the site (ALAEDDINOGLU and CAN 2011). Tourism in relation to places far away from settlements, in relatively natural places is defined as nature-based tourism (PRISKIN 2001).

Most of the year-round accommodation places were in the shore zone of lakes Czos and Probarskie. A clear correlation was observed between the level of residential development in the shore zone of the studied lakes and the number of all-year-round beds. The lowest number of all-year-round (and at the same time the highest number of seasonal) beds was recorded in the shore zone of Lake Mokre. The Probarskie Lake was the second lake in terms of the number of seasonal beds in the shore zone. The shore zone of the lake was covered by 35% forest and 20% by rural buildings. The development index of other tourist facilities (*Bo*) was very similar for all the studied lakes. This fact indicates that the development of the other tourist facilities is proportional to the accommodation base. At each lake there were more than two other tourist facilities (2.2–2.6) per 100 beds (no distinction was made between year-round and seasonal beds). The location of four villages around Probarskie Lake results in uniform spatial distribution of the shore zone tourism load. The presence of a bicycle path around Lake Mokre was the reason for the lack of completely undeveloped areas. However, the tourism impact on the shore zone of this lake was uneven. Tourist objects had been concentrated mainly on its southern edge in the vicinity of a typical tourist village Zgon (which in itself is a tourist attraction due to the nature of its architecture). Two thirds of the eastern shore of Lake Juksty was occupied by a turtle refuge. The highest level of tourism load was noted at both ends of this guttering lake, situated longitudinally. The southern, undeveloped edge of Lake Czos was outside the city border. The city border at this place run along the lake shoreline. In this part of the shore zone there were completely

undeveloped areas (V class of load). Such areas were also located in the south-western part of the shore zone of the lake, already within the boundaries of the municipality of Mrągowo. At the same time, the lake was the only one with areas in its shore zone that belong to the highest tourism load class (I).

The research showed the influence of the type of land use/cover (LU/LC) around the lakes on the level of tourism load on their shore zone. The proposed method makes it possible to study the tourism load on the shore zone of lakes in relation to the LU/LC type around the lakes. The set of indices enables a reliable assessment of the impact of tourism on the shore zone of lakes and monitoring its changes over time. The studies aimed at monitoring the impact of tourism on the natural environment, especially the lake shore zone, which is a vulnerable ecotone, are scarce. The paper fills a gap in the literature by presenting a useful tool for such analyses. The methodology used in this paper can be used in the study of most glacial lakes in Europe and other regions of the world to monitor the threats arising from the tourist development of the lake shore zone.

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References

ALAEDDINOGLU F., CAN A.S. 2011. *Identification and classification of nature-based tourism resources: western Lake Van basin*, Turkey. The 2nd International Geography Symposium GEOMED 2010. Procedia Social and Behavioral Sciences, 19: 198–207, doi: 10.1016/j.sbspro.2011.05.124.

ASHA T.E. 2013. *Impact of tourism on environment: responding to global challenges*. Pauline Journal of Research and Studies, 1(1): 169–182.

Atlas jezior Polski, vol. III. *Jeziora Pojezierza Mazurskiego i Polski Południowej*. 1999. Ed. J. Jańczak. Bogucki Wydawnictwo Naukowe S.C., Poznań.

ATZORI R., FYALL A., MILLER G. 2018. *Tourist responses to climate change. Potential impacts and adaptation in Florida's coastal destinations*. Tourism Management, 69: 12–22, doi: 10.1016/j.tourman.2018.05.005

BARNETT M.J., JACKSON-SMITH D., HAEFFNER M. 2018. *Influence of recreational activity on water quality perceptions and concerns in Utah. A replicated analysis*. Journal of Outdoor Recreation and Tourism, 22(1): 26–36, doi: 10.1016/j.jort.2017.12.003.

Beck M., Vondracek B., Hatch L.K., Vinje J. 2013. *Semi-automated analysis of high-resolution aerial images to quantify docks in glacial lakes*. ISPRS Journal of Photogrammetry and Remote Sensing, 81: 60–69, doi: 10.1016/j.isprsjprs.2013.04.006.

Biagi B., Ladu M.G., Meleddu M., Royuela V. 2019. *Tourism and the city. The impact on residents' quality of life*. International Journal of Tourism Research, 1–14, doi: 10.1002/jtr.2326.

Bielinis E., Łukowski A., Omelan A., Boiko S., Takayama N., Grebner D.L. 2019. *The effect of recreation in a snow-covered forest environment on the psychological wellbeing of young adults: randomized controlled study*. Forests, 10: 827, doi: 10.3390/f10100827.

Bielinis E., Takayama N., Boiko S., Omelan A., Bielinis L. 2018. *The effect of winter forest bathing on psychological relaxation of young Polish adults*. Urban Forestry & Urban Greening, 29: 276–283, doi: 10.1016/j.ufug.2017.12.006.

Brida J.G., Zapata S. 2009. *Cruise tourism. Economic, socio-cultural and environmental impacts*. International Journal of Leisure and Tourism Marketing, 1: 205–226, doi: 10.1504/IJLTM.2010.029585.

Buckley R. 2011. *Tourism and environment*. Annual Review of Environment and Resources, 36: 397–416, doi: 10.1146/annurev-environ-041210-132637.

Burak S., Dogan E., Gazioglu C. 2004. *Impact of urbanization and tourism on coastal environment*. Ocean & Coastal Management, 47, 9: 515–527, doi: 10.1016/j.ocecoaman.2004.07.007.

Burgin S., Hardiman N. 2011. *The direct physical, chemical and biotic impacts on Australian coastal waters due to recreational boating***.** Biodiversity and Conservation, 20: 683–701, doi: 10.1007/s10531-011-0003-6.

Caric H., Mackelworth P. 2014. *Cruise tourism environmental impacts and the perspective from the Adriatic Sea*. Ocean & Coastal Management, 102: 350–363, doi: 10.1016/j.ocecoaman.2014.09.008.

Choiński A. 2006. *Katalog jezior Polski*. Wydawnictwo Naukowe UAM.

Dávid L., Baros Z., Patkós C., Tuohino A. 2012. *Lake tourism and global climate change: an integrative approach based on Finnish and Hungarian case-studies*. Carpathian Journal of Earth and Environmental Sciences, 7(1): 121–136.

Deja W. 2001. *Przydatność rekreacyjna i atrakcyjność jezior Pojezierza Iławskiego*, In: *Przydatność rekreacyjna strefy brzegowej jezior Polski*, Bogucki Wydawnictwo Naukowe S.C., pp. 7–16.

Dokulil M.T. 2014. *Environmental impacts of tourism on lakes*. In: *Eutrophication. Causes, consequences and control*. Eds. A.A. Ansari, S.S. Gill. Springer Science + Business Media Dordrecht, pp. 81–88, doi: 10.1007/978-94-007-7814-6_7.

Dornier R., Mauri C. 2018. *Overview. Tourism sustainability in the Alpine region. The major trends and challenges*. Worldwide Hospitality and Tourism Themes, 10, 2: 136–139, doi: 10.1108/WHATT-12-2017-0078.

Dustin D.L., Jacobson P.C. 2015. *Predicting the extent of lakeshore development using GIS datasets*. Lake and Reservoir Management, 31, 3: 169–179, doi.org/10.1080/10402381.2015.1053010.

Folgado-Fernández J.A., Di-Clemente E., Hernández-Mogollón J.M., Campón-Cerro A.M. 2019. *Water tourism. A new strategy for the sustainable management of water-based ecosystems and landscapes in Extremadura (Spain)*. Land, 8: 2, doi: 10.3390/land8010002.

Furgała-Selezniow G., Jankun M., Hakuć-Błażowska A. 2019. *Analiza funkcji turystycznej wybranych miejscowości nad jeziorem Jeziorak*. (*Analysis of a tourist function in towns and villages on the lakeshore of Lake Jeziorak*). Prace i Studia Geograficzne, 64, 2: 35–47.

Furgała-Selezniow G., Jankun-Woźnicka M. Mika M. 2020. *Lake regions under human pressure in the context of socio-economic transition in Central-Eastern Europe. The case study of Olsztyn Lakeland, Poland*. Land Use Policy, 90: 104350. doi: 10.1016/j.landusepol.2019.104350.

Furgała-Selezniow G., Sankiewicz D., Skrzypczak A., Mamcarz A. 2010. *The impacts of tourism and recreation on lake shores: a case study of Limajno and Stobajno Lakes in north-eastern Poland*. In: *Lake tourism research. Towards sustaining communities and lake environments*. Eds. N. McIntyre, R. Koster, H. Lemelin. Lakehead University, Centre for Tourism & Community Development Research. Occasional Research Publication, 99–111.

GÖSSLING S. 2002. *Global environmental consequences of tourism*. Global Environmental Change, 12: 283–302, doi: 10.1016/S0959-3780(02)00044-4.

HADWEN W.L., BUNN S.E., ARTHINGTON A.H., MOSISCH T.D. 2005. *Within-lake detection of the effects of tourist activities in the littoral zone of oligotrophic dune lakes*. Aquatic Ecosystem Health Management, 8, 159e173, doi: 10.1080/14634980590953211.

JENNINGS G. 2003. *Marine tourism*. In: *Sport and adventure tourism*. Ed. S. Hudson. New York, Haworth Hospitality Press, pp. 125–164

JENNINGS G. 2007. *Water-based tourism, sport, leisure, and recreation experiences*, pp. 1–20, doi: 10.4324/9780080468310.

KUENZI C., MCNEELY J. 2008. *Nature-based tourism*. In: *Global risk governance. Concept and practice using the IRGC Framework*. Eds. O. Renn, K. Walker. Springer: Berlin/Heidelberg, Germany, pp. 155–178.

Lake tourism. An integrated approach to lacustrine tourism systems. Aspects of tourism. 2006. Eds. C.M. Hall, T. Härkönern. Channel View Publications, Clevedon, Buffalo, Toronto, pp. 3–26.

LANFRANCHI M., GIANNETTO C., DE PASCALE A. 2014. *The role of nature-based tourism in generating multiplying effects for socio economic development of rural areas*. Calitatea Access to Success Bucharest, 15, 140: 96–100.

LI G., YANG X.J., LIU Q., ZHENG F. 2014. *Destination island effects. A theoretical framework for the environmental impact assessment of human tourism activities*. Tourism Management Perspectives, 10: 11–18, doi: 10.1016/j.tmp.2013.12.001.

LIJEWSKI T., MIKUŁOWSKI B., WYRZYKOWSKI J. 1998. *Geografia turystyki Polski*. Polskie Wydawnictwo Ekonomiczne, Warszawa.

LIU S., CHEUNG L.T.. LO A.Y., FANG W. 2018. *Livelihood benefits from post-earthquake nature-based tourism development: a survey of local residents in rural China*. Sustainability, 10, 699, doi: 10.3390/su10030699.

ŁAPKO A., PANASIUK A. 2019. *Water tourism as a recipient of transport services on the example of Szczecin*. Transportation Research Procedia, 39: 290–299, doi: org/10.1016/j.trpro.2019.06.031.

MACNEILL T., WOZNIAK D. 2018. *The economic, social, and environmental impacts of cruise tourism*. Tourism Management, 66: 87–404, doi: 10.1016/j.tourman.2017.11.002.

MIKA M. 2004. *Turystyka a przemiany środowiska przyrodniczego Beskidu Śląskiego*. Instytut Geografii i Gospodarki Przestrzennej UJ, Kraków.

PRISKIN J. 2001. *Assessment of natural resources for nature-based tourism. The case of the Central Coast Region of Western Australia*. Tourism Management, 22, 6: 37–48, doi: 10.1016/S0261-5177(01)00039-5.

RAMAZANOVA M., BULAI M., URSU A., TORTELLA B., KAKABAYEV A. 2019. *Effects of tourism development on surface area of main lakes of Shchuchinsk-Burabay resort area, Kazakhstan*. European Journal of Tourism Research, 21: 69–86.

ROCHELLE S., MORRISON C., CASTLEY J.G. 2015. *Birdwatching and avitourism. A global review of research into its participant markets, distribution and impacts, highlighting future research priorities to inform sustainable avitourism management*. Journal of Sustainable Tourism, 23, 8–9: 1257–1276, doi: 10.1080/09669582.2014.924955.

SCHMIEDER K. 2004. *European lakeshores in danger – concepts for a sustainable development*. Limnologica, 34: 3–14, doi: 10.1016/S0075-9511(04)80016-1.

SCHNAIBERG J., RIERA J., TURNER M.G., VOSS P.R. 2002. *Explaining human settlement patterns in a recreational lake district: Vilas County*, Wisconsin, USA. Environmental Management, 30: 24–34, doi: 10.1007/s00267-002-2450-z.

SMITH S. 2003. *Lake tourism research. Themes, practice and prospects*. In: *International Lake tourism Conference 2–5 July*. 2003. Ed. T. Härkönen, Savonlinna, Finland. Publication of the Savonlinna Institute for Regional Development and Research. Joensuun Yliopistopaino, Savonlinna, pp. 13–25.

SPENCELEY A. 2017. *Tourism and protected areas: comparing the 2003 and 2014 IUCN World Parks Congress*. Tourism and Hospitality Research, 17, 1: 8–23, doi: org/10.1177/1467358415612515.

Strategia rozwoju społeczno-gospodarczego województwa warmińsko-mazurskiego do roku 2025. 2013. Sejmik Województwa Warmińsko-Mazurskiego, https://strategia2030.warmia.mazury.pl/strategia/, access: 20.11.2019.

TANDYRAK R., PARSZUTO K. GROCHOWSKA J. 2016. *Water quality of Lake Ełk as a factor connected with tourism, leisure and recreation on an urban area.* Quaestiones Geographicae, 35, 3: 51–59.

TAKAYAMA N., MORIKAWA T., BIELINIS E. 2019. *Relation between psychological restorativeness and lifestyle, quality of life, resilience, and stress-coping in forest settings*. International Journal of Environmental Research and Public Health, 16, 1456, doi:10.3390/ijerph16081456.

TRANCOSO GONZÁLEZ A. 2018. *Venice. The problem of overtourism and the impact of cruises*. Investigaciones Regionales. Journal of Regional Research, 42: 35– 51.

TUOHINO A. 2015. *In search of the sense of Finnish lakes. A geographical approach to lake tourism marketing*. Oulu, Nordia Geographical Publications, 44: 5.

Tourism and global environmental change ecological. Economic, social and political interrelationships. 2006. Eds. S. Gössling, M.C. Hall. London Routledge.

VENOHR M., LANGHANS S.D., PETERS O., HÖLKER F., ARLINGHAUS R., MITCHELL L., WOLTER C. 2018. *The underestimated dynamics and impacts of water-based recreational activities on freshwater ecosystems*. Environmental Reviews, 26, 2: 199–213, doi: 10.1139/er-2017-0024.

WALSH S.E., SORANNO P.A., RUTLEDGE D.T. 2003. *Lakes, wetlands, and streams as predictors of land use/cover distribution environmental management*, 31, 2: 198–214, doi: 10.1007/s00267-002-2833-1.

WHITELAW P.A., KING B.E.M., TOLKACH D. 2014. *Protected areas, conservation and tourism – financing the sustainable dream*. Journal of Sustainable Tourism, 22, 4: 584–603,doi: org/10.1080/09669582.2013.873445.

WOLF I.D., CROFT D.B., GREEN R.J. 2019. *Nature conservation and nature-based tourism: a paradox?* Environments, 6, 9: 104, doi: 10.3390/environments6090104.

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THE REASONS OF CULLING OF CATTLE IN DAIRY COWS HERD – A REVIEW

***Małgorzata Dziekiewicz-Mrugasiewicz*[1], *Małgorzata Wierzbicka*[2]**

[1] ORCID: 0000-0003-3823-4446
[2] ORCID: 0000-0002-9086-3058
Department of Large Animal Diseases with Clinic, Faculty of Veterinary Medicine
University of Life Sciences, Warsaw, Poland

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Abstract

The culling of cows is an important element in dairy farming, which has a huge impact on the progress of breeding. Culling of animals in the herd is understood as the removal of the animal from the herd as a result of selling the animal to another farm, slaughter and death or euthanasia of animal. The reasons for culling can be divided into voluntary and involuntary or the division into economic and biological reasons. Economic culling (voluntary) is a conscious action of the breeder aimed at increasing the breeding and functional value of the herd, while biological culling is connected with occurrence of metabolic and infectious diseases as well as reproduction and limbs problems or sudden deaths of the animal. In recent years, biological culling has caused significant economic losses to the herd. Improving this can be achieved through intensive prevention and herd health monitoring.

Introduction

The culling of cows is an important element in dairy farming, which has a huge impact on the progress of breeding. At the same time, it can cause significant economic losses (OLECHNOWICZ et al. 2011). The elimination of cows from the herd is a topic rarely undertaken by veterinarians, but very important to breeders. A veterinarian has a great influence on the decision on culling in the herd. The aim of the study was to present the main reasons for the culling of dairy cows and the factors influencing the decision. Knowing the causes of culling by a veterinarian in a herd allows to know the main problems occurring in a herd, which enables effective treatment and prevention of problems. This information can improve the welfare and performance of the cows.

Address: Małgorzata Dziekiewicz-Mrugasiewicz, Warsaw University of Life Science, Nowoursynowska St. 100, 02-797 Warszawa, Poland, e-mail: malgorzata_dziekiewicz_mrugasiew@sggw.edu.pl

Culling definition

Culling of animals in the herd is understood as the removal of the animal from the herd as a result of selling the animal to another farm, slaughter and death or euthanasia of animal (FETROW et al. 2006). The decision to eliminate animals from the herd is one of the most important decisions for the breeder, affecting the economics of production and breeding progress. The reasons for culling can be divided into voluntary and involuntary or the division into economic and biological reasons (FETROW et al. 2006). Economic culling (voluntary) is a conscious action of the breeder aimed at increasing the breeding and functional value of the herd, while biological culling (involuntary) is connected with occurrence of metabolic and infectious diseases as well as reproduction and limbs problems or sudden deaths of the animal (POKORSKA et al. 2012).

According to world data, the level of cows culling in the herd should be between 25-35% (ROGER et al. 1988), which is confirmed by the results in the publications: United States 35% (BASCOM et al. 1998), Canada 19.9% (CRAMER et al. 2009), Great Britain-Scotland 33.7% (CHIUMA et al. 2013), 25% (Bell et al. 2001), 31.6% in Finland (RAJALA-SCHULTZ et al. 1999). In literature there are different data about the level of culling of cows in Poland, from 7.31% to 26.8% (POKORSKA et al. 2012); 20–35% (OLECHNOWICZ et al. 2011); to 26.4% (ZIĘTARA et al. 2013). Culling for biological reasons occurs much more often than for economic reasons. The biological to economic reasons ratio is 68% to 32% (BELL et al. 2010) or 74% to 26% (ANSERI-LARI et al. 2012).

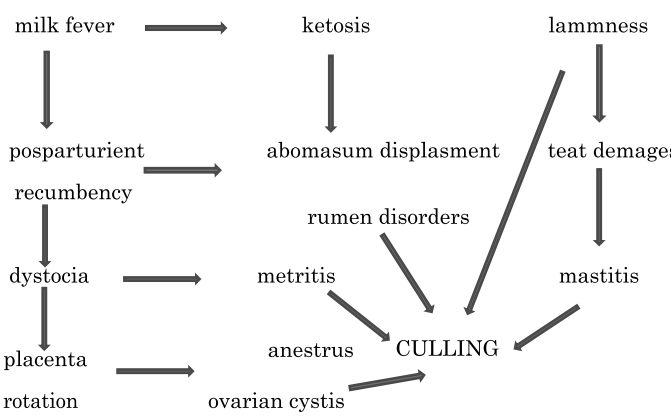

Fig. 1. Relationships between the occurrence of diseases and the relationship between these diseases and culling

Source: own study based on RAJALA-SCHULTZ et al. (1999).

These proportions have changed in recent decades. In the 1980s, the elimination of cows for economic reasons (low production) was the cause of culling four times more often than in the 1990s and in the 21st century. In recent years, biological (involuntary) culling has prevailed. High-yield cows are more often eliminated for health reasons (WEIGEL et al. 2003). This may be related to the fact that for many years increasing the milk yield was the most important in breeding. Selecting only one trait contributed to increase in culling cows as a result of health problems – biological culling.

Factors influencing cows culling

The cows culling decision is complex. Many factors are taken into account, such as: age, number of lactations, stage of lactation, milk yield, health status, reproductive indicators. The elimination of a cow from the herd is also related to economic indicators such as: milk price, livestock purchase price and cost of introducing new animals to the herd. The decision of culling of cows in herd is made individually by the breeder. Sometimes, two farmers having a cow with the same problem may make different culling decisions (BASCON and YOUNG 1998).

The age of the cow and the lactation number associated with that is an important factor influencing culling decisions. Many studies have shown that the older the cow and the greater number of lactation is, the greater is the likelihood that the animal will be eliminated from the herd (ALHMAN et al. 2011, BELL et al. 2010, RILANDO et al. 2020). On the other hand, the analyses of ARMENGOL and FRAILE (2018) and RAJALA-SCHULTZ et al. (1999) showed that the culling increased after the first and second lactation especially, it could be due to elimination of cows with very low yields in first lactation. The high percentage of culling during and after the first lactation may be also related to the fact that in this age group there are the most random cases, understood as accidents and injuries (RILANDO et al. 2020). The poor condition of primiparous cows may be related to the negative influence of diseases during the rearing period (SZULC et al. 1983). Again, the risk of eliminating animals from the herd increases above sixth lactation.

The analysis of Polish authors (POKORSKA et al. 2012) also indicated that the highest percentage of culling is observed in the first and second lactations and amounted to 23.2% (ZIĘTARA et al. 2013) and 23% (JUSZCZAK et al. 2003), respectively.

It should be emphasized that the early elimination of animals generates high economic losses for the farm. Currently, the period of use of cows

in dairy herds is relatively short, averaging 3–4 years and usually not exceeding 6 years (ADAMCZYK et al. 2017). Recently, there has been an increasing tendency in breeding to extend the useful life of cows. Genetic selections in this direction are already underway. It may be related to the strong development of organic farming. In Sweden, organic farming already occupies 6% of the dairy market (ALHMAN et al. 2011).

Another factor influencing the elimination of animals from the herd is the stage of lactation – the day of lactation. Many authors (HERTL et al. 2010, PINEDO et al. 2010, RILANDO et al. 2020) noted in their studies that the highest percentage of elimination of animals from the herd occurs in the first and last months of lactation. It is associated with the occurrence of postpartum complications, metabolic diseases and diseases of the digestive system. Moreover, random accidents in primiparous cows occurred in the first 100 days of lactation and amounted 61.4% of animals (JUSZCZAK et al. 2003). Lactation usually lasts for 305 days, a significant extension of the lactation time contributing to an increase in culling because it affected fertility. Cows culling due to infertility and diseases of the reproductive system systematically increased along with the extension of the lactation period, reaching 57.02% of culling over the 485th day of lactation (SAWA et al. 2012). However, as it was shown by the studies of Guliński et al. and Krzyżewski et al. (2003), extending the lactation time did not reduce the milk production, it even increased the results, reaching an increase of 55.5% with the extension of lactation by 180 days.

Cows culling may depend on the climate and temperature. Cows are more often eliminated in the summer season from May to October (ARMENGOL and FRAILE 2018, PINEDO et al. 2010, SCHNEIDER et al. 2007, ANSARI-LARI et al. 2012). In southern countries such as Spain, the south of the USA the highest percentage of culling is mainly during the summer months, while in northern Europe – in Sweden and Finland, in September – October. Interestingly, it has been noticed that falls in the summer season are higher on farms in France (SEEGERS et al. 1998) and in the USA (WILLIAMS et al. 2015) without heat stress management systems.

Economic reasons for culling

The main cause of economic culling in dairy cows is low milk yield, high somatic cell count or age of cows and disturbances in animal body structure. In the literature, low yield appears as the main cause of economic culling. The percentage of culling for this reason varies in Poland and around the world and ranges from 23.5% in studies in Spain (ARMEN-

GOL and FRAILE 2018) to 2.0 % in Poland (ZIĘTARA et al. 2013). The primiparous cows were most often culled in the first months of lactation (ARMENGOL and FRAILE 2018, POKORSKA et al. 2012). In the following lactations, the percentage of elimination of cows from the herd decreased, increasing successively after 5th lactation. Taking into account the productivity of individual cows, the culling concerned mainly the least productive cows, whose results differed from the average. The culling percentage decreased along with the increase in productivity. Interestingly, after reaching the productivity above 9000 liters culling started to increase again (ZIĘTARA et al. 2013), which could be due to the occurrence of reproductive problems and metabolic diseases in these cows.

Another reason for culling is the age of the cow – old age of the cow, this group including cows over 6 years of age (ALHMAN et al. 2011). In Iran, due to old age, cows over 10 years of age are eliminated, the culling rate for this reason is 8.1% per year (ANSARI-LARI et al. 2012).

Biological reasons for culling

The most common reasons for the biological culling of cows in a herd are reproductive problems: infertility, perinatal disorders, placental retention, miscarriages, as well as mastitis and teat injuries. The next group consists of digestive system and metabolic diseases, such as: abomasal displacement, diarrhea, ketosis, postpartum paralysis, and hypomagnesaemia. In addition, limb problems such as lameness and hoof disease are another reason why cows are eliminated from the herd. The last groups of diseases that affect culling are infectious diseases and other or unrecognized causes, and sudden falls. Figure 1 shows the relationship between the occurrence of diseases and the relationship between these diseases and culling.

Biological reasons – reproductive disorders

According to the literature data, reproductive disorders are the main cause of the elimination of cows from the herd. In Poland, this problem is related to infertility and ranges from 23.4% (POKORSKA et al. 2012), 39.6% (ADAMCZYK et al. 2017) to 44.4% (ZIĘTARA et al. 2013). Worldwide, culling for this reason is also one of the most important, but it varies from 12.5% (RILANDO et al. 2020) to 32.6% (ANSARI-LARI et al. 2012).

Infertility is a separate reproductive problem affecting culling decisions. The presence of anestrus and the presence of ovarian cysts may

contribute to it (BASCON et al. 1998, GRÖHN et al. 1998). The reasons for the elimination of cows from the herd, in addition to sterility, may be: miscarriages, difficult delivery, retention and torsion of the placenta, metritis, vaginal or uterine prolapse and lack of estrus. The probability of eliminating animals for reproductive reasons increases with the age and the milk yield. It is significantly higher above the third lactation (ARMENGOL and FRAILE 2018).

The deterioration of fertility may be influenced by many factors, among the reasons are management and feeding errors, too high milk yield and the animal's genetic characteristics. In addition, infertility may be affected by the occurrence of metabolic factors, such as ketosis or subclinical rumen acidosis.

Culling due to heavy labor, placental retention or torsion, and uterine inflammation are completely time-dependent and due to this, culling occurs in the first month after delivery. Frequency of culling due to metritis as a cause increases at the end of the lactation (RAJALA-SCHULC et al. 1999, GRÖHN et al. 1998), although in the studies of Gröhn et al. (1998) the incidence of uterine inflammation did not have a significant influence on the culling decisions. In addition, sterility was more often the cause of cows' culling on large-scale farms than on smaller organic farms (AHLMAN et al. 2011).

Biological reasons – injuries and inflammation of the mammary gland

Within the pathology of the mammary gland, mastitis is often considered to be the most common disease in dairy cows which contribute to significant economic losses in dairy farming. Teat injuries are the second reason why cows are eliminated from the herd as it causes incread risk of infection which can, in turn, cause subclinical and clinical symptoms of mastitis. For this reason, in Poland culling amounts in 16.13%, and in the world from 22.26% (RILANDO et al. 2020) in Estonia, to 24% in Sweden (SCHREIDER et al. 2007). The problems with the mammary gland were the main cause of cows culling on organic farms in Sweden, amounting to 26.75% (AHLMAN et al. 2011). Clinical mastitis caused by Gram-negative bacterias (*E. coli, Klebsiella*, *Citrobacter, Pseudomonas*) or others such as *Arcanobacterium pyogenes* (now *Trueperella pyogenes*), *Mycobacterium*, fungi and algae may contribute to the loss or death of the animals (HERTL et al. 2011). Mammary gland infections caused by *E. coli* are usually acute or peracute, which can even lead to the death of the animal.

Trueperella pyogenes is a pathogenic agent, which in combination with other bacteria causes purulent inflammation of the mammary gland, called summer mastitis (SMULSKI et al. 2011). Mastitis caused by *S. aureus* is difficult to treat and control because of its ability to spread and penetrate protective barriers, the ability to adhere, to produce enzymes and toxins, and survive in the mammary gland in encysted abscesses. Therefore, one of the ways to combat this microorganism in the herd is to eliminate infected cows. Mastitis as the cause of culling was more common in older cows than in the primiparous cows, it was most common during the second lactation (SCHREIDER et al. 2007). Moreover, HERTL et al. (2011) noticed that a greater number of deaths and losses of a primiparous cows occurs in the first as well as in the 6th and 7th month of lactation because of mastitis. In multiparous cows, he noticed that cows are eliminated due to mastitis most often in the first month of lactation; in the second month this number significantly decreases, while after the third month it systematically increases. He also noted that the cow deaths were more frequent in spring and summer. In the primiparous cows, the most frequently isolated bacteria were *E. coli* and *Streptococcus* spp. In multiparous cows the isolates mainly consisted of Gram-negative bacteria.

In the studies of SEEGERS et al. (1998), cows were eliminated from the herd mostly at the beginning of lactation due to problems with the mammary gland. The highest percentage of cows being removed from the herd for this reason occurred in the 4–6th lactations. When making decisions about culling due to mastitis, the type of treatment, the effectiveness of treatment and the status of pregnancy are taken into account. It was noted that the age of first calving influences the decision to cure (SEEGERS et al. 1998).

Biological reasons – problems with limbs and hooves

Lameness in cows is one of the most common diseases of dairy cattle. The incidence of hoof diseases can be as high as 70–80% of animals in the herd (BEDNARSKI 2013). The elimination of cows due to hoof disease is an increasingly common cause of culling, ranging from 3.6% in Spain (ARMENGOL and FRAILE 2018) to 19% in Japan (GOTO et al. 2015) or 26.24% in Estonia (RILANDO et al. 2020), in other countries, the percentage of cows elimination for this reason is at a similar level: 5.9% in Sweden (AHLMAN et al. 2011), 8.62% in Poland (POKORSKA et al. 2011), 8.1% in USA (PINEDO et al. 2012). Lameness is a disease with a multifactorial etiology, which includes genetic and technological aspects – related to the farm equipment

and the way of keeping and caring for animals, including the feeding of cows. The occurrence of hoof disorders may also be influenced by the presence of metabolic disorders, disturbances of the acid-base balance and mineral deficiencies. Hoof diseases can be non-infectious or infectious. The most common non-infectious hoof diseases in cattle are: laminitis, interdigital dermatitis, plantar and bulb ulcer, extravasation of the hoof capsule, fissures-cracks in the wall of the hoof, white line disease, white line abscess, nails and finger fractures.

The non-infectious diseases of the skin of the fingers and hoof material can become infected and this can even lead to purulent inflammation of the area. The disease is caused by pyogenic and necrotic bacteria – *A. pyogenes, F. necrophorum,* staphylococci, micrococci and *Pseudomonas aeruginosa* and others (DIRKSEN et al. 2009, MORDAK et al. 2008). Major among the infectious diseases are foot rot, caused by *Fusobacterium necrophorum* (DIRKSEN et al. 2009, SIKORA 2013) and papillomatous dermatitis (Mortellaro's disease – the so-called "strawberry" disease or foot warts) caused by *Treponema* spp., *Fusobacterium* spp., *Bacterioides* spp., *Dichelobacter nodosus* (SLAWUTA et al. 2005).

It has been found that non-infectious diseases or injuries are more likely to be the cause of cows culling than infectious diseases (CRAMER et al. 2009). The presented hoof diseases may be the direct or indirect cause of culling. They can cause reduced milk production and its lack of suitability for consumption, weight loss due to lower food intake and poorer feed utilization, fertility disorders, shortening the period of use and increasing the herd repair (OLECHNOWICZ et al. 2011).

Initially, hoof disease may be asymptomatic – without marked lameness or gait disturbance, but they are uncomfortable and painful. This contributes to the reduction of animal welfare, reduces production, and increases the occurrence of metabolic and reproductive disorders. Often, the relationship between metabolic disorders, reproductive disorders, mastitis and hoof diseases is not recognized, and a cause other than hoof disease is mentioned as the cause of culling.

Additionally, it has been noticed that in farms smaller than 20 cows, the culling problem due to hoof problems is smaller and increases with farm size (RILANDO at al. 2020). On large farms, free-standing rearing is predominantly used, which increases the risk of limb diseases (COOK et al. 2003), additionally, walking on hard, contaminated ground in the barn, and long standing during milking increases the risk of hoof diseases. It is believed that culling due to hoof disease can occur throughout the lactation period, while the studies by RAJALA-SCHULTZ and GRÖHN (1999) show that cows are culled for this reason mostly in the second lactation.

Biological reason – metabolic disorders or diseases of the digestive system

The most common metabolic and digestive system disorders causing cows to be culled are: displacement, bloat and/ or torsion of the abomasum, diarrhea, ketosis, fatty liver syndrome, hypocalcemia, hypomagnesaemia, acidosis, and downer cow syndrome (ARMANGOL and FRAILE 2018). These diseases can account for up to 18.9% of the diseases in a herd (GOTO et al. 2015). The rate of elimination of cows due to metabolic diseases and digestive system problems is 7.2% in Spain (ARMANGOL and FRAILE 2018), 2.2% in Sweden (ALHMAN et al. 2011) and 7.88% in Poland (POKORSKA et al. 2012).

These disorders occur much more often in multiparous cows than in the primiparous ones, and the level of culling for these reasons increases with the efficiency and number of lactation and milk yield (RILANDO et al. 2020). Moreover, the occurrence of these diseases depends on the stage of lactation, and most often occurs during the first 30–60 days of lactation.

The occurrence of postpartum paralysis (hypocalcaemia) is directly related to the increased demand for calcium due to the production of large amounts of colostrum immediately after delivery. A poorly balanced diet during the dry period and after parturition leads to severe hypocalcemia (calcium level below 1.5 mmol/L) (BEDNARSKI 2013), which contributes to the occurrence of clinical symptoms such as decreased appetite, weakness, lack of motor coordination. In the next stage recumbence and rumen bloat are observed. Failure to implement timely treatment may result in the death of the animal. The disease occurs up to 48 hours after delivery, therefore culling for this reason occurs in the first 30 days of lactation (RAJALA-SCHULTZ and GRÖHN 1999).

An insufficient amount of magnesium in the feed can lead to the occurrence of grass tetany (hypomagnesaemia). Disease is common especially in pasture-raised cows in the spring season when the grass grows rapidly and contains a small amount of magnesium. Clinical signs such as shakiness, stiff gait, muscle stiffness, muscle tremors can be observed. The next stage of the disease is lateral recumbence and opisthotonus. As in the case of postpartum paralysis, if the treatment is not applied, the animal will die, late treatment may be ineffective, and the administration of magnesium alone may lead to cardiac arrest (BEDNARSKI 2013). The occurrence of hypomagnesaemia contributes to the death of the animal or it is the reason for culling of the animal from the herd, mainly on smaller farms where pasture rearing is prevalent (RAJALA-SCHULTZ and GRÖHN 1999).

The abomasal displacement occurs in the postpartum period as a result of a sudden decrease in the volume of the uterus. The so-called "empty space" created in the cow promotes the displacement of the abomasum. In addition, poor diet, hypocalcaemia, stress, metabolic disorders, and toxaemia predispose to the occurrence of this disease (BEDNARSKI et al. 2013). Symptoms of abomasal displacement involve: decreased appetite and milk yield, weight loss and recumbence. Conservative treatment often does not lead to healing; it is advisable to perform surgery, which generates additional costs. Therefore, the occurrence of this disease may predispose to culling of animal from the herd. Culling due to this reason was around 5.3% Ketosis as the cause of cows culling occurs at a similar level and amounts to 5% (GRÖHN et al. 1998).

Clinical metabolic diseases as a cause of cows culling occur more often in small or ecological farms. In large-scale farms, culling for this reason occurs sporadically, and subclinical forms are much more common.

Biological reasons – respiratory system diseases and other diseases

Diseases of the respiratory system as the reason of culling are rare and their causes are often classified as other, in published studies the percentage of elimination for this reason was 0.7% and it is most common in the primiparous cows. The culling for this reason is usually statistically insignificant (ARMANGOL and FRAILE 2018). Cows that are positive for diseases under infectious disease control programs such as neosporosis, BVD, paratuberculosis or bovine leukemia are eliminated. The culling for infectious diseases is about 1–2%. It was shown that the most common culling for this reason took place in the 4th lactation and was as high as 6.6%. (ARMANGOL and FRAILE 2018).

Biological reasons – accidents on the farm and unknown reasons

Another cause of culling can be severe injuries to equipment, tools or buildings on the farm. This group also includes errors related to wrong administration of drugs, poisoning with chemical compounds. The elimination rate for cows for this reason can be as high as 7.7% (ARMANGOL and FRAILE 2018). Other reasons for the elimination of cows are often understood as: chronic diarrhea, emaciation, arthritis, traumatic reticulo-perito-

nitis, endocarditis, myopathies or septicemia (ANSARI-LARI et al. 2012, WALDNER et al. 2009). In these cases, the exact cause can be established by post-mortem examination

Sudden deaths of cows constitute a separate group in the culling, additional tests are often skipped and the cause of the cow's death cannot be precisely determined. The cause of the cows's death may be one specific factor or it is the result of multiple health problem with the animal. The proportion of undiagnosed causes of cows culling or dying is approximately 4–5% (ARMANGOL and FRAILE 2018, PINEDO et al. 2010, POKORSKA et al. 2012). In the studies of PINEDO et al. (2010) conducted in the United States in the Mississippi region, sudden deaths for unknown reasons occurred in dairy herds of cows more often than infertility and accidents.

Conclusions

High cow productivity has been the main focus of selection in many dairy herds in recent times, leading to adverse effects such as reproductive problems, metabolic diseases and mastitis. This contributed to an increase in biological (involuntary) culling at the expense of economic (voluntary) culling.

Reproductive disorders, in particular infertility, are the main reason for eliminating cows from the herds in Poland and generally in the other countries. Mastitis and teat injuries are the second cause of cows culling from the herd. The elimination of cows due to hoof disease is an increasingly common cause of culling. Metabolic disorders or disease of the digestive system can also be cause of culling, but the elimination of cows for this reason is less than 10%. Diseases of the respiratory system, infectious diseases or others diseases such as: diarrhea, arthritis, endocarditis, myopathies and sepsis, can all be causes of culling, but are not significant.

The biological (involuntary) culling causes significant losses in the dairy farming. To avoid the economic losses for this reason is it very important to conduct monitor and prevention of the herd. Reduction of excessive culling should based on caring for animal welfare by proper feeding of cows in the perinatal period and early lactation, prevention of metabolic diseases and infectious diseases as well as systematic and correct hoof trimming. In addition, very important aspect of biosecurity and infectious diseases prevention is keeping animals in closed system, without any transfers of animals into the herd, using only heifers born in the herd.

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References

ADAMCZYK K., MAKULSKA J., JAGUSIAK W., WĘGLARZ A. 2017. *Associations between strain herd size, age at first calving, culling reason and lifetime performance characteristics in Holstein--Friesian cows*. Animal, 11: 327–334.

ALHMAN T., BERGLUND B., RYDHMER L., STRANDBERG E. 2011. *Culling reasons in organic and conventional dairy herds and genotype by environmental interaction for longevity*. J. Dairy Sci., 94: 1568–1575.

ANSARI-LARI M., MOHEBBI-FANI M., ROWSHAN-GHASRODASHTI A. 2012. *Causes of culling in dairy cows and its relation to age at culling and interval from calving in Shiraz, Southern Iran*. Vet. Res. Forum, 3(4): 233–237.

ARMENGOL R., FRAILE L. 2018. *Descriptive study for culling and mortality in five high-producing Spanish dairy cattle farms (2006–2016)*. Acta Vet. Scand., 60: 45.

BASCOM S., S., YOUNG A.J.: 1996. *A summary of the reason why farmers cull cows*. J. Dairy Sci., 81: 2299–2305.

BEDNARSKI M. 2013. *Choroby bydła podstawy diagnostyki i terapii*. Apra-wetpress s.c. – Lecznica Dużych Zwierząt, pp. 312.

BELL M.J., WALL E., RUSSELL G., ROBERTS D.J., SIMM G. 2010. *Risk factor for culling in Holstein – Friesian dairy cows*. Vet. Record, 167: 238–240.

CHIUMA D., CHAGUND M.G., MACRAE A.I., ROBERTS D.J. 2013. *Predisposing factors for involuntary culling in Holstein-Friesian dairy cows*. J. Dairy Res., 80(1): 45–50.

COOK N.B. 2003. *Prevalence of lameness among dairy cattle in Wisconsin as a function of housing type and stall surface*. J. Am. Vet. Med. Assoc., 233: 1324–1328.

CRAMER G,. LISSEMORE K.D., GUARD C.L, LESLIE K.E., KELTON D.F. 2009. *The association between foot lesion in Ontario Holstein cows*. J. Dairy Sci., 92: 2572–2579.

DIRKSEN G., GRÜNDER H.D., MATTHAEUS S. 2009. *Choroby wewnętrzne i chirurgia bydła*. Galaktyka, pp. 1071–1076.

FETROW J., NORDLUND K.V., NORMAN H.D. 2006. *Invided Review. Culling. Nomenclature, definition and recommendation*. J. Dairy Sci., 89: 1896–1905.

GOTO A., NAKANA K., KATAMOTO H. 2016. *The association of culling and death rate within 30 days after calving with productivity or reproductive performance in dairy herds in Fukuoka, South Japan*. J. Vet. Med. Sci., 78(4): 587–592.

GRÖHN T.Y., EICKER W., DUCROCQ V., HERTL J.A. 1998. *Effect of diseases on the culling of Holstein dairy cows*. J. Dairy Sci., 81: 966–978.

HERTL J.A., SCHUKKEN Y.H., BAR D., BENNETT G.J., GONZALES R.N., RAUCH B.J., WELCOME F.L., TAUER L.W., GROHN Y.T. 2011. *The effect of recurrent episodes of clinical mastitis caused by gram-positive and gram-negative bacteria and other organism on mortality and culling in Holstein dairy cows*. J. Dairy Sci., 94: 4863–4877.

JUSZCZAK J., HIBNER A., ZIEMIŃSKI R., TOMASZEWSKI A. 2003. *Przyczyny i konsekwencje przedwczesnego brakowania krów*. Med. Weter., 59(5): 432–435.

KRZYŻEWSKI J., REKLEWSKI Z. 2004. *Wpływ przedłużonych laktacji krów na wydajność, skład chemiczny i jakość mleka oraz wskaźniki reprodukcyjne*. Med. Weter., 60: 76–79.

MORDAK R. 2008. *Kulawizny u krów – wieloprzyczynowy problem zdrowotny*. Życie Wet., 83(4): 288–291.

OLECHNOWICZ J., JAŚKOWSKI J.M. 2011. *Reason for culling due to lameness and economic losses in dairy cows*. Med. Weter., 67(6): 618–621.

PINEDO P.J., DE VRIES A., WEBB D.W. 2010. *Dynamic of culling risk with disposal codes reported by dairy herd improvement dairy herds*. J. Dairy Sci., 93: 2250–2261.

POKORSKA J., KUŁAJ D., ORMIAN M. 2012. *Przyczyny brakowania krów rasy polskiej holsztyńsko--fryzyjskiej odmiany czarno-białej użytkowanych w fermie wielkotowarowej*. Rocz. Nauk. PTZ, 8(2): 17–24.

ROGER G.W., DANIL M.C., DENTINE M.R., NORMAN H.D. 1989. *Relation among proofs for survival, culling, among yields in first and later lactation*. J. Dairy Sci., 72: 528–532.

SCHNEIDER M. P., STRANDBERG E., EMMANUELSON U., GRANDINSON K., ROTH A. 2007. *The effect of veterinary-treated clinical mastitis and pregnancy status on culling in Swedish dairy cows*. Prevent. Vet. Med., 80: 179–192.

SEEGERS H., BEAUDEAU C., FOURICHON C., BAREILLE N. 1998. *Reasons for culling in French Holstein cows*. Prevent. Vet. Med., 36: 257–271.

SIKORA J. 2013. *Wybrane choroby bydła*. SIMA WLW., pp. 187–189.

SLAWUTA P., NICPOŃ J., MRÓZ K., NICPOŃ J. 2005. *Etiopatogeneza, diagnostyka i terapia wybranych nieurazowych chorób palców u bydła*. Med. Weter., 61(11): 1221–1224.

SMULSKI S., MALINOWSKI E., KACZMAROWSKI M., LASSA H. 2011. *Występowanie, postacie i czynniki etiologiczne zapaleń wymienia w Polsce w zależności od wielkości gospodarstwa*. Med. Weter., 57(3): 190–193.

SZULZ T., DUBICKI A., NOWICKA M. 1983. *Choroby cieląt i ich wpływ na późniejszą użytkowość mleczną*. Zesz. Nauk. AR Wrocław, 25: 13–19.

RAJALA-SCHULTZ P.J., GROHN Y.T. 1999. *Culling of dairy cows. Part I. Effects of diseases on culling in Finnish Ayrshire cows*. Preventive Vet. Med., 41: 195–208.

RILANTO T., REIMUS K., ORRO T., EMANUELSON U., VILTROP A., MOTUS K. 2020. *Culling reasons and risk factors in Estonian dairy cows*. BMV Vet. Res., 16(173): 1–16.

ROGER G.W., VAN ARENDONK J.A.M., MC DANIEL B.T. 1988. *Influence of production and prices on optimum culling rates and annualized net revenue*. J. Dairy Sci., 71: 3453–62.

ROGER G.W., VAN ARENDONK J.A.M., MC DANIEL B.T. 1988. *Influence of production and prices on optimum culling rates and annualized net revenue*. J. Dairy Sci., 71: 3453–62.

WEIGEL K.A., PALMER R.W., CARAVIELLO D.Z. 2003. *Investigation of factors affecting voluntary and involuntary culling in expanding dairy herds in Winsconsin using survival analysis*. J. Dairy Sci., 86: 1482–1486.

WILLIAMS K.E.B., BERKE O., PEARL D.L., HAND K., KELTON D.F. 2015. *Heat stress related dairy cow mortality during heat waves and control periods in rural Southern Ontario from 2010–2012*. BMC Vet. Res., 11: 219.

ZIĘTARA W., ADAMSKI M., MIRKOWSKA Z. 2013. *Rzeczywisty a optymalny okres użytkowania krów mlecznych*. Rocz. Naukowe Ekonomii Rol. i Rozwoju Obszarów Wiejskich, 100(3): 90–100.