UNIVERSITY OF WARMIA AND MAZURY IN OLSZTYN

Polish Journal of Natural Sciences

38

(1/2023)

PUBLISHER UWM
OLSZTYN 2023

The Polish Journal of Natural Sciences is indexed and abstracted
in Biological Abstracts and Biosis Previews

The print edition is the primary version of the Journal

The Journal is also available in electronic form on the websites
http://www.uwm.edu.pl/polish-journal/ (home page)
https://czasopisma.uwm.edu.pl/index.php/pjns/about (electronic platform; submissions)

PL ISSN 1643-9953

Publisher UWM Olsztyn
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

Edition 65 copies; publisher's sheets 8.0; number of printed sheets 6.5
Print Zakład Poligraficzny UWM w Olsztynie
order number 656

TABLE OF CONTENTS

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 38(1): 5–18, Y. 2023

DOI: 10.31648/pjns.8724

PROSPECTS FOR TRADITIONAL LIVESTOCK BREEDING OF POLISH RED CATTLE WITH THE AGREEMENT OF BIODIVERSITY PROTECTION*

***Anna J. Korzekwa*[1], *Marta Siemieniuch*[2], *Julia Kaczmarczyk*[3], *Władysław Kordan*[4]**

[1] ORCID: 0000-0002-1044-9288
[2] ORCID: 0000-0002-5117-9659
[3] ORCID: 0000-0003-4685-0775
[4] ORCID: 0000-0001-6842-932X

[1,3] Department of Biodiversity Protection, Institute of Animal Reproduction and Food Research Polish Academy of Sciences, Olsztyn, Poland
[2] Research Station, Institute of Animal Reproduction and Food Research Polish Academy of Sciences, Popielno, Poland
[4] Department of Animal Biochemistry and Biotechnology, Faculty of Animal Bioengineering University of Warmia and Mazury in Olsztyn, Poland

Key words: welfare, native cattle, grazing, meat quality, Polish Red cattle, biodiversity protection.

Abstract

Meat from conservation breeding programs receives higher scores for juiciness, texture and aroma. Polish Red cattle kept on low-input farms where livestock are raised extensively (pastured in summer and fed farm-made fodder in winter) and highly appropriate for breeding due to moderate productivity and the high quality of milk and meat. In the past, Polish native cattle breeds were extensively grazed to preserve bird habitats in the same ecosystem. The demand for beef from "grass-fed" cattle is on the rise because consumers have a growing interest in animal health and well-being, environmental sustainability, and meat products with a modified nutritional profile and lower fat content. Two-thirds of the energy supplied by feed is required to maintain body functions of cattle. Energy expenditure increases in response to both low and high temperatures that differ from the thermal optimum of each species. Cattle are sensitive to both heat and winter stress, and the maintenance of thermal homeostasis requires additional energy when animals are exposed to suboptimal temperatures. Based on the knowledge, the aim of this review is understanding that to ensure high levels of animal welfare, the grazing of traditional native cattle breeds should be planned by calculating nutrient yields in pasture grass and basal diets, not only for cows, but also for other free-ranging herbivores.

Address: A.J. Korzekwa, Polish Academy of Sciences, ul. Tuwima 10, 10-748 Olsztyn, Poland, e-mail: a.korzekwa@pan.olsztyn.pl

* The research was financed from the statutory funds of Department of Biodiversity Protection, Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olsztyn, Poland.

Characteristics and history of Polish Red cattle

Italy and France are leaders in the conservation of native cattle breeds. The conservation of local livestock breeds in those countries is highly promoted by the Food and Agriculture Organization of the United Nations with the aim of upholding agricultural traditions and the local culture.

Polish Red, Polish Red-and-White, White-Backed, and Polish Black-and-White cattle represent only a small percentage of Polish native cattle breeds that increase biodiversity (LITWIŃCZUK et al. 2012). Polish Red cattle originated from small wild brachycephalic (short-horned) cattle in Central Europe and Scandinavia. These animals have red to dark red coloring, and they were introduced to Polish territory at the beginning of the 16th century. Between the two world wars, Polish Red cattle accounted for 25% of the domestic cattle population. Before World War II, the Polish Red had the status of an international breed, and by the mid-20th century, it was found in nearly all Polish regions. The breed accounted for 22% of the entire Polish cattle population in the 1950s and for 18% in the 1960s (2 million head). In 1973, Polish Red was reared exclusively in three districts in southern Poland as part of a regionalization scheme. A conservation breeding program for Polish Red cattle was introduced in Nowy Sącz Voivodeship at the end of 1975 to maintain this valuable breed. The regionalization scheme and the conservation breeding program were abandoned in 1982, and Polish Red cattle were replaced by more productive breeds and animals crossbred with foreign breeds, mainly the Angler (*Bydło. Ochrona ras…* 2023).

Historical and current grazing conditions of Polish Red cattle

Until the 1970s, grazing played an important role in Polish Red cattle breeding, but uncontrolled bark chewing and trampling caused significant damage to tree stands. However, grazing improved light access and the supply of animal manure, which led to changes in the structure of the local biocenosis. Cattle grazing led to the formation of thermophilic oak forests which emerged as one of the most floristically rich forest communities in Poland (LIBBERT 1933) and became protected as a priority habitat under the Natura 2000 program (JAKUBOWSKA-GARBARA 2004). Widespread cattle and pig grazing in forests induced permanent changes in the structure of plant communities because excessive foraging on the undergrowth and overgrowth eliminated hornbeam and lime trees from the stand.

Undergrowth density was reduced to 5–10% of the original value, which enabled light-loving or even xerothermic species to develop in the undergrowth layer. Sparse oak crowns were easily penetrated by light and rain, which promoted the growth of lush and diverse vegetation on the forest floor. However, the absence of livestock and anthropogenic pressure led to rapid changes in the phytocenosis. Oak forests emerged as a result of planned forest management activities (JAKUBOWSKA-GARBARA 1991), as well as the pressure exerted by foraging deer. Light oak disappeared from Białowieża Forest due to changes in the abundance and hierarchy of herbivores feeding on hornbeam trees (KWIATKOWSKA 1994). However, herbivores populations were controlled not only by natural predators, but also, at least periodically, by humans, which suggests that herbivores pressure was a natural as well as an anthropogenic factor. The impact of large herbivores on woodlands led to the formation of "pasture forests" in Europe already before the development of agriculture (BORKOWSKI 2011).

Conservative cattle breeding in Poland

The Polish Red is the oldest Polish cattle breed with a single body color, and it is characterized by adaptability to unfavorable environmental conditions, resistance to diseases (such as mastitis, leukemia, and hoof disorders), very high fertility, calving ease, and extraordinary longevity. Polish Red cattle can be raised under unsupportive environmental conditions, and they are able to effectively utilize low-quality roughage. The popularity of traditional breeds has increased, especially on smaller farms, and Polish breeders are entitled to subsidies. The Polish Red presently accounts for 1% of the domestic cattle population and only 0.29% of the registered population. According to the annual monitoring data of the Agricultural Advisory Center, approximately 3500 head of Polish Red cattle were reared in 320 herds as part of the breed protection program in 2021 (Table 1).

Table 1

Breeding history of the Red Polish Cow in Poland based on number of individuals according to reports of „Statistic Poland"

Year	Number of individuals
1960	2 000 000
1999	150
2010	2091
2015	2388
2021	3500

Polish Red and White-Backed cattle are dual-purpose breeds that have long been used for milk production. In comparison with other cattle breeds, the Polish Red and White-Backed are characterized by moderate productivity and very high milk and meat quality. Upon the breeders' request, the National Research Institute of Animal Production (NRIAP) introduced breed assessment programs based on meat production traits for Polish Red cattle in 2017 (Polish Red Cattle Conservation Program, NRIAP, 2022) and for White-Backed cattle in 2019 (Polish White-Backed Cattle Conservation Program, NRIAP 2022).

The average milk yield of Polish Red cattle is 3,786 kg (4.26% fat content and 3.39% protein content). The superior performance of Polish Red cows can be attributed to their genetic ancestry which contributes to the breed's high conservation value. This valuable genetic resource must be protected because it plays an important biological role in agriculture and organic farming, and contributes to the preservation of landscapes, local traditions and culture. Cattle are registered for the needs of the genetic resource conservation program coordinated by the National Research Institute of Animal Production in Poland (NRIAP) in accordance with the applicable animal welfare regulations. According to recommendations, White-Backed, Polish Red, Polish Red-and-White, and Polish Black-and-White cattle should be kept in a pasture system in summer and indoors outside the grazing season (Polish Red Cattle Conservation Program, NRIAP, 2022).

The role of large herbivores in habitat restoration

In the 20th century, many livestock species were deprived of their natural habitats, which led to a dramatic decline or even the extinction of some animal populations (GOLONKA and JAWORSKI 2002). The establishment of nature conservation areas would protect endangered species, increase their population, and, in some cases, facilitate the reinstatement of these populations in the original habitats. Native cattle and horse breeds can be grazed extensively to protect indigenous avifauna. Before Polish Red cattle and Konik Polski horses were used to protect ornithofauna habitats through grazing, the Polish Society for the Protection of Birds had advocated for extensive wetland grazing to restore valuable bird habitats at the turn of the 19th and 20th centuries (KALSKI 2019). In recent years, a seasonal grazing program involving Polish Red cattle and Konik Polski horses was launched by the Polish Society for the Protection of Birds in the Narew River Valley (more than 290 ha) to restore local avi-

fauna habitats. During the program, Konik Polski horses from Popielno and Polish Red Cattle were grazed between June and October/November (*Ptaki. Podsumowanie…* 2023).

A greater increase in the breeding populations of *Charadriiformes,* including corncrake (*Crex crex*), spotted crake (*Porzana porzana*), northern lapwing (*Vanellus vanellus*), black-tailed godwit (*Limosa limosa*), ruff (*Calidris pugnax*), and common redshank (*Tringa tetanus*), was observed in grazed meadows than in the remaining areas. Grazing and trampling effects improved vegetation structure and foraging conditions for wading birds in wetland habitats (*Ptaki. Podsumowanie...* 2023). The animals were reunited with their herds at the end of the program. The grazing scheme could not be prolonged due to periodic flooding of meadows in the Narew River Valley. Konik Polski horses and Polish Red cattle had to be returned to their respective farms to undergo obligatory veterinary preventive care and other maintenance procedures, such as hoof correction. In Poland, only Konik Polski horses can be kept in semi-open breeding systems in natural reserves. However, the above applies only to individuals born in the wild.

Energy expenditure during cattle grazing

Generally, in seasonal grazing cattle milk yield is lower than in indoor breeding. The stress associated with herding cattle, changes of the diet and weather conditions cause periodic drops in the milk yield of these animals. Whereas energy expenditure during grazing increases in response to both very high and very low temperatures which fall outside the optimal thermal range of a given species. Cattle are sensitive to both heat and winter stress, and in addition to meeting basic physiological needs, the energy derived from feed has to accommodate the animals' thermal needs in unfavorable climatic conditions. On winter rangelands at northern latitudes, forage intake is often limited by low temperature and poor-quality pastures (ARNOLD 1985). The diets of grazing beef cattle are supplemented with protein to increase the intake of dormant forages for their nutritional needs and improve productivity (BODINE et al. 2001). In winter, protein supplementation strategies are introduced on the assumption that all animals consume a daily target quantity and that deviations from the targeted intake negatively impact their health and performance (BOWMAN and SOWELL 1997, WYFFELS et al. 2020). In winter, prolonged exposure to low temperatures and cold winds in northern grazing environments increases the resting metabolic rate and the overall energy expenditure to

maintain homeothermy (KEREN and OLSON 2006). Changes in energy requirements can affect the intake of dietary supplements during winter months. Short-term behavioral responses showed by thermal stress may be critical to the energy balance of domestic animals under extreme weather conditions (SENFT and RITTENHOUSER 1985). Moreover according to THORNOTON et al. (2021), anthropogenic climate change has major impacts on domesticated livestock, including increased heat stress in animals in both intensive and extensive livestock systems.

The influence of grazing on meat quality and palatability

Low-input livestock production systems, also known as extensive or traditional farming systems, promote animal welfare, sustainable development, and the production of safe foods with health promoting properties (MARINO et al. 2011, RAZMINOWICZ et al. 2006). However, the nutritional profile of meat produced in pasture-based feeding systems in countries with a long growing season, such as Brazil, Argentina, Uruguay, Australia, and New Zealand, is not directly equivalent to the nutritional profile of beef marketed as "grass-fed" in the US. Differences in pasture type and availability, cattle breed and age can influence the nutritional composition, quality, palatability, and digestibility of meat (LUCIANO et al. 2011, MIR et al. 2006). The fatty acid profile of beef is most effectively modified through diet. Forage such as grass, clover, and haylage is a rich source of α-linolenic acid (18: 3n-3), whereas cereal-based concentrates and maize silage are abundant in linoleic acid (18: 2n-6). The meat of cattle fed fodder from permanent grasslands was characterized by a more desirable ratio of n-6/n-3 fatty acids than the meat of cattle fed concentrates (NUERNBERG et al. 2005). In Poland, breeders of native cattle are entitled to subsidies under agricultural and environmental programs (FLOREK et al. 2017).

The influence of green fodder on the fatty acid profile of beef should not be generalized because the content and proportions of fatty acids in meat appear to be affected by both breed and type of grass (VAN ELSWYK and MCNEILL 2014). For example, the saturated fatty acid (SFA) profile of beef from two US cattle breeds, Angus and Simmental, differed in animals fed annual and perennial grasses. When the results were adjusted for differences in intramuscular fat content, Angus cattle grazed on annual pastures (rye grass, red clover, lotus) deposited more stearic acid in the polar lipid fraction of intramuscular fat than Simmental cattle grazed on annual pastures (ITOH et al. 1999). The fatty acid profile of individual muscles also differed in grass-fed or grain-finished cattle (LORENZENL et al. 2007). Car-

cass fat content was also found to affect the fatty acid composition of beef. Regardless of diet, the meat of "lean" breeds (such as double-muscled breeds) is more abundant in polyunsaturated fatty acids (PUFAs) than the meat of other breeds (DESMET et al. 2004) because PUFAs are accumulated mainly in muscle membrane phospholipids.

The above examples indicate that that meat quality is determined by numerous factors; therefore, the extent to which the transition from a standard diet to year-round grazing will affect the quality and nutritional value of Polish Red beef is difficult to predict. Research has shown that nutrition and genetics have no influence on the protein content or amino acid profile of beef (SCOLLAN et al. 2006). A comparison of the protein content of meat from grass/forage-fed and grain-finished cattle revealed no significant or practical differences (protein content ranged from 20% to 23%) (DUCKETT et al. 2009, 2013, LEHESKA et al. 2008). Meat aroma and palatability may be associated with individual preferences or cultural norms. For example, American consumers enjoy the flavor of beef from traditionally fed cattle, whereas consumers in other countries prefer beef from grass-grazed cattle (SCOLLAN et al. 2006, SITZ et al. 2005). Meat flavor is influenced by forage type and maturity, cattle breed, fat content, and marbling score, which is why the flavor of beef from grass/forage-fed and grain-finished cattle is difficult to compare (VAN ELSWYK and MCNEILL 2014). It should also be noted that the content of macro- and microelements is similar in the meat of Polish Red, Simmental, and Polish Holstein-Friesian cattle (DOMARADZKI et al. 2016).

Grazing by ruminants in different countries

In many US regions, beef cattle that had been raised under intensive farming systems are increasingly often grazed in pastures. Cattle are usually grazed in summer when fodder crops are more nutritious and palatable. Seasonal grazing strategies are also implemented to improve animal welfare and performance as well as for economic reasons. Consumers are becoming increasingly aware that grazing promotes animal health and well-being, contributes to environmental sustainability, and that the meat of pasture-grazed cattle is leaner and more abundant in health-promoting fatty acids (VAN ELSWYK and MCNEILL 2014). Feed is the most significant cost in livestock farming, and supplemental feeding accounts for 65% of the annual costs in cow-calf production (ARTHUR et al. 2004, VAN DER WESTHUIZEN et al. 2004, MEYER et al. 2008). Two-thirds of the energy derived from feed is utilized for physiological processes (FERRELL and

JENKINS 1984, 1988) and is an important consideration in cattle production (ARTHUR et al. 2001, NKRUMAH et al. 2006, CROWLEY et al. 2010). Feed constitutes the largest production input, which is why the selection of breeds characterized by lower feed intake and high productivity could substantially improve profitability (MEYER et al. 2008).

Livestock grazing influences landscape composition and plays a significant role in most European countries. In the Netherlands, the importance of grazing, including in forests, is widely recognized as a factor that contributes to animal welfare. Livestock grazing promotes habitat biodiversity and leads to changes in the size and composition of plant communities (LINDENMAYER et al. 2018). High-quality fodder from pastures is also a rich source of macro- and microelements, protein, energy, and vitamins (BARSZCZEWSKI et al. 2015). In the Netherlands, the number of grazed animals has decreased due to a rise in the cattle population. For example, 92% of the animals are grazed in herds with 40 head, but only 42% are grazed in herds of 160 or more animals. Average herd size continues to increase, which implies that fewer animals will be grazed (VAN DEN POL-VAN DASSELAAR et al. 2015). In recent years, the uncontrolled increase in the population of large herbivores in the Oostvaardersplassen nature reserve in the Netherlands has stirred considerable controversy. Oostvaardersplassen spans an area of around 56 square kilometers, and it is an experiment in rewilding. Due to uncontrolled reproduction and overcrowding, nearly 3300 deer, horses, and cattle died of starvation in the winter of 2017–2018 (Givetash 2018,Barkham 2018).

Conventional grazing requires access to areas with high ecological and landscape value, including forests. Forest grazing is prohibited in many countries, but its popularity is on the rise in Europe, despite the critical opinions of forest rangers who have argued that grazing cattle disrupt hunters, damage seedlings, the soil environment, and tree stands (VARGA et al. 2015). In Poland, rotational grazing is the most popular grazing strategy. The pasture is divided into paddocks, and cattle are moved to different portions of the pasture. Paddocks should have a similar size, and they should be enclosed by trenches, roads, or forests. Pastures should be rotated and effectively managed to maximize productivity. All four Polish native cattle breeds (Polish Red, White-Backed, Polish Black-and-White, and Polish Red-and-White) are kept on low-input farms where livestock are pastured in summer and fed farm-made fodder (silage and grass) in winter (LITWIŃCZUK et al. 2014). Beef from such production systems has higher nutritional value (CABRERA and SAADOUN 2014). The mineral content of beef is influenced by cattle breed, age, diet, production system, and meat processing (CABRERA and SAADOUN 2014, DOMARADZKI et al. 2016).

In Poland, ruminants are grazed between May and mid-October (around 160–170 days), and the grazing season ends shortly before the first ground frost (TWARDY and BARSZCZEWSKI 2015, BARSZCZEWSKI et al. 2015). Forest grazing is generally forbidden in Poland, but the forest division in Strzałowo (north-eastern Poland) authorized supervised grazing of Konik Polski horses in oak forests to restore ecosystem biodiversity (BOREK 2015). However, the project was not prolonged.

In Scandinavian countries, clear cuts in boreal forests play an important in livestock grazing (TOFASTRUD et al. 2019), but the availability of *Picea* and *Abies, Larix,* and *Pinus* species (GAUTHIER et al. 2015), as well as flora has been greatly reduced (KELMAN WIEDER et al. 2006). Tofastrud et al. (2019, 2020) determined the number of beef cattle grazing in boreal forests of Norway. The grazing season lasted 80-120 days, from late May to early September. Northern Scandinavian forests are valuable grazing areas for adult cattle and calves, but livestock compete for food with moose, which decreases the availability of feeding resources for wild animals. Moreover, changes in forage composition also decrease foraging activities (TOFASTRUD et al. 2019, 2020). Although forests offer a good alternative to livestock pastures, in many cases, they can be used only as supplementary pastures (10–20% of total grazing; VARGA et al. 2020). Forest grazing is not always recommended. In comparison with pasture forage, forest forage is low in protein and energy, thus it does not provide sufficient nourishment for livestock (MERCKER 2019). Low-energy diets can lead to metabolic diseases (KOWALSKI 2010), and a negative energy balance is associated with ketosis in cattle (LITTLEDIKE et al. 1981). According to CANTÓN et al. (2021), the low energy content of winter diets can also contribute to hypomagnesaemia.

Ten to forty acres of forest or one acre of improved pasture are typically required to provide the same number of grazing days. Around one hundred plant species growing in forests can have toxic effects for livestock (MERCKER and SMITH 2019). Forest grazing can decrease cattle productivity due to the adverse effects of poisonous herbs and reduced availability of forage during the grazing season (TOFASTRUD et al. 2019). According to HAN et al. (2021), forest grazing contributes to the extinction of rare plant species.

Attempts have been made to introduce Polish Red cattle to the natural habitat of Konik Polski horses and wildlife herbivores (*Naukowcy zbadają…* 2023). At present, the conservation herd of Konik Polski horses consists of twenty-one mares of reproductive age, four stallions, and their annual offspring. The herd is grazed on an area of approximately 1,620 ha, including 57 ha of forest meadows. This area is also inhabited by wild

ruminants such as red deer, fallow deer, and roe deer. According to annual hunting data, more than 100 cervids were identified in Popielno in March of 2021 (Table 2).

Table 2

Number of ruminants living on the Popielno Peninsula according to annual counting (March 2021)

Species	Number of individuals
Konik Polski (*Equus caballus*)	32
– stallions	4
– mares	21
– foals	7
Red deer (*Cervus elaphus*)	57
– bulls	15
– hinds	32
– calves	10
Fallow deer (*Dama dama*)	12
– bulls	4
– hinds	5
– calves	3
Roe deer (*Capreolus capreolus*)	32
– bucks	12
– does	14
– calves	6

In recent years, local meadows were considerably damaged by wild boars (damaged turf and strong weed infestation), which reduced the availability of forage for large herbivores, including cattle. Plans had been made to clear 100 ha of forests, establish meadows, and replace forest trees with oaks, but these efforts require time and effective management to ensure a good feed base. Despite the disadvantages of forest grazing, native cattle breeds have well adapted to pasture grazing without human involvement. Pasture grazing can raise environmental awareness and attract tourists, but it can also compromise long-term biotope stability, especially in habitats with rare and protected-plant species (CHODKIEWICZ 2020). The demand for healthy beef products has increased in recent years. Research has demonstrated that grass-fed beef delivers health benefits due to its desirable fatty acid profile and antioxidant content (DALEY et al. 2010). However, poorly managed forest grazing areas can compromise the health of ecosystems by contributing to erosion, reducing vegetation cover, decreasing organic matter content, soil moisture and compaction (MERCKER and SMITH 2019).

Conclusion

Polish Red cattle are characterized by high adaptability to unsupportive environments, high disease resistance, high fertility, extraordinary longevity, and high meat quality. In countries with a temperate climate (such as Poland), this breed should not be pasture-grazed throughout the year because low temperatures compromise the animals' health and welfare.

Accepted for print 28.07.2023

References

ARNOLD G.W. 1985. *Regulation of forage intake. Bioenergetics of wild herbivores.* CRC Press, Boca Raton, FL: 81–102.

ARTHUR P.F., ARCHER J.A., HERD R.M. 2004. *Feed intake and efficiency in beef cattle: Overview of recent Australian research and challenges for the future.* Australian Journal of Experimental Agriculture, 44: 361–369.

ARTHUR P.F., ARCHER J.A., JOHNSTON D.J., HERD R.M., RICHARDSON E.C., PARNELL P.F. 2001. *Genetic and phenotypic variance and covariance components for feed intake, feed efficiency, and other postweaning traits in Angus cattle.* Journal of Animal Science, 79: 2805–2811.

BARKHAM P. 2018. *Dutch rewilding experiment sparks backlash as thousands of animals starve*, https://www.theguardian.com/environment/2018/apr/27/dutch-rewilding-experiment-backfires-as-thousands-of-animals-starve, The Guardian, 27.04.2018, access: 28.09.2023.

BARSZCZEWSKI J., WASILEWSKI Z., MENDRA M. 2015. *Racjonalne użytkowanie pastwisk niżowych.* Materiały Informacyjne-Instytut Technologiczno-Przyrodniczy, Wydawnictwo ITP, pp. 1–24.

BODINE T.N., PURVIS H.T. II, LALMAN D.L. 2001. *Effects of supplement type on animal performance, forage intake, digestion, and ruminal measurements of growing beef cattle.* Journal of Animal Science, 79: 1041–1051.

BOREK R. 2015. *Agroforestry systems in Poland a preliminary identification.* Papers on Global Change IGBP, 22: 37–51.

BORKOWSKI J. 2011. *Jak wyglądały lasy pierwotne Europy pod presją dużych roślinożerców?* Leśne Prace Badawcze, 72: 183–190.

BOWMAN J.G., SOWELL B.F. 1997. *Delivery method and supplement consumption by grazing ruminants: A review.* Journal of Animal Science, 75: 543–550.

Bydło. Ochrona ras rodzimych, *Bydło. Bioróżnorodność. Instytut Zootechniki. Państwowy Instytut Badawczy w Krakowie,* http://bydlo.bioroznorodnosc.izoo.krakow.pl, access: 22.08.2023.

CABRERA M.C., SAADOUN A. 2014. *An overview of the nutritional value of beef and lamb meat from South America.* Meat Science, 98: 435–444.

CANTÓN G.J., FERNANÁNDEZ E.L., POO J.I., SPÄTH E.J.A., ODRIOZOLA E.R., MONTERUBBIANESI M.G., MORENO F.C. 2021. *Hypomagnesemia in beef cattle from the central region of Argentina: retrospective study.* Ciência Rural, 52: 1–9.

CHODKIEWICZ A. 2020. *Advantages and disadvantages of Polish primitive horse grazing on valuable nature areas A review.* Global Ecology and Conservation, 21: 1–11.

CROWLEY J.J., MCGEE M., KENNY D.A., CREWS JR D.H., EVANS R.D., BERRY D.P. 2010. *Phenotypic and genetic parameters for different measures of feed efficiency in different breeds of Irish performance-tested beef bulls.* Journal of Animal Science, 88: 885–894.

DALEY C.A., ABBOTT A., DOYLE P.S., NADER G.A., LARSON S. 2010. *A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef.* Nutrition Journal, 9: 10.

De Smet S., Raes K., Demeyer D. 2004. *Meat fatty acid composition as affected by fitness and genetic factors. A review.* Animal Research, 53: 81–98.

Domaradzki P., Florek M., Staszowska A., Litwińczuk Z. 2016. *Evaluation of the mineral concentration in beef from Polish native cattle.* Biological Trace Element Research, 171: 328–332.

Duckett S.K., Neel J.P.S., Fontenot J.P., Clapham W.M. 2009. *Effects of winter stocker growth rate and finishing system on: III. Tissue proximate, fatty acid, vitamin, and cholesterol content.* Journal of Animal Science, 87: 2961–2970.

Duckett S.K., Neel J.P.S., Lewis R.M., Fontenot J.P., Clapham W.M. 2013. *Effects of forage species or concentrate finishing on animal performance, carcass and meat quality.* Journal of Animal Science, 91: 1454–1467.

Elswyk M.E. van, McNeill S.H. 2014. *Impact of grass/forage feeding versus grain finishing on beef nutrients and sensory quality: The U.S. experience.* Meat Science, 96: 535–540.

Ferrell C.L., Jenkins R.G. 1988. *Influence of biological types on energy requirements.* Beef Research Program Progress Report, 3: 86–90.

Florek M., Litwińczuk Z., Domaradzki P., Chabuz W., Żółkiewski P., Jankowski P. 2017. *Rodzime rasy bydła podstawą produktów regionalnych z wołowiny.* Wiadomości Zootechniczne, 5: 123–133.

Gauthier S., Bernier P., Kuuluvainen T., Shvidenko A.Z., Schepaschenko D.G. 2015. *Boreal forest health and global change.* Science, 349: 819–822.

Givetash L. 2018. *Slaughter of starving wild horses divides the Netherlands. NBC News*, https://www.nbcnews.com/news/world/slaughter-starving-wild-horses-divides-netherlands-n895156, access: 28.09.2023.

Golonka M., Jaworski Z. 2002. *Reintrodukcja koni Przewalskiego.* Zeszyty Naukowe. Przegląd Hodowlany, 1: 19–21.

Han G., Cheong E., Park W., Kim S. 2021. *Impact of the disturbances for forest grazing on flora composition in a natural forest.* Forests, 12: 872.

Itoh M., Johnson C.B., Cosgrove G.P., Muir P.D., Purchas R.W. 1999. *Intramuscular fatty acid composition of neutral and polar lipids for heavy-weight Angus and Simmental steers finished on pasture or grain.* Journal of the Science of Food and Agriculture, 79: 821–827.

Jakubowska-Gabara J. 1991. *Recesja zespołu świetlistej dąbrowy Potentillo albae-Querce-tum Libb. 1933 w rezerwacie Trębaczew*. Parki Narodowe i Rezerwaty Przyrody, 10: 69–79.

Jakubowska-Gabara J. 2004. Świetlista dąbrowa *Potentillo albae-Quercetum.* W: J. Herbich (ed.). *Lasy i bory. Poradnik ochrony siedlisk i gatunków Natura 2000 – podręcznik metodyczny*, 5: 261–265.

Kalski R. 2019. *Konik polski pośród orlików, bocianów i rycyków – 19 lat hodowli Polskiego Towarzystwa Ochrony Ptaków. Konferencja naukowa, Mazurska hodowla koników polskich – przeszłość, teraźniejszość, przyszłość.* Wierzba, Poland, 5–7.09.2019, 37.

Kelman Wieder R., Vitt D.H., Benscoter B.W. 2006. *Peatlands and the Boreal Forest*. In: Wieder, R.K., Vitt, D.H. (eds). *Boreal peatland ecosystems. Ecological Studies*, Springer, Berlin, Heidelberg, 188: 1–8.

Keren E.N., Olson B.E. 2006. *Thermal balance of cattle grazing winter range: Model application.* Journal of Animal Science, 84: 1238–1247.

Kowalski Z.M. 2010. *Wpływ żywienia na płodność krów mlecznych.* Życie Weterynaryjne, 85: 830–834.

Kwiatkowska A.J. 1994. *Changes in the species richness, Spatial pattern and species frequency associated with the decline of Oak Forest.* Vegetatio, 112: 171–180.

Leheska J.M., Thompson L.D., Howe J.C., Hentges E., Boyce J., Brooks J.C., Shriver B., Hoover L., Miller M.F. 2008. *Effects of conventional and grass-feeding systems on the nutrient composition of beef.* Journal of Animal Science, 86: 3575–3585.

Libbert W. 1933. *Die Vegetationseinheiten der neumärkischen Staubeckenlandschaft unter Berücksichtigung der angrenzenden Landschaften.* Botanischer Verein der Provinz Brandenburg, 75: 229–348.

LINDENMAYER D.B., BLANCHARD W., CRANE M., MICHAEL D., SATO C. 2018. *Biodiversity benefits of vegetation restoration are undermined by livestock grazing*. Restoration Ecology, 26: 1157–1164.

LITTLEDIKE E.T., YOUNG J.W., BEITZ D.C. 1981. *Common metabolic diseases of cattle: Ketosis, milk fever, grass tetany, and downer cow complex*. Journal of Dairy Science, 64: 1465–1482.

LITWIŃCZUK Z., BARŁOWSKA J., CHABUZ W., BRODZIAK A. 2012. *Nutritional value and technological suitability of milk from cows of three Polish breeds included in the genetic resources conservation programme*. Annals of Animal Science, 1: 423–432.

LITWIŃCZUK Z., ŻÓŁKIEWSKI P., FLOREK M., CHABUZ W., DOMARADZKI P. 2014. *Semi-intensive fattening suitability and slaughter value of young bulls of 3 Polish native breeds in comparison with Polish Holstein-Friesian and Simmental*. Annals of Animal Science, 2: 453–460.

LORENZEN C.L., GOLDEN J.W., MARTZ F.A., GRÜN I.U., ELLERSIECK M.R., GERRISH J.R., MOORE K.C. 2007. *Conjugated linoleic acid content of beef differs by feeding regime and muscle*. Meat Science, 75: 159–167.

LUCIANO G., MOLONEY A.P., PRIOLO A., RÖHRLE F.T., VASTA V., BIONDI L., LOPEZ-ANDRES P., GRASSO S., MONAHAN F.J. 2011. *Vitamin E and polyunsaturated fatty acids in bovine muscle and the oxidative stability of beef from cattle receiving grass or concentrate-based rations*. Journal of Animal Science, 89: 3759–3768.

MARINO R., ALBENZIO M., CAROPRESE M., NAPOLITANO F., SANTILLO A., BRAGHIERI A. 2011. *Effect of grazing and dietary protein on eating quality of Podolian beef*. Journal of Animal Science, 89: 3752–3758.

MERCKER D., SMITH J. 2019. *Grazing Livestock in Woodlands*. University of Tennessee, Institute of Agriculture, pp. 1–12.

MEYER A.M., KERLEY M.S., KALLENBACH R.L. 2008. *The effect of residual feed intake classification on forage intake by grazing beef cows*. Journal of Animal Science, 86: 2670–2679.

MIR P.S., BITTMAN S., HUNT D., ENTZ T., YIP B. 2006. *Lipid content and fatty acid composition of grasses sampled on different dates through the early part of the growing season*. Canadian Journal of Animal Science, 86: 279–290.

Naukowcy zbadają jak wypas krów prymitywnych ras w lesie wpłynie na jakość i smak mięsa. 2021. Instytut Rozrodu Zwierząt i Badań Żywności Polskiej Akademii Nauk w Olsztynie, https://pan.olsztyn.pl/2021/06/naukowcy-zbadaja-jak-wypas-krow-prymitywnych-ras-w-lesie-wplynie-na-jakosc-i-smak-miesa/, access: 22.08.2023.

NKRUMAH J.D., OKINE E.K., MATHISON G.W., SCHMID K., LI C., BASARAB J.A., PRICE M.A., WANG Z., MOORE S.S. 2006. *Relationships of feedlot feed efficiency, performance, and feeding behavior with metabolic rate, methane production, and energy partitioning in beef cattle*. Journal of Animal Science, 84: 145–153.

NUERNBERG K., DANNENBERGER D., NUERNBERG G., ENDER K., VOIGT J., SCOLLAN N., WOOD J.D., NUTE G.R., RICHARDSON R.I. 2005. *Effect of a grass-based and a concentrate feeding system on meat quality characteristics and fatty acid composition of longissimus muscle in different cattle breeds*. Livestock Production Science, 94: 137–147.

POL VAN DEN-DASSELAAR A. VAN., VLIEGHER A. DE, HENNESSY D., ISSELSTEIN J., PEYRAUD J.L. 2015. *The future of grazing: Proceedings, 3rd Meeting of the EGF Working Group 38 'Grazing' (report 906 ed.)*. Wageningen UR Livestock Research, pp. 1–39.

Ptaki. Podsumowanie projektu 'Ochrona ptaków wodno-błotnych w Dolinie Górnej Narwi PLB200007', https://www.ptop.org.pl/ochrona/ptaki/1574-podsumowanie-projektu-ochrona-ptakow-wodno-blotnych-w-dolinie-gornej-narwi-plb200007.html, access: 21.08.2023.

RAZMINOWICZ R.H., KREUZER M., SCHEEDER M.R.L. 2006. *Quality of retail beef from two grass-based production systems in comparison with conventional beef*. Meat Science, 73: 351–361.

SCOLLAN N., HOCQUETTE J.F., NUERNBERG K., DANNENBERGER D., RICHARDSON I., MOLONEY A. 2006. *Innovations in beef production systems that enhance the nutritional and health value of beef lipids and their relationship with meat quality*. Meat Science, 74: 7–33.

SENFT R.L., RITTENHOUSE L.R. 1985. *A model of thermal acclimation in cattle*. Journal of Animal Science, 61: 297–306.

Sitz B.M., Calkins C.R., Feuz D.M., Umberger W.J., Eskridge K.M. 2005. *Consumer sensory acceptance and value of domestic, Canadian, and Australian grass-fed beef steaks*. Journal of Animal Science, 83: 2863–2868.

Thornton P., Nelson G., Mayberry D., Herrero M. 2021. *Increases in extreme heat stress in domesticated livestock species during the twenty-first century*. Global Change Biology, 27: 5681–6003.

Tofastrud M., Devineau O., Zimmermnn B. 2019. *Habitat selection of free-ranging cattle in productive coniferous forests of south-eastern Norway*. Forest Ecology and Management, 437: 1–9.

Tofastrud M., Hessle A., Rekdal Y., Zimmermann B. 2020. *Weight gain of free-ranging beef cattle grazing in the boreal forest of southeastern Norway*. Livestock Science, 233: 103955.

Twardy S., Barszczewski J. 2015. *Racjonalne użytkowanie pastwisk górskich*. Materiały Informacyjne, Instytut Technologiczno-Przyrodniczy, Wydawnictwo Itp., pp. 1–24.

Westhuizen R.R. van der, Westhuizen J. van der, Schoeman S.J. 2004. *Genetic variance components of residual feed intake and feed conversion ratio and their correlations with other production traits in beef bulls*. South African Journal of Animal Science, 34: 257–264.

Varga A., Demeter L., Ulicsni V., Öllerer K., Biró M., Babai D., Molnár Z. 2015. *Prohibited, but still present: local and traditional knowledge about the practice and impact of forest grazing by domestic livestock in Hungary*. Journal of Ethnobiology and Ethnomedicine, 16: 1–12.

Wyffels S.A., Parsons C.T., Dafoe J.M., Boss D.L., McClain T.P., Carter B.H., DelCurto T. 2020. *The influence of age and winter environment on Rumax Bovibox and Bovibox HM supplement intake behavior of winter grazing beef cattle on mixed-grass rangelands*. Translational Animal Science, 4: 37–42.

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 38(1): 19–29, Y. 2023

DOI: 10.31648/pjns.7409

SHIGA, TOXIN-PRODUCING *ESCHERICHIA COLI* O157:H7 IN PACKAGED DRINKING-WATER IN ABEOKUTA, NIGERIA

***Abiola Ajibola*[1], *Olufunke Shittu*[2], *Grace Oluwasanya*[3], *Abidemi Ojo*[4], *Paul Akinduti*[5], *Aderonke Akintokun*[6]**

[1] ORCID: 0000-0002-4592-7585
[2] ORCID: 0000-0001-5000-0015
[3] ORCID: 0000-0003-0731-2042
[4] ORCID: 0000-0001-6359-4086
[5] ORCID: 0000-0003-0697-8176
[1,2,4,6] Department of Microbiology, College of Biosciences
Federal University of Agriculture, Abeokuta (FUNAAB), Nigeria
[3] Department of Water Resources Management and Agricultural Meteorology
College of Environmental Resources Management, FUNAAB, Nigeria
[5] Department of Biological Sciences
College of Science and Technology, Covenant University, Ota, Nigeria

Key words: drinking-water quality, *Escherichia coli,* molecular identification, packaged sachet water, Shiga toxin.

Abstract

There is a need to carry out regular microbiological analysis of packaged sachet water (PSW) sold in Nigeria to determine whether they comply with drinking-water quality standards. One hundred and fifty three PSW samples from fifty-one packaged sachet water facilities (PWMFs) in Abeokuta were collected from retail outlets between April – July, 2014. Samples were analyzed for *Escherichia coli* using standard culture media. Latex agglutination serological test; sequencing of 16S rRNA gene and sequence similarity analysis yielded *E. coli* O157:H7 from two PSW (1.32%) from one PWMF (1.96%). Both *E. coli* O157:H7 isolates were Shiga toxin (stx1) positive but stx2 negative. The two *E. coli* O157:H7 strains exhibited different resistance patterns to ten (10) antibiotics belonging to seven (7) different classes. Each *E. coli* O157:H7 strain showed resistance to more than two classes of antibiotics with MIC ≥ 8 μg/ml. This paper showed that the not all packaged sachet water analyzed in this study are fit for drinking, and can even be source of multidrug resistant and Shiga, toxin-producing *E. coli*.

Introduction

Packaged water has become another source of drinking water in most countries of the world (Oyedeji et al. 2010). In Nigeria, many people are currently involved in the production of PSW using different complex tech-

Address: Abiola Ajibola, Federal University of Agriculture, Abeokuta (FUNAAB), Nigeria, phone: +234 8032068108, e-mail: oladotunat@funaab.edu.ng

niques such as ozonization and reverse osmosis, however, many producers fail to adhere to standards for the production of packaged sachet water (PSW). Findings from quality assessment using *Escherichia coli* as faecal indicator revealed that there are some PSW with microbiological limits exceeding WHO drinking-water guidelines of zero coliform count per 100 ml of water (WHO 2002, 2017). Although most *E. coli* strains are harmless, but some, such as serotype O157:H7, can cause serious food poisoning in humans and are occasionally responsible for product recalls (TARR et al. 2005).

Shiga-like toxin-producing *Escherichia coli* (STEC) O157:H7 are known zoonotic, food and water-borne pathogens and toxin-producing strains associated with human illness (GYLES 2007) and STEC also harbor the phage-encoded genes *stx1* and *stx2* coding for potent cytotoxins that cause severe tissue damage with fatal consequences (WALKER et al. 2012).

In Nigeria, especially the southwest, a lot of work has been done on the microbiological quality of vended sachet water (OLUYEGE et al. 2014, ONILUDE et al. 2013, OYEDEJI et al. 2010) with *E. coli* as an indicator of water quality. There is no index study on the serotype of *E. coli* isolated from such vended sachet water. Also in Nigeria, *E. coli* 0157:H7 have been reported from clinical and food samples (OKEKE et al. 2003, OLATOYE 2010), tap water and surface water (CHIGOR et al. 2010, LUGA et al. 2007, SMITH et al. 2009) but none from packaged sachet water. There is a need to subject suspected *Escherichia coli* from sachet water to further analysis (phenotypic, serotyping and molecular). As such, this study investigates the presence of Shiga, toxin-producing *Escherichia coli* O157:H7 in packaged sachet water samples in Abeokuta, Nigeria, with the goal to assess the possible risk of human exposure to *Escherichia coli* O157:H7 through consumption of sachet water, which are packaged essentially as drinking-water in the study area.

Methodology

The study area, Abeokuta, southwest Nigeria, has three local government areas (LGA). Samples (PSW) were collected over a period of 4 months from April to July 2014 from 39 different locations within the study area. The main selection criterion is population density. As such, the selected locations are highly populated areas (in terms of residential and business activities) within each of the three LGA.

In Nigeria, The National Agency for Food and Drug Administration and Control (NAFDAC) is the agency saddled with regulation of food and

water that is packaged for commercial purposes. As at April 2014, the number of registered PSW companies in Abeokuta was 72. There were only sixty registered PSW companies that were in operation as at the time of collection of samples. Using Yamane's formula (YAMANE 1967) the sample size was determined as follows:

$$n = N/1 + Ne2,$$

where:
n – required sample size
N – population size which is 60
e = 0.05 at 95% confidence interval.

The calculation gives a total of 51 which was used as the sample size. Samples were purchased thrice. Therefore, in total there was one hundred and fifty-three (153) packaged sachet water samples from 51 different packaged water manufacturing facilities (PWMFs) purchased randomly from retailers across the city of Abeokuta at the thirty nine different locations. All samples were transported in ice packs to the laboratory for microbiological analysis within 6 hours (h).

One ml from each sample was pre-enriched in Brain Heart Infusion (BHI) broth at ratio 1:9 for 3 h at 35°C. An aliquot (1 ml) of the pre-enrichment was transferred to 9 ml Tryptone Soya broth (TSB) and incubated at 44°C for 20 h followed by subsequent culture on Eosin-methylene blue (EMB) agar, incubated at 37°C for 24 h (Pathogenic *Escherichia coli*... 2006). Both gram and biochemical reactions were carried out. Typical green metallic sheen (presumptive *E. coli*) colonies which were gram negative and indole positive were subjected to further characterization such as test for sorbitol fermentation using Sorbitol MacConkey Agar (SMAC) incubated at 37°C for 18–24 h. Non-sorbitol fermenting *E. coli* colonies were serologically confirmed as O157:H7 using *E. coli* latex agglutination serotyping kit (Dryspot *E. coli* O157 latex test) for *E. coli* O157 (Oxoid, Basingstoke, UK). Colonies that agglutinated to the antisera were considered to be *E. coli* O157:H7.

Each of the isolates (*Escherichia coli*) was subjected to antibiotic sensitivity testing by the disk diffusion method (KIRBY-BAUER et al. 1966). The diameter of the zone of clearance (including the diameter of the disk) was measured to the nearest whole millimeter and interpreted using Clinical and Laboratory Standards Institute guidelines (CLSI 2012). The antibiotics used are: penicillin, cefixime, streptomycin, erythromycin, amoxicillin, ampicillin, tetraxycline, co-trimoxazole, gentamycin, ciprofloxacin. The antibiotic disks were produced by Randox laboratories, UK.

Extraction of genomic DNA was conducted at Biotechnology Centre, Federal University of Agriculture, Abeokuta, Nigeria. Extraction was per-

formed using DNA extraction kit (Norgen, Canada) following manufacturer's instruction. The extracted DNA was viewed by agarose gel electrophoresis followed by PCR amplification obtained with universal primer for detection of bacteria (5pMol forward (5′-CGAGCAGCCGCGGAATACG -3′) and backward primer (5′ ATCGGTACCTTGTACGACTTC -3′)) (RAHMANI et al. 2006).

Bi-directional sequences were edited and aligned to generate a consensus sequence using BioEdit Sequence Alignment Editor (version 7.1.9). Consensus sequences were then subjected to similarity searches on National Centre for Biotechnological Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) to establish identities of the bacteria strains (both sequences have been submitted with accession numbers (OQ311321 and OQ311322). Also, PCR amplification for *stx*1 and *stx*2 gene was carried out (WILLNER et al. 2012) USING universal selective primers. Table 1 shows the PCR primers used for Shiga toxin detection in this study.

Table 1

Genomic sequence of primers for detection of Siga toxin genes in *Escherichia coli* O157:H7 strains from sachet water packaged as drinking-water in Abeokuta, Nigeria

Primer	Primer sequence (5'- 3')	Target gene	Size of amplicon (base pair, bp)	Reference
*Stx*1-F	ACACTGGATGATCTCAGTGG	Stx-1	614	(FERENS and HOVDE 2011)
*Stx*1-R	CTGAATCCCCCTCCATTATG	–	–	–
*Stx*2-F	CCATGACAACGGACAGCAGTT	Stx-2	779	(FERENS and HOVDE 2011)
*Stx*2-R	CCTGTCAATGAGCAGCACTTTG	–	–	–

Abbreviations: F – Forward; R – Reverse

Results

Occurrence of *Escherichia coli* in packaged sachet water samples, phenotypic reaction of isolated *E. coli* and serotyping

Out of 153 packaged sachet water samples analyzed, *E. coli* was isolated from only two sachet water samples (1.32%). The organism (*E. coli*) was isolated from the same PSW collected at different months (two months apart, with different batch number) from the same PWMF. The two Isolates were confirmed to be *Escherichia coli* O157:H7 with sorbitol fermentation and latex agglutination test as shown by the clumps in plate (Figure 1).

Table 2 shows that there was a positive reaction for the 2 isolates in terms of fermentation and gas production at 37° and 44°C respectively, which shows that the *E. coli* isolates are from feacal origin. The isolates were negative for tube haemagglutination. The hemolytic action of the isolates on blood agar shows that the isolates were not able to cause any hemolytic action on the blood agar (γ-hemolysis) while the isolates were pale coloured (negative reaction) on sorbitol MacConkey agar which shows that they were non-sorbitol fermenters.

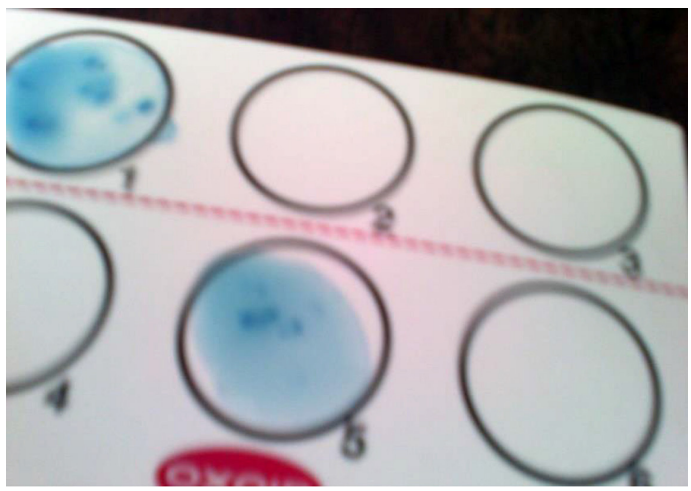

Fig. 1. Agglutination reaction of *E. coli* from packaged water sold in Abeokuta, Ogun State, Nigeria to oxoid O157:H7 antiserum

Table 2

Phenotypic characterization of *E. coli* isolates in packaged water sold in Abeokuta, Ogun State, Nigeria

Phenotypic conditions	Isolates	
	Escherichia coli[a]	*Escherichia coli*[b]
Growth at 37°C	+	+
Growth at 44°C	+	+
Haemmaglutination assay	–	–
Hemolytic reaction	–	–
Sorbitol fermentation	–	–

Abbreviations: + positive; – negative

Antibiotics Sensitivity Test (AST)

Table 3 shows the pattern of each bacteria strain to different classes of antibiotics used. Strain A showed resistance to five antibiotics of four classes while *E. coli* strain B showed resistance to seven antibiotics belonging to six classes which indicate that the antibiotic resistance pattern differed in the two strains.

Table 3

Minimum inhibitory concentration and minimum bactericidal concentration of *E. coli* isolates in packaged sachet water sold in Abeokuta, Ogun State, Nigeria

Antibiotics	*Escherichia coli*[a]	*Escherichia coli*[b]
PEN	R	S
CEFX	S	R
STREP	R	S
ERY	R	R
AMX	S	R
AMP	S	R
TET	S	R
COT	S	R
GEN	R	R
CIPX	R	S

Abbreviations: [a] – first *E. coli* isolate; [b] – second *E. coli* isolate; S – sensitive; R – resistance; Antibiotics (CLSI, 2012): PEN – Penicillin; CEFX – Cefixime; STREP – Streptomycin; ERY – Erythromycin; AMX – Amoxicillin; AMP – Ampicillin; TET – Tetracycline; COT – Co-trimoxazole; GEN – Gentamycin; CIPX – Ciprofloxacin

Molecular characterization of *E. coli* O157:H7 isolates

Figure 2 described a gel picture showing the successful extraction of deoxyribonucleic acid (DNA) material of isolates.

STX genes in *Escherichia coli* O157:H7 strains

Gel electrophoresis of PCR amplified Shiga toxin genes shows that both *E. coli* strains produced *stx*1 with 503 bp (Figure 3) but none had *stx*2 (Figure 4).

Fig. 2. Gel picture of extracted genomic DNA of *E. coli* O157:H7 from packaged water sold in Abeokuta, Ogun State, Nigeria. Abbreviations: *M* – DNA marker; *A* – *E. coli* isolate *A*; *B* – *E. coli* isolate B; - – control

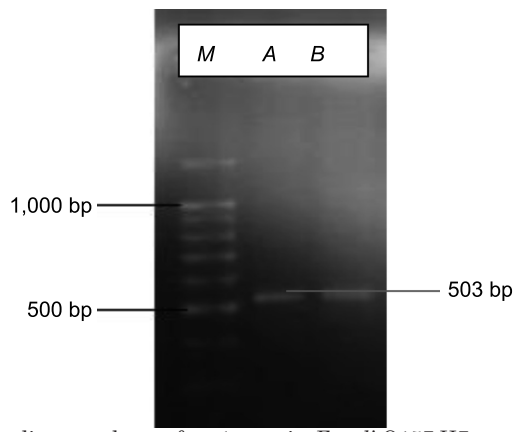

Fig. 3. PCR amplicon products of *stx1* gene in *E. coli* O157:H7 strains from sachet waterpackaged as drinking-water in Abeokuta, Nigeria. Abbreviations: *M* – molecular marker; *A* – *Escherichia coli* strain A; *B* – *Escherichia coli* strain B

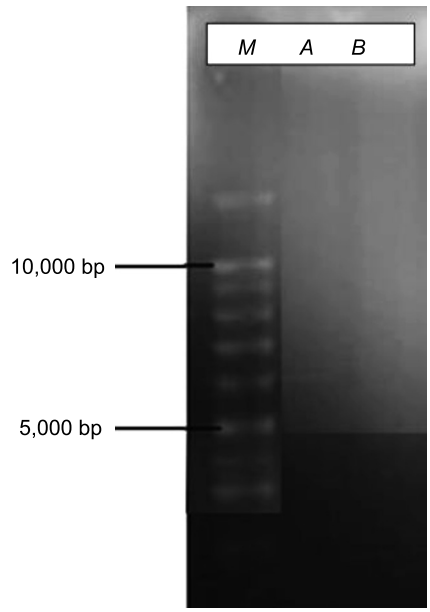

Fig. 4. PCR amplicon products of *stx2* gene in *Escherichia coli* O157:H7 strains isolated from sachet water packaged as drinking-water in Abeokuta, Nigeria. Abbreviations: *M* – molecular marker; *A* – *Escherichia coli* strain A; *B* – *Escherichia coli* strain B

Discussion

Shiga, toxin-producing Escherichia coli O157:H7 in packaged drinking-water: implications for public health

This study isolated *Escherichia coli* O157:H7 from sachet water that are essentially packaged as drinking-water in Abeokuta, southwest Nigeria, at a prevalence of 1.3% from one brand of PWMF. Arguably, the percentage prevalence of 1.3 is low, however, it should be noted that the object of study (Packaged sachet water) is treated water and should, according to WHO and NAFDAC, have zero coliforms cfu/100ml of water. Also, though the percentage prevalence is low (1.32%), the strain of *E. coli* (0157:H7) that was isolated from the samples is of public health interest considering the bacterial pathogenicity, low infectious dose and ability to survive in extra-intestinal environments (AIBINU et al. 2007). Similarly, the population at risk (more than 60% of over 170 Million Nigerian population take sachet water) when factored in makes the presence of coliforms a public health concern to a wide human population regardless of the low prevalence rate.

Serological and molecular techniques confirmed the strain of *E. coli* as O157:H7. Although, this strain has been isolated in tap water and surface water in Nigeria (CHIGOR et al. 2010, LUGA et al. 2007, SMITH et al. 2009), but it not been isolated from PSW, due to the assumption that PSW, being treated water should be free from bacteria not to talk of the highly pathogenic *E. coli* O157:H7. Therefore, most research on PSW in Nigeria are benchmarked at biochemical characterization.

The two strains from this study were positive for PCR detection of Shiga toxin (stx1) confirming their virulence though stx2 was not detected. Shiga toxin is the critical virulence factor in STEC diseases. The isolated *E. coli* O157:H7 strains were also multidrug resistant, which is not a new occurrence in Nigeria (AIBINU et al. 2007). Multi-drug resistant Shiga toxin-producing *E. coli* O157:H7 constitutes a significant public health problem in Nigeria (SOUZA et al. 2011). In addition, the occurrence of *E. coli* O157:H7 from packaged sachet water in this study is of public health significance, particularly, considering the percentage of households that depend on sachet water for drinking in Nigeria. The people that are most at risk of infection are the very young, the elderly and people with an already weakened immune system (KALGI and MARTINS 2015), pregnant women and HIV/AIDS patients (ABONGO et al. 2008, REUBEN and GYAR 2015). Therefore, packaged sachet water in Nigeria should be given serious attention by carrying out more studies on the pathotypes of *E. coli* isolated in water beyond the sole focus given to the occurrence of *E. coli* as an indicator for water quality.

Furthermore, urgent attention should be geared towards the enforcement of the adoption and development of water safety interventions by drinking-water suppliers to prevent outbreaks of waterborne diseases, particularly, the application of Water Safety Plans (WSP). Water Safety Plans are preventive, comprehensive and systematic assessment of drinking-water supply systems from source to tap, using risks assessments and management tools (WHO 2017). Enforcing the application of water safety planning is to ultimately ensure water and public health protection.

Conclusion

Shiga toxin producing multi-drug resistant *Escherichia coli* O157:H7 is identified as a microbiological hazard transmitted to a wide population through sachet water that is packaged essentially for drinking in Abeokuta, Nigeria.

Acknowledgements

All members of staff of the Microbiology laboratory, College of Veterinary medicine, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.

Funding

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

Accepted for print 23.02.2023

References

ABONGO B.O, MOMBA M.N.B., MALAKATE V.K., MWAMBAKANA J.N. 2008. *Prevalence of Escherichia coli O157:H7 among diarrhoeic HIV/AIDS patients in the eastern cape province-South Africa.* Pak. J. Biol. Sci., 11:1066–1075.

AIBINU I.E., PETERS R.F., AMISU K.O., ADESIDA S.A., OJO M.A., ODUGBEMI T. 2007. *Multidrug resistance in Escherichia coli O157 strains and the public health implication*. Am. J. Sci., 3: 22–33.

BAUER A.W., KIRBY W.M.M., SHERRIS J.C., TURCK, M. 1966. *Antibiotic susceptibility testing by a standard single disc diffusion method*. Am. J. Clin. Pathol., 45: 493–496.

CHIGOR V.N., UMOH V.J., SMITH S.I., IGBINOSA E.O., OKOH A.I. 2010. *Multidrug resistance and plasmid patterns of Escherichia coli O157 and other E. coli isolated from diarrheal stools and surface waters from some selected sources in Zaria, Nigeria*. Int. J. Environ. Res. Public Health., 7: 3831–3841.

Clinical and Laboratory Standard Institute (CLSI) 2012. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-second informational Supplement. CLSI document M100-S22. Clinical & Laboratory Standards Institute. Wayne, PA, USA.

FERENS W.A., HOVDE C.J. 2011. *Escherichia coli O157:H7: animal reservoir and sources of human infection.* Foodborne Pathog. Dis., 8(4): 265–487.

GYLES C.L. 2007. *Shiga toxin-producing Escherichia coli: an overview*. J. Anim. Sci., 85: E45–E62. http://doi.org/10.2527/jas.2006-508.

KALGI B., MARTENS M. 2015. *Preventing Escherichia coli O157:H7 infection in pregnancy*. Obstet. Gynaecol., 2 (2): 029.

LUGA I.I., AKODU L.I., MHOMGA L., ALLAM U., AJOGI I., UMOH V.J., KWAGA J.P.K. 2007. *Antimicrobial resistance of Shiga toxin producing Escherichia coli O157: NM isolates from water fed to cattle in Northern-western Nigeria.* Asian J. Anim. Vet. Adv., 2(4): 205–211.

OKEKE I.N., OJO O., LAMIKANRA A., KAPER J.B. 2003. *Etiology of acute diarrhea in adults in South Western Nigeria.* J. Clin. Microbiol., 41: 4525–4535.

OLATOYE I.O. 2010. *The incidence and antibiotics susceptibility of Escherichia coli O157:H7 from beef in Ibadan municipal, Nigeria.* Afr. J. Biotechnol., 9(8): 1196–1199.

OLUYEGE J.O., OLOWOMOFE T.O., ABIODUN O.R. 2014. *Microbial contamination of packaged drinking-water in Ado-Ekiti Metropolis, South Western Nigeria.* Am. J. Res. Commun., 2(10): 231–246.

ONILUDE A.A., ADESINA F.C., OLUBOYEDE O.A., ADEYEMI B.I. 2013. *Microbiological quality of sachet packaged water vended in three local governments of Oyo State, Nigeria*. AJFST., 3(9): 195–200.

OYEDEJI O., OLUTIOLA P.O., MONINUOLA M.A. 2010. *Microbiological quality of packaged drinking water brands marketed in Ibadan metropolis & Ile-Ife city in South Western Nigeria.* Afr. J. Microbiol. Res., 4: 96–102.

Pathogenic Escherichia coli Network (PEN). Methods for detection and molecular characterisation of pathogenic Escherichia coli. 2006. Eds. J.O. Sullivan., D.J. Bolton, G. Duff, C. Baylis, R. Tozzoli, Y. Wasteson, S. Lofdahl. Co-ordination Action Food-CT-2006-036256., Ashtown Food Research Centre, Teagasc, Ashtown, Dublin 15, Ireland, pp. 11.

RAHMANI S., FOROZANDEH M., MOSAVP M., REZAEY A. 2006. *Detection of bacteria by amplifying the 16S RNA gene with universal primers and restriction fragment length polymorphism*. Med. J. Islam Repub. Iran, 19(4): 333–338.

REUBEN C.R., GYAR S.D. 2015. *Isolation and antibiogram of Shiga toxin-producing Escherichia coli O157:H7 from diarrheic HIV/AIDS patients in Lafia, Central Nigeria.* Int. Res. J. Microbiol., 6(2): 020–026.

SMITH S.I., BELLO O.S., GOODLUCK H.A., OMONIGBEHIN E.A., AGBOGU V.N., ODEIGAH P. 2009. *Prevalence of EHEC O157:H7 from human and environmental samples from Lagos and Zaria.* Pak. J. Med. Sci., 25(3): 398–403.

SOUZA R.L., CARVALHAES J.T.A., NISHIMURA L.S., ANDRADE M.C., GUTH B.E.C. 2011. *Hemolytic uremic syndrome in pediatric intensive care units in Sao Paulo, Brazil.* Open Microbiol. J., 5: 76–82.

TARR P.I., GORDON C.A., CHANDLER W.L. 2005. *Shiga-toxin-producing Escherichia coli and haemolytic uraemic syndrome*. Lancet, 365: 1073–1086.

WALKER C.L., APPLEGATE J.A., BLACK R.E. 2012. *Haemolytic uremic syndrome as a sequela of diarrheal disease.* J. Health Popul. Nutr., 30(3): 257–261.

WILLNER D., DALY J., WHILEY D., GRIMWOOD K., WAINWRIGHT C.E., HUGENHOLTZ P. 2012. *Comparison of DNA extraction methods for microbial community profiling with an application to pediatric bronchola lveolar lavage samples.* PLoS One, 7(4): e34605.

World Health Organization. 2017. Guidelines for drinking-water quality, 4th edition, Incorporating the 1st addendum.

World Health Organization. 2002. *Heterotrophic plate count measurement in drinking water safety management. WHO Public Health Expert Report*, Geneva, Switzerland. http://www.who.int/water_sanitation_health/dwq/WSH02.10.pdf, access: 15.01.2015.

YAMANE T.1967. *Statistics: An introductory analysis.* 2nd Ed., New York, Harper and Row.

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 38(1): 31–42, Y. 2023

DOI: 10.31648/pjns.8607

DETECTION OF POTENTIALLY PATHOGENIC BACTERIA IN BUTCHER SHOPS: FIRST REPORT FROM AL MANDAQ CITY, SAUDI ARABIA

Mohammad Melebari

ORCID: 0000-0001-6312-181X
Department of Biology, Faculty of Science
Al-Baha University, Al-Baha, Saudi Arabia

Key words: food safety, pathogenic bacteria, butcher shop, meat, local market, *E. coli*.

Abstract

Food-borne pathogens cause significant economic losses and affect the quality of life. Butcher shops act as a perishable commodity and contribute to the possible spread of food-borne pathogens. Due to the absence of knowledge and studies related to food safety in Al Mandaq city, Saudi Arabia, the main objective of this study was to initiate and establish a database about the possibilities of the existence of pathogenic bacteria in butcher shops in the city. From each local shop at the city (n = 6), 14 samples were collected from various spots, and the potential bacterial pathogens were identified on specific media (blood agar, MacConkey agar, Hicrome *Staphylococcus* selective agar, and Eosin methylene blue agar). Based on the number of presumed pathogens, Shop 1 was the most contaminated (n = 57), followed by Shop 2 (n = 51). Among the collected samples, lamb meat contained the highest number (25) of all pathogens, followed by beef, the floor, and the fridge (22 each). The prevalence of *Staphylococcus aureus* was 29%, followed by *Salmonella* spp. (26%) and *Escherichia coli* (24%) in all the samples of the six butcher shops. All 84 samples were less contaminated with *Salmonellae* spp. (10%) compared with other isolated pathogens. The increased frequency of these potential pathogens in meat shows the appallingly unhygienic and unsanitary techniques used, from the slaughterhouse, during transportation to butcher shops, and during processing at the butcher shops. As a result, the current investigation, which is the first of its type in Al Mandaq, demonstrates that meat is highly contaminated with potential bacterial pathogens. Minimizing meat contamination in markets and using good sanitation and inspection techniques are crucial.

Introduction

Food-borne diseases (FBDs) are one of the most critical global public health issues and should be addressed on a priority basis to ensure a healthy environment (KHAN et al. 2022). Around the world, one person out of 10 (600 million people) is a victim of FBDs. Each year, 420,000 peo-

Address: Mohammad Melebari, Al-Baha University, Al-Baha 65431 Saudi Arabia, phone: + 966556558455, e-mail: melebari@bu.edu.sa

ple die due to food poisoning. It is reported that 70% of FBDs are caused by microorganisms (WHO 2015). Bacteria play a crucial role in the development of FBDs due to either the production of toxins or the colonization of these bacteria to the epithelial cells of the gastrointestinal tract (KIRK et al. 2015).

Meat is a perishable food and provides a favorable environment for the growth of pathogenic bacteria and microorganisms, which causes it to spoil (BIRHANU et al. 2017). Meat is exposed to a variety of contaminations, from the production stage to the point at which it is available to consumers (ARAFA et al. 2022). Such contamination is the prime cause of illness and, occasionally, death upon ingestion due to the persistence of pathogenic microorganisms (WHO 2007). Mainly, FBDs result from ingesting pathogenic bacteria and microbial toxins (BANNON et al. 2016).

Many reports depict the isolation of bacterial pathogens from fresh meat, which plays an important role in the onset of diseases in humans (BANNON et al. 2016, BANTAWA et al. 2018, CASTELLANO et al. 2008, MESHAAL et al. 2021, MOR-MUR and YUSTE 2009, UKUT et al. 2010). These pathogens include *E. coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, etc. The main sources of these pathogenic bacteria in butcher shops are slaughtered animals, workers' gloves, knives, meat storage, contaminated water, tables, cutting boards, and weighing scales (BIRHANU et al. 2017).

With prolonged favorable environments, such as acidity, moisture, temperature, and availability of nutrients, the microbial load in meat increases (NIYONZIMA et al. 2015). *E. coli*, *Listeria monocytogenes, Salmonella* spp., *Staphylococcus aureus*, and *Campylobacter* spp. are considered the main contaminants of meat and can cause not only diarrhea but also other gastrointestinal disorders (ORPIN et al. 2018). One of the mesophilic commensal microbes present in the digestive tract of both humans and animals is *Escherichia coli* (DUBREUIL 2012). Gastroenteritis and extra-intestinal infections such as urinary tract infections (UTIs) are caused by pathogenic strains of *E. coli* (BANNON et al. 2016).

Determination of the bacterial load in the meat indicates the hygienic quality of the meat. Factors such as poor facilities in butcher shops, slaughtering of diseased animals, poor handling of the remains after slaughtering, and contaminated environments contribute to the high bacterial count, which in turn is a threat to humans (BIRHANU et al. 2017).

Without a hygienic environment at butcher shops and abattoirs, the availability of pathogen-free meat is minimal. Control measures are taken to ensure the hygiene and quality of meat, particularly in the catering industry (TAVAKOLI and RIAZIPOUR 2008). Few reports are available on handling practices and hygiene status of the meat at butcher shops in

some parts of Saudi Arabia, keeping these gaps in view, the purpose of this study was to establish a study and to assess the potential existence of pathogenic bacteria in local butcher shops at Al Mandaq city, Saudi Arabia, particularly *Staphylococcus aureus*, *Staphylococcus epidermis*, *E. coli*, *Salmonella* spp., and *Shigella* spp.

Materials and Methods

Samples collection

Samples were collected from instruments/apparatus used in butcher shops at different locations in Al Mandaq, located in the southwestern region of Saudi Arabia. These samples were classified into two categories: Abiotic samples and biotic samples (Table 1). A total of 84 different samples were collected aseptically using cotton swabs from six butcher shops (14 samples from each shop). All samples were stored in an icebox and then brought to the lab in sterilized containers.

Table 1

Types of collected abiotic and biotic samples from each butcher shop

Biotic samples	Abiotic samples
butchers' hands	meat storage
lamb meat	cutting knives
beef meat	cutting boards
–	scales
–	sinks
–	masks
–	floors
–	gloves
–	fridges
–	doors
–	saws
–	mincers

Isolation and identification of bacterial strains

Swabs were streaked on different media using the protocol of MELEBARI et al. (2022). Used media were blood agar, MacConkey agar, Hicrome *Staphylococcus* selective agar, and Eosin methylene blue (EMB) agar, and

incubated at 37°C for 24 hours. After 24 hours of incubation, morphologically distinct colonies were picked and purified. All the isolates were identified using specific characters on a selective medium. Salmonella-Shigella agar medium was used to identify *Salmonella* spp. and *Shigella* spp. The *E. coli* strains were identified by producing a metallic sheen on the EMB medium. Hicrome *Staphylococcus* selective agar was used to identify *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Staphylococcus aureus* and *Staphylococcus epidermidis* were identified based on the production of green and blue colonies on said medium. Gram staining and catalase activity of the isolated strains were performed.

Basic confirmation tests

Gram staining of the isolated strain was performed using the protocol of SMITH and HUSSEY (2005). Catalase activity of the isolated strains was conducted using 3% H_2O_2 on the bacterium colony, and bubble formation was noted. The hemolytic potential of the isolated strains was checked on blood agar (nutrient agar + 10% sheep blood). Pathogens were streaked on blood agar and incubated at 37ºC for 24 hours. Hemolysis was checked based on the clear zone and color of colonies.

Statistical analysis

Frequencies and percentages were used to represent the data. To compare the groups, a chi-square test was performed using SPSS 26. A substantial impact was judged to be demonstrated by a p-value of >0.05.

Results

Six butcher shops were targeted for the isolation of pathogenic bacterial strains with standard procedure. Samples were collected from different tools and storage used in the butcher shop, including meat storage, cutting knives, cutting boards, scales, sinks, masks, floors, gloves, fridges, doors, saws, mincers, the butchers' hands, lamb meat, and beef meat. These strains were *Staphylococcus aureus, Staphylococcus epidermidis, Salmonella* spp., *Shigella* spp., and *E. coli*, based on identification on respective media. The highest pathogenic load was found in shop 1, where the total number of pathogens was 57, followed by shop 2 (51), shop 3 (46), shop 6 (45), shop 4 (43), and shop 5 (37) – Figure 1.

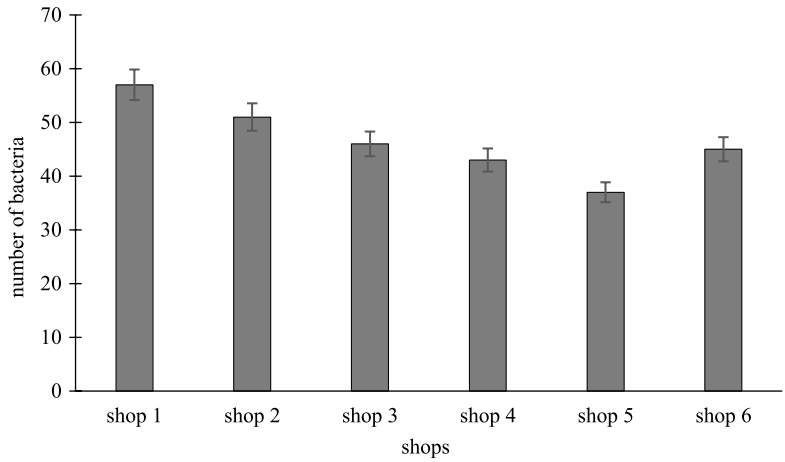

Fig. 1. Pathogenic load in collection site

Out of 57 bacterial strains from Shop 1 from all the samples, the number of each strain was ≥10, among which *S. aureus* was the highest (13), followed by *S. epidermidis* and *E. coli* (each 12), *Salmonella* spp. (10), and *Shigella* spp. (10). From Shop 2, 14 *S. aureus*, 13 *S. epidermidis*, 10 *E. coli*, and seven *S. aureus* were isolated. From Shop 3, 14 *S. aureus*, 12 *S. epidermidis*, 13 *E. coli*, two *Salmonella* spp., and five *Shigella* spp. were isolated. *Salmonella* spp. and *Shigella* spp. were isolated less in number (three each). From Shop 5, *S. aureus* (14) was found more than the others: *S. epidermidis* (10), *E. coli* (11), *Salmonella* spp. (1), and *Shigella* spp. (1). The highest number of pathogenic strains found in Shop 6 was *S. aureus* (14), followed by *S. epidermidis* (12), *E. coli* (10), *Shigella* spp. (5), and *Salmonella* spp. (4) – Table 2.

Table 2

Bacterial pathogens isolated from collection site

Shop	Pathogens [%]				
	S. aureus	*S. epidermidis*	*E. coli*	*Salmonella* spp.	*Shigella* spp.
Shop 1	23	21	21	18	18
Shop 2	27	25	20	14	14
Shop 3	30	26	28	4	11
Shop 4	30	33	23	7	7
Shop 5	38	27	30	3	3
Shop 6	31	27	22	9	11

Fourteen samples were selected for the isolation of bacterial strains. The collective number of bacteria from each sample was calculated from the total shops. Biotic samples contained higher numbers of pathogens than abiotic samples. The most contaminated was lamb's meat, from which 25 strains were isolated. Twenty-two strains were isolated each from beef meat, mincers, floors, and fridges. The bacterial count isolated from boards, scales, and gloves was 19 for each sample. From each sample of saws and sinks, 21 bacteria were isolated from all six shops. The lowest bacterial load was observed for masks, from which 14 bacterial isolates were recovered (Figure 2).

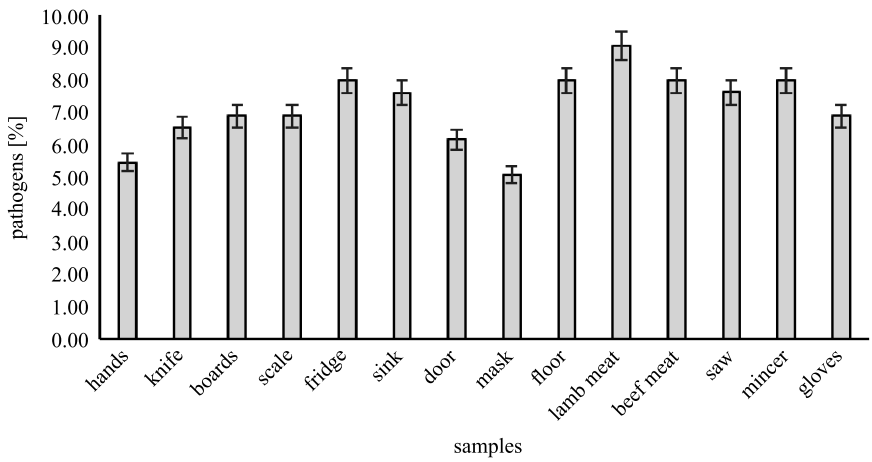

Fig. 2. Bacterial load with respect to samples from all the shops

Pearson chi-square analysis was performed to determine the association of the pathogens to the total observation (84) from all six butcher shops. From statistical analysis, a significant association between the sampling location and *S. aureus* ($p = 0.535$), *S. epidermidis* ($p = 0.353$), and *E. coli* ($p = 0.696$) count was not observed ($p > 0.05$). However, such association was found for *Shigella* spp. and *Salmonella* spp., where the p-values were 0.008 and 0.002 ($p < 0.05$), respectively (Table 3).

The total number of pathogens isolated from all the shops with respect to samples was calculated. The most isolated pathogen in all the shops was *S. aureus* ($n = 82$). Almost all 14 samples contained this pathogen. However, this pathogen was not present in the door sample of Shop 1 and the mincer sample of Shop 4 (Figure 3). This pathogen contributed 29% to the overall load of pathogens (Figure 4). The second most abundant pathogen in all the samples from all shops was *S. epidermidis* ($n = 70$).

Table 3

Chi-square analysis of the isolated pathogens from all the shops

Specification	*S. aureus*			*S. epidermidis*			*E. coli*			*Salmonella* spp.			*Shigella* spp.		
	value	df	*p*-value	value	df	*p*-value	value	df	*p*-value	value	df	*p*-value	value	df	*p*-value
Pearson Chi-Square	4.098[a]	5	0.535	5.544[a]	5	0.353	3.024[a]	5	0.696	18.830[a]	5	0.002	15.556[a]	5	0.008
Likelihood Ratio	4.493	5	0.481	6.810	5	0.235	3.331	5	0.649	19.347	5	0.002	16.582	5	0.005
Linear-by-Linear Association	0.694	1	0.405	0.434	1	0.510	0.506	1	0.477	10.211	1	0.001	10.979	1	0.001
N of Valid Cases	84	–	–	84	–	–	84	–	–	84	–	–	84	–	–
a) 6 cells (50.0%) have an expected count of less than 5; the minimum expected count is 0.88				a) 6 cells (50.0%) have an expected count of less than 5; the minimum expected count is 1.83			a) 6 cells (50.0%) have an expected count of less than 5; the minimum expected count is 2.83			a) 6 cells (50.0%) have an expected count of less than 5; the minimum expected count is 4.50			a) 0 cells (0%) have an expected count of less than 5; the minimum expected count is 5.00		

From Shop 1, this strain was isolated from all the samples except door and mask samples. From the hand sample of Shop 2 and mask and glove samples from Shop 3, *S. epidermidis* was not observed. All the samples from Shop 4 contained this strain. The fridge, sink, floor, and saw samples of Shop 5 were free of *S. epidermidis*. All the samples of Shop 6 contained these pathogens except mask and beef meat samples (Figure 3). This pathogen contributed 26% of the pathogens' total load from all the samples collected from the six butcher shops (Figure 4). *E. coli* was the third most abundant pathogen isolated from the samples (n = 67). Except for hand samples, all the other samples of Shop 1 contained *E. coli*. This pathogen was absent in samples of hands, knives, boards, and masks of Shop 2. *E. coli* was recovered from all the samples of Shop 3 except the mask sample. Knife, scale, and lamb meat samples for Shop 4 were not contaminated with the pathogen. The glove and mask samples of Shops 5 and 6 were free of *E. coli*. This pathogen was absent from the floor of Shop 5. Hands and fridges were contaminated with this pathogen in Shop 6 (Figure 3). This pathogen contributed 24% to the total load of the pathogens from all the samples collected from all six butcher shops (Figure 4). *Shigella* spp. was the fourth most abundant pathogen among the pathogens isolated from all the samples of all the shops (n = 31). Hands, board, door, and mask samples of Shop 1 were free of this pathogen. Scale, sink, door, floor, lamb meat, beef meat, and glove samples from Shop 2 were positive for the presence of *Shigella* spp. Board, fridge, sink, beef meat, and mincer samples from Shop 3 contained this pathogen. All the samples of Shop 5 were free of this pathogen except for the lamb meat. Samples of hand, floor, lamb meat, and saw from Shop 6 were contaminated with *Shigella* spp. (Figure 3). This pathogen contributed 11% to the total load of the patho-

gens from all the samples collected from all six butcher shops (Figure 4). *Salmonella* spp. was the fifth most abundant pathogen among the pathogens isolated from all the samples of all the shops (n = 27). Hands, sink, door, and mask samples from Shop 1 were free of *Salmonella* spp. Scales, sinks, floors, lamb meat, beef meat, mincers, and gloves from Shop 2 were positive for the presence of this pathogen. Fridge and sink samples collected from Shop 3 were contaminated with *Salmonella* spp. No sample from Shop 4 contained this pathogen except the fridge, sink, and mincer. All the samples from Shop 5 were free of *Salmonella* spp., except for lamb meat. Samples of the floor, lamb meat, and saws from Shop 6 showed the presence of this bacterium (Figure 3). This pathogen contributed 10% to the total load of pathogens from all the samples collected from all six butcher shops (Figure 4).

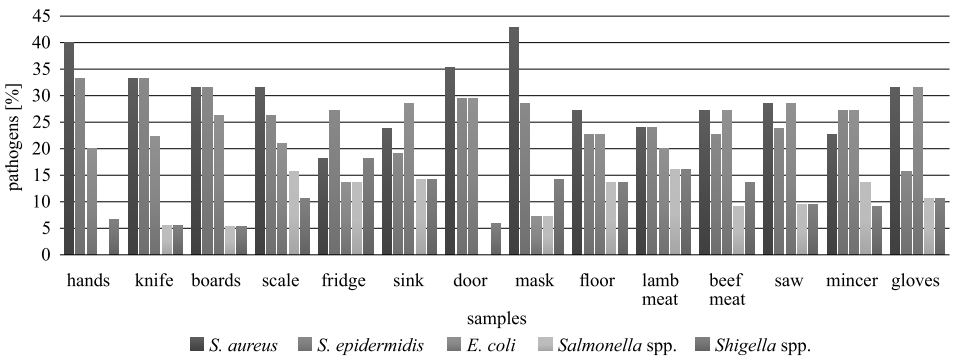

Fig. 3. Number of each pathogen from all the shops

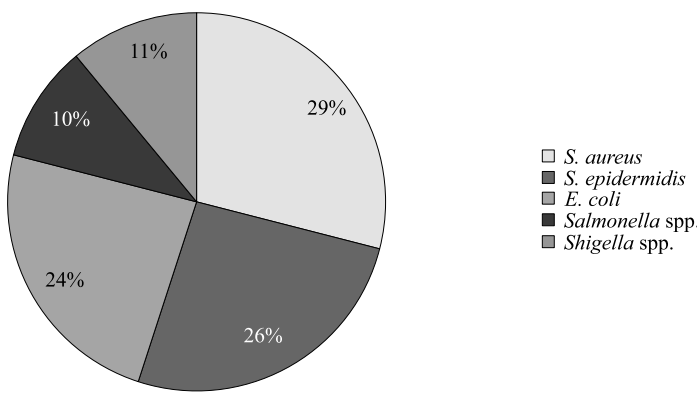

Fig. 4. Distribution of pathogens [%]

Discussion

Meat provides an excellent environment for pathogenic bacteria (DAS et al. 2019). Generally, meat is consumed cooked, but in some recipes, meat is used raw or partially cooked, which gives rise to the problem of food poisoning (HENNEKINNE et al. 2015). The butcher shop is a component of the food industry that can contribute to the spread of food-borne pathogens, toxins, and other contamination (BANNON et al. 2016). Using such contaminated food with pathogens or toxins results in diarrhea, which can lead to death. Each year, 3 million deaths occur worldwide due to food poisoning (WHO 2007).

An unhygienic butcher shop is considered a source of pathogens unless the SOPs of hygienic practices are employed (ROBERTS et al. 2009). From the current study, it is clear that such a load of pathogenic bacteria from all the samples results from a lack of SOPs and personal hygiene. Workers' hygienic conditions impact the possible contamination of the meat. Diseased and unclean workers, equipment, dressing type, and dressing process all accounted for pathogenic spread and storage (SAMUEL et al. 2011). A general recommendation for workers is to use contamination-free clothes, gloves, protective coats, and hair cover while processing the meat (MOAE 2010). In our study, *S. aureus*, *S. epidermidis*, *E. coli,* and *Shigella* spp. were recovered from the hands of the workers which is in agreement with the findings of BERSISA et al. (2019), who reported that cross-contamination could also happen while handling food with contaminated hands.

In the current study, gloves and masks are considered part of clothing. We isolated all the types of pathogens from these samples, indicating the unhygienic clothing of the workers. The workers themselves may be a potential source of contamination due to disease in addition to their clothing. It was advised that new hires be given a clinical and bacteriological examination before hiring and regularly afterward (BERSISA et al. 2019). The examination should include a medical history to ascertain any prior infections, focusing on venereal and skin diseases, dysentery, typhoid, and paratyphoid fevers (WHO 2004). Handling money and touching carcasses with the same unclean hands could be important contamination sources (BERSISA et al. 2019)). Another important consideration is that most abattoirs are located on the side of the road, where they are subjected to wind and vehicle-generated dust, which might contaminate them with the organisms prevalent there. The samples used, unclean methods of transportation, handling, and processing, an unhygienic atmosphere, and practices such as employing dirty cutting boards, knives, or utensils may

all account for the variation in the overall bacterial counts. Our results are in line with the results of ADEBOWALE et al. (2010), who reported that such variation in number might be due to cutting, cleaning, and storing practices. This study determined a possible variety of pathogens on knives and cutting boards. This is because of the contamination of meat with pathogens or contaminated water or previous persistence of the pathogens on the surface of other tools used in butcher shops. The same finding was reported by GURMU and GEBRETINSAE (2013). This study aimed to isolate bacterial pathogens, particularly *Salmonella* spp., *Shigella* spp., *E. coli, S. aureus*, and *S. epidermidis*. Our results align with KUMAR et al. (2014), who isolated the same pathogens from meat.

Conclusion

In summary, this study aimed to isolate bacterial pathogens, particularly *Salmonella* spp., *Shigella* spp., *E. coli, S. aureus,* and *S. epidermidis* using different kinds of culture media. From different local butcher shops, all locations showed the potential existence of pathogenic bacteria. The most isolated pathogen in all different shops was *S. aureus* (29%), and *Salmonella* spp. was the least (10%) among the pathogens isolated from all the samples of all the shops. The increased frequency of these pathogens in meat shows the appallingly unhygienic and unsanitary techniques used in the slaughterhouse, during transportation to butcher shops, and during processing at the butcher shops. Advanced techniques such as molecular identification of all the isolated strain-based 16S rRNA genes are recommended to know the exact taxonomic position in addition to antimicrobial susceptibility testing.

Accepted for print 7.03.2023

References

ADEBOWALE O., ALONGE D., AGBEDE S., ADEYEMO O. 2010. *Bacteriological assessment of quality of water used at the Bodija municipal abattoir, Ibadan, Nigeria*. Sahel J. Vet. Sci., 9(2): 63–67.

ARAFA S.H., ALSHEHRI W.A., ORGANJI S.R., ELBANNA K., OBAID N.A., ALDOSARI M.S., ASIRI F.H., AHMAD I., ABULREESH H.H. 2022. *Antimicrobial resistance, virulence factor-encoding genes, and biofilm-forming ability of community-associated uropathogenic in western Saudi Arabia*. Pol. J. Microbiol., 71(3): 325–339, doi: 10.33073/pjm-2022-029.

BANNON J., MELEBARI M., JORDAO JR C., LEON-VELARDE C.G., WARRINER K. 2016. *Incidence of top 6 Shiga toxigenic Escherichia coli within two Ontario beef processing facilities: challenges in screening and confirmation testing*. Aims Microbiol., 2(3): 278–291.

BANTAWA K., RAI K., SUBBA LIMBU D., KHANAL H. 2018. *Food-borne bacterial pathogens in marketed raw meat of Dharan, eastern Nepal*. BMC Research Notes, 11(1): 618, doi: 10.1186/s13104-018-3722-x.

Bersisa A., Tulu D., Negera C. 2019. I*nvestigation of bacteriological quality of meat from abattoir and butcher shops in Bishoftu, Central Ethiopia*. Int. J. Microbiol., 2019, Article ID 6416803*:* 1–8, doi: 10.1155/2019/6416803.

Birhanu W., Weldegebriel S., Bassazin G., Mitku F., Birku L., Tadesse M. 2017. *Assesment of microbiological quality and meat handling practices in butcher shops and abattoir found in Gondar town, Ethiopia*. Int. J. Microbiol. Res., 8(2): 59–68.

Castellano P., Belfiore C., Fadda S., Vignolo G. 2008. *A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina*. Meat Sci., *79*(3): 483–499, doi: 10.1016/j.meatsci.2007.10.009.

Das A. K., Nanda P.K., Das A., Biswas S. 2019. *Hazards and safety issues of meat and meat products*. In: *Food safety and human health*. Eds. R.L. Singh, S. Mondal, Academic Press, Elsevier Inc., pp. 145–168, doi: 10.1016/B978-0-12-816333-7.00006-0.

Dubreuil J.D. 2012. *The whole shebang: the gastrointestinal tract, Escherichia coli enterotoxins and secretion*. Curr Issues Mol Biol., 14(2): 71–82.

Gurmu E.B., Gebretinsae H. 2013. *Assessment of bacteriological quality of meat contact surfaces in selected butcher shops of Mekelle city, Ethiopia*. J. Environ. Occup. Sci., 2(2): 61–66.

Hennekinne J.A., Herbin S., Firmesse O., Auvray F. 2015. *European food poisoning outbreaks involving meat and meat-based products*. Procedia Food Sci., 5: 93–96, doi: 10.1016/j.profoo.2015.09.024.

Khan J.A., Rathore R.S., Ahmad I., Gill R., Husain F.M., Arshad M., Alam P., Albalawi T., Al-Kheraif A., Akhtar J., Albarakaty F. M., Neyaz L. A., Elbanna K., Abulreesh H.H. 2022. *Assessment of foodborne bacterial pathogens in buffalo raw milk using polymerase chain reaction based assay*. Food-borne Pathogens and Disease, 19(11): 750–757, doi: 10.1089/fpd.2022.0044.

Kirk M.D., Pires S.M., Black R. E., Caipo M., Crump J.A., Devleesschauwer B., Döpfer D., Fazil A., Fischer-Walker C.L., Hald T. 2015. *World Health Organization estimates of the global and regional disease burden of 22 food-borne bacterial, protozoal, and viral diseases, 2010: a data synthesis*. PLoS medicine, 12(12): e1001921.

Kumar P., Rao J., Haribabu Y. 2014. *Microbiological quality of meat collected from municipal slaughter houses and retail meat shops from Hyderabad Karnataka region, India*. APCBEE procedia, 8: 364–369.

Melebari M., Alpakistany T., Taha T.M. Alsalman A.S. 2022. *Prevalence of pathogenic bacteria on face masks from wet markets in Makkah during the COVID-19 pandemic*. Sci. J. King Faisal Univ., 23(2): 33–8, doi: 10.37575/b/sci/220040.

Meshaal A.K., Hetta H.F., Yahia R., Abualnaja K. M., Mansour A.T., Al-Kadmy I.M.S., Alghamdi S., Dablool A.S., Emran T.B., Sedky H., Batiha G.E., El-Kazzaz W. 2021. *In vitro antimicrobial activity of medicinal plant extracts against some bacterial pathogens isolated from raw and processed meat*. Life, 11(11): 1178.

MOAE. 2010. Animal and Plant Health Regulatory Directorate, Meat Handlers Personal Hygiene Guideline for Abattoir and Airport Cargo Terminal Worker*s*. Addis Ababa, Ethiopia.

Mor-Mur M., Yuste J. 2009. *Emerging bacterial pathogens in meat and poultry: an overview*. Food Bioproc Tech, 3(1): 24, doi: 10.1007/s11947-009-0189-8.

Niyonzima E., Ongol M.P., Kimonyo A., Sindic M. 2015. *Risk factors and control measures for bacterial contamination in the bovine meat chain: a review on Salmonella and pathogenic E. coli*. J. Food Res., 4(5): 98–121, https://nru.uncst.go.ug/xmlui/handle/123456789/129, access: 12.05.2023.

Orpin J., Mzungu I., Osuji C. 2018. *Isolation and identification of bacteria associated with suya (roasted meat product) sold in Dutsinma Local Government Area, Kastina State*. J. Adv. Biol. Biotechnol., 20(2): 1–8.

Roberts H., de Jager L., Blight G. 2009. W*aste-handling practices at red meat abattoirs in South Africa*. Waste Manag. Res., 27(1): 25–30, doi: 10.1177/0734242X07085754.

Samuel L., Marian M., Apun K., Lesley M., Son R. 2011. *Characterization of Escherichia coli isolated from cultured catfish by antibiotic resistance and RAPD analysis*. Int. Food Res. J., 18(3): 971– 976.

SMITH A.C., HUSSEY M.A. 2005. *Gram stain protocols*. American Society for Microbiology, 1, 14.
TAVAKOLI H.R., RIAZIPOUR M. 2008. *Microbial quality of cooked meat foods in Tehran university's restaurants*. Pak. J. Med. Sci., 24(4): 595–599.
UKUT I., OKONKO I., IKPOH I., NKANG A., UDEZE A., BABALOLA T., MEJEHA O., FAJOBI E. 2010. *Assessment of bacteriological quality of fresh meats sold in Calabar metropolis, Nigeria*. Elec. J. Env. Agricult. Food Chem., 9(1): 89–100.
WHO. 2004. *Developing and maintaining food safety control systems for Africa: current status and prospects for change*. In: *Second FAO/WHO Global Forum of Food Safety Regulators*, https://www.researchgate.net/publication/278000382_Developing_and_maintaining_national_food_safety_control_systems_Experiences_from_the_WHO_African_region, access: 6.01.2023.
WHO. 2007. *Food-borne hazards in basic food safety for health workers*. http://www.who.int/mediacentre/factsheets/fs237/en/, access: 6.01.2023.
WHO. 2015. *Informe Estimación de la carga mundial de las enfermedades de transmisión alimentaria*. Ginebra [Internet]. 2015 [Updated: 2017]. http://www.who.int/mediacentre/news/releases/2015/foodborne-disease-estimates/es/, access: 12.12.2017.

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 38(1): 43–50, Y. 2023

DOI: 10.31648/pjns.8423

SURVIVAL AND GROWTH OF SILVER RASBORA (*RASBORA ARGYROTAENIA*) FED ARTEMIA ENRICHED WITH *SARDINELLA LEMURU* FISH OIL

***Vira Yuniar*[1], *Darmawan Setia Budi*[2], *Asep Permana*[3], *Ahmad Shofy Mubarak*[4]**

[2] ORCID: 0000-0001-9508-1437
[3] ORCID: 0000-0002-7759-3022
[4] ORCID: 0000-0003-1654-0397
[1,2] Study Program of Aquaculture,
School of Health and Life Science, University of Airlangga, Surabaya, East Java, Indonesia
[3] Research Center for Conservation of Marine and Inland Water Resources
National Research and Innovation Agency, Jakarta, Indonesia
[4] Department of Marine, Faculty of Fisheries and Marine
University of Airlangga, Surabaya, East Java, Indonesia

Key words: fish species, artemia nauplii, life feed enrichment, larvae feeding.

Abstract

Silver rasbora (*Rasbora argyrotaenia*) is a new fish species cultured economically for consumption and aquascape. The purpose of this study was to know the effect and determine the optimal concentration of *Sardinella lemuru* fish oil enrichment on artemia to the survival rate and growth of silver rasbora larvae. Fish larvae were feed by enriched artemia nauplii with different doses per liter water. Larvae feeding artemia enrichment with *Sardinella lemuru* fish oil in different doses not affect ($P > 0.05$) on survival, but all growth parameters significantly affected ($P < 0.05$). Optimal artemia enrichment dose for feeding practical of silver rasbora larvae is 0.5 ml fish oil + 0.5 ml egg yolk. Estimate doses that leads to the maximum length gain is 0.6035 ml fish oil + 0.6035 ml egg yolk.

Introduction

Silver rasbora (*Rasbora argyrotaenia*) is a tropical freshwater fish spread in Asian region (Mekong, Chao Phraya and Mae Khlong basins, Malay Peninsula to Borneo, Java and Sumatra in Indonesia), and occurs in rivers and enters flooded fields mainly (CAPULI and BAILLY 2019). The decline in silver rasbora wild population due to high capture for consumption (ROSADI et al. 2014) needs aquaculture production to overcome this problem. As newly cultured species (ADAWIYAH et al. 2019), silver rasbora farming continues to grow and several studies on hatcheries and grow-ups have been carried out.

Address: Darmawan Setia Budi, University of Airlangga, Surabaya, Wijaya Kusuma Street No. 113, Banyuwangi 68425, East Java, Indonesia, e-mail: darmawansetiabudi@fpk.unair.ac.id

The fry availability affects the reliability of silver rasbora farming as a limiting factor (BUDI et al. 2020). Thus, increasing survival and growth must be one of the study goals for many species during the larval phase (SLATER et al. 2018). The quality and quantity of suitable feed in the early larval life can affect the growth and survival of fish (QIN et al. 1997). As the first feed for larvae, many hatcheries still depend on the zooplankton in spite of little or absence of nutrients (KOTANI 2017). Although deficient in certain important nutrients, artemia is important zooplankton as early feed used in larviculture, and enrichment is thus common with nutrients and other functional additives (LANES et al. 2012).

Fish oil is one of additives used in artemia enrichment as life food (LANES et al. 2012). As the major lipid source, the fish oil used to increase energy content and essential fatty acids (EFA) in fish diets (HOSSEINI et al. 2010). EFA are important for early development and survival of fish larvae (IZQUIERDO 1996). *Sardinella lemuru* fish oil is a by-product produced in the fish canning industry located in Banyuwangi, East Java, Indonesia. It has been used for feed supplementation in silver rasbora juvenile (AGUSTIN et al. 2020, AYUNDA et al. 2020, DEWI et al. 2020, MARINI et al. 2020), and potentially use for silver rasbora larvae. The purpose of this study was to know the effect and determine the optimal concentration of *Sardinella lemuru* fish oil enrichment on Artemia to the survival rate and growth of larvae of silver rasbora.

Materials and Methods

Larvae origin

This study was conducted from January to February 2019 in Universitas Airlangga, Banyuwangi Campus. The research was carried out in accordance with the ethical standards of the Law of the Republic of Indonesia Number 18 of 2002 about the National System of Research, Development, and Application of Science and Technology.

Approximately 1-year old brood fish of silver rasbora were imported from Technical Implementation of Development Unit of Freshwater Aquaculture of Umbulan (Pasuruan, East Java, Indonesia). Breeding of brood fish were done using 6 males (4.62 ±0.07 g of body weight and 7.50 ±0.40 cm of total length) and 2 females (5.20 ±0.14 g body weight and 8.60 ±0.56 cm total length). Palm tree fibers were used as a substrate of brood fish natural mating and spawning (BUDI et al. 2020) in a glass aquarium (40 x 50 x 50 cm^3). Brood fish were transferred from the aquarium and the eggs stick to the substrate incubated in this media approximately

2 days for hatching. The dissolved oxygen (DO), temperature, pH, and total ammonia nitrogen (TAN) measured were 6.82 ±0.38 ppm (range 6.2–7.5 ppm), 24.94 ±0.6ºC (range 24–27ºC), 7.24 ±0.32 (range 6.9–7.5), and 0.012 ±0.001 ppm (range 0.010–0.013 ppm), respectively.

Artemia culture and enrichment

A total 1 g of Artemia cysts were cultured in 1.5 l aerated conical plastic bottle using 1 l salt water (30 ppt). After 24 hours, artemia nauplii were transferred to enriched media (100 individual/ml) with various *Sardinella lemuru* fish oil concentrations based on treatments for 6 hours. Before enrichment process, chicken egg yolk was mixed with fish oil (ratio 1:1) to formed emulsion in enrichment media.

Experimental design and rearing

A total of 900 silver rasbora larvae (1.08 ±0.06 mg body weight, 3.14 ±0.17 mm total length) aged 4 days after fertilization (DAF) were assigned to 18-cylinder plastic tank (10 l capacity; 30 cm diameter) with 5 l water volume with maintain dissolved oxygen level using gentle aeration. Fish larvae were feed by enriched artemia nauplii based on the treatments with doses per liter media 0 ml fish oil (P_0), 1 ml egg yolk (P_1), 0.25 ml fish oil + 0.25 ml egg yolk (P_2), 0.5 ml fish oil + 0.5 ml egg yolk (P_3), 0.75 ml fish oil + 0.75 ml egg yolk (P_4), and 1 ml fish oil + 1 ml egg yolk (P_5). There were 3 repetitions for each treatment. Feed was given ad satiation and estimated 20 artemia nauplius per day per larvae with feeding frequency 4 times per day which is 06.00 AM, 12.00 AM, 05.00 PM, and 09.00 PM. Water quality parameters of larvae rearing can be seen in Table 1.

Table 1

Water quality parameters of silver rasbora larvae fed artemia enriched with *Sardinella lemuru* fish oil for 21 days

Treatments*	Dissolved oxygen [mg/l]	Temperature [ºC]	pH	Total ammonia nitrogen [mg/l]
P_0	7.2–8.5	25–27	7–7.4	0–0.029
P_1	7.5–8.5	25–27	6.9–7.4	0–0.002
P_2	7.2–8.4	25.5–27	6.9–7.3	0–0.027
P_3	7.8–8.5	25.5–27	6.9–7.3	0–0.035
P_4	7.8–8.4	25–27	7–7.4	0.038–0.069
P_5	7.8–8.4	25.5–27	6.9–7.3	0–0.073

*Treatments doses per liter media 0 ml fish oil (P_0), 1 ml egg yolk (P_1), 0.25 ml fish oil + 0.25 ml egg yolk (P_2), 0.5 ml fish oil + 0.5 ml egg yolk (P_3), 0.75 ml fish oil + 0.75 ml egg yolk (P_4), and 1 ml fish oil + 1 ml egg yolk (P_5).

Observation and measurements of larvae

At least 10% (n = 5) of larvae number at each treatment were sampled randomly in 4 DAF, 11 DAF, 18 DAF, and 25 DAF. Rapid cooling anesthetic method was used for larvae sampling procedure (CHEN et al. 2013). Larvae total body length (mm) was measured using a micrometer under a stereo-microscope and larvae body weight was measured using a digital scale with 0.1 mg precision.

Observed parameters

The effects of feeding artemia enriched with *Sardinella lemuru* fish oil on survival and growth of silver rasbora larvae were calculated the following parameters. Survival rates (SR, %) was calculated following formula:

$$SR\ [\%] = [(N_f / N_i) \cdot 100],$$

where:

N_i – the initial larvae numbe
N_f – the final larvae number.

Length gain (LG, mm) was calculated based on formula:

$$LG = TL_f - Tl_i,$$

where:
TL_i and TL_f are initial and final average total length [mm].

Weight gain (WG, mm) was calculated based on formula:

$$WG = W_f - W_i,$$

where:
W_i and W_f are initial and final average weight [mg].

Growth rate (GR, mg/days) was calculated following formula:

$$GR = (W_f - W_i)/ D,$$

where:
W_i and W_f are initial and final average weight [mg]
D – rearing times [days].

The specific growth rate (SGR, %/day) was calculated by formula:

$$SGR = [(\ln BW_f - \ln BW_i) / D) \cdot 100],$$

where:
BW_i and BW_f are the initial and final body weights of fish
D – rearing times [days].

Data analysis

The each analyzed data parameters were confirmed normal distribution and homogeneity of variances. Statistically data analysis was used ANOVA test with 95% confidence level and continued with Duncan Multiple Range Test (DMRT) using SPPS 17.0 software. *Sardinela lemuru* fish oil doses that leads to the maximum length gain of silver rasbora (*Rasbora argyrotaenia*) was estimated using polynomial contrast (regression) (YOSSA and VERDEGEM 2015).

Results

The survival and growth of silver rasbora larvae fed artemia enriched with *Sardinella lemuru* fish oil are showed in Table 2. Larvae feeding with artemia enriched with *Sardinella lemuru* fish oil affect significantly ($P < 0.05$) on all growth parameters, but not so with survival. Overall growth data showed the same tendency; where LG, WG, GR, and SGR tends to be increase from P_0 to maksimum in P_3; and then tends to be decrease to P_5 as last treatment. *Sardinela lemuru* fish oil doses that leads to the maximum length gain of silver rasbora (*Rasbora argyrotaenia*) estimated using polynomial contrast (regression) was 0.6035 ml/l.

Table 2

Survival rate (SR), length gain (LG), weight gain (WG), growth rate (GR), and specific growth rate (SGR) of silver rasbora larvae fed artemia enriched with *Sardinella lemuru* fish oil for 21 days

Treatments*	SR [%]	LG [mm]	WG [mg]	GR [mg/days]	SGR [%/days]
P_0	88.3 ±7.8	7.53^{c} ±0.46	13.7^{b} ±1.71	0.65^{b} ±0.08	12.73^{c} ±0.55
P_1	87.3 ±5.8	7.59^{c} ±0.20	15.5^{ab} ±0.68	0.74^{ab} ±0.03	13.29^{abc} ±0.20
P_2	88.7 ±1.3	8.11^{bc} ±0.54	15.7^{ab} ±0.76	0.75^{ab} ±0.04	13.35^{abc} ±0.22
P_3	91.8 ±2.1	8.73^{a} ±0.14	18.0^{a} ±1.80	0.86^{a} ±0.09	13.96^{a} ±0.46
P_4	90.0 ±5.4	8.51^{ab} ±0.10	16.2^{ab} ±1.04	0.77^{ab} ±0.05	13.48^{ab} ±0.29
P_5	89.8 ±3.3	8.14^{abc} ±0.19	13.8^{b} ±1.80	0.66^{b} ±0.09	12.89^{bc} ±0.39

*Treatments doses per liter media 0 ml fish oil (P_0), 1 ml egg yolk (P_1), 0.25 ml fish oil + 0.25 ml egg yolk (P_2), 0.5 ml fish oil + 0.5 ml egg yolk (P_3), 0.75 ml fish oil + 0.75 ml egg yolk (P_4), and 1 ml fish oil + 1 ml egg yolk (P_5). Values are means ± SD. Superscript letters denote significant differences ($P < 0.05$) between treatments.

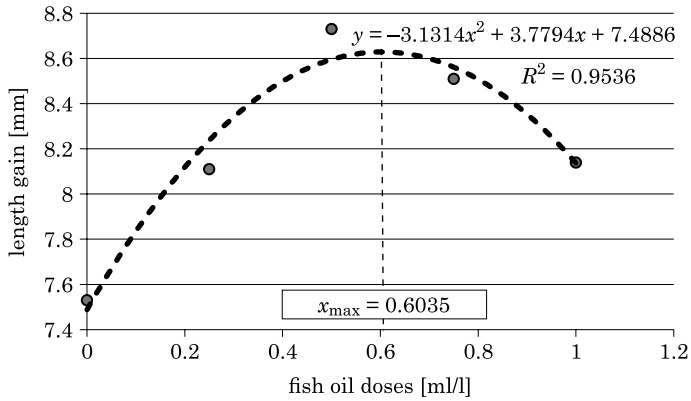

Fig. 1. Estimate *Sardinela lemuru* fish oil doses (0.6035 ml/l) that leads to the maximum length gain of silver rasbora (*Rasbora argyrotaenia*) using polynomial contrast (regression)

Discussion

Enrichment diets have been successfully used in artemia as natural feed for fish larvae (OZUSAGLAM et al. 2013). Fatty acid profiles of sardine oil emulsions increase levels of PUFA in enriched artemia (ARULVASU and MUNISWAMY et al. 2009). Larvae feeding artemia enrichment with *Sardinella lemuru* fish oil in different doses not affect ($P > 0.05$) on survival. Meanwhile, interestingly, all growth parameters significantly affected ($P < 0.05$) and increase compare to control. Similar result was also obtained in previous study with artemia fed on gelatin-acacia microcapsules containing cod liver oil, which supported faster growth rate significantly in post-larval gobies than control (JONES et al. 1984) and using sardine oil in artemia enrichment as natural food was also increased growth in *Poecillia latipinna* fry (ARULVASU and MUNISWAMY et al. 2009). *Sardinella lemuru* fish oil contain essential fatty acids (EFA) including EPA and DHA (MARINI et al. 2020) that have important role in fish growth (IZQUIERDO 1996) and deficiency of EFA causing some non-infectious diseases, low immunity, and increase abnormality rate (NOGA 2010).

In present study, the higher growth was obtained in P_3 (enrichment doses 0.5 ml fish oil + 0.5 ml egg yolk) and tends to be decrease in higher doses. It shows that excessive of *Sardinella lemuru* fish oil in enrichment artemia is not good for larvae growth. *Sardinella lemuru* fish oil as source of fatty acid (lipids) contain high energy that use for growth. Excess of fatty acid means that energy also excessive. Similar case was also obtained in other previous excessive dietary of fatty acid; excessive levels of DHA significantly reduced total weight of summer flounder larvae (BISBAL

and BENGSTON 1991), also growth of African catfish larvae tended to be reduced by excess in cod fish oil and coton seed oil in the diet (LEGENDRE et al. 1995). Based on the study, we recommend 0.5 ml fish oil + 0.5 ml egg yolk as optimal artemia enrichment dose for feeding practical of silver rasbora larvae.

Larvae feeding artemia enrichment with *Sardinella lemuru* fish oil in different doses not affect ($P > 0.05$) on survival, but all growth parameters significantly affected ($P < 0.05$). Optimal artemia enrichment dose for feeding practical of silver rasbora larvae is 0.5 ml fish oil + 0.5 ml egg yolk. Estimate doses that leads to the maximum length gain is 0.6035 ml fish oil + 0.6035 ml egg yolk.

Acknowledgements

There are no grant funding institutions in this study. The authors would like to thank all colleagues at the Faculty of Fisheries and Marine, Universitas Airlangga.

Accepted for print 14.06.2023

References

ADAWIYAH L.A., SULMARTIWI L., BODUR T., BUDI D.S. 2019. *Induction of spermiation using ovaprim^{TM} with topical gill method in the silver rasbora (Rasbora argyrotaenia)*. Theriogenology, 126: 172–176.

AGUSTIN L., SULMARTIWI L., MUBARAK A.S. 2020. *Addition of lemuru fish oil in feed on the gonadal maturity level of female silver barb, Rasbora argyrotaenia*. IOP Conf. Ser. Earth Environ. Sci., 441: 012047.

ARUVALSU C., MUNUSWAMY N. 2009. *Survival, growth and composition of Poecilia latipinna fry fed enriched Artemia nauplii*. Current Sci., 96:114–119.

AYUNDA R., SULMARTIWI L., MUBARAK A.S. 2020. *Addition of Lemuru fish oil to protein retention and feed utilization efficiency of silver barb Rasbora argyrotaenia.* IOP Conf. Ser. Earth Environ. Sci., 441: 012047.

BISBAL G.A., BENGSTON D.A. 1991. *Effect of dietary (n-3) HUFA enrichment on survival and growth of summer flounder, Paralichthys dentatus, larvae*. Larvi, 91:56–57.

BUDI D.S., RIZKYAN I., PRAYOGO. 2020. *The effect of stocking density on the survival and growth of silver rasbora (Rasbora argyrotaenia) larvae*. Polish J. Nat. Sci., 35(1): 87–95.

CAPULI E.E., BAILLY N. 2019. *Rasbora argyrotaenia (Bleeker, 1849), Silver rasbora*. FishBase. URL https://www.fishbase.in/summary/Rasbora-argyrotaenia.html (Accessed 27 August 2019).

CHEN K., WANG C.Q., FAN Y.Q., XIE Y.S., YIN Z.F., XU Z.J., ZANG H.L., CAO J.T., HAN Z.H., WANG Y., SONG D.Q. 2013. *The evaluation of rapid cooling as an anesthetic method for the zebrafish*. Zebrafish, 11:71–75.

DEWI S.A., SULMARTIWI L., MUBARAK A.S., MUKTI, A.T. 2020. *Growth and survival rate of silver barb, Rasbora argyrotaenia under different concentrations of sardinelle fish oil addition in fish feed*. IOP Conf. Ser. Earth Environ. Sci., 441: 012047.

HOSSEINI S.V., KENARI A.A., REGENSTEIN J.M., REZAEI M., NAZARI R.M., MOGHADDASI M., KABOLI S.A., GRANT A.A.M. 2010. *Effects of alternative dietary lipid sources on growth performance*

and fatty acid composition of beluga sturgeon, Huso huso, juveniles. J. World Aquacult. Soc., 41: 471–489.

IZQUIERDO M.S. 1996. *Essential fatty acid requirements of cultured marine fish larvae*. Aquacult. Nut., 2: 183–191.

JONES D.A., HOLLAND D.L., JABBORIE S. 1984. *Current status of microencapsulated diets for aquaculture*. Applied Biochem. Biotechnol., 10: 275–288.

KOTANI T. 2017. *Enrichment of rotifers and its effect on the growth and survival of fish larvae*. In: *Rotifers: Aquaculture, Ecology, Gerontology, and Ecotoxicology*. Eds. A. Hagiwara, T. Yoshinaga. Springer Nature Singapore Pte Ltd. and the Japanese Society of Fisheries Science, pp. 56–71.

LANES C.F.C., BOLLA S., FERNANDES J.M.O., NICOLAISEN O., KIRON V., BABIAK I. 2012. *Nucleotide enrichment of live feed: a promising protocol for rearing of atlantic cod Gadus morhua larvae*. Marine Biotechnol., 14: 544–558.

LEGENDRE M., KERDCHUEN N., CORRAZE G., BERGOT P. 1995. *Larval rearing of an African catfish Heterobranchus longifilis (Teleostei, Clariidae): effect of dietary lipids on growth, survival and fatty acid composition of fry*. Aquat. Living Resour., 8: 355–363, doi: 10.1051/alr:1995040.

MARINI A., MUBARAK A.S., MUKTI A.T. 2020. *Addition of lemuru fish oil in the diet on the fat retention and fatty acid profile of silver barb (Rasbora argyrotaenia)*. IOP Conf. Ser. Earth Environ. Sci., 441: 012047.

NOGA E.J. 2010. *Fish disease: diagnosis and treatment*. John Wiley & Sons.

OZUSAGLAM M.A., CAKMAK Y.S., KAYA M. 2013. *The biotechnological potential of Artemia salina fatty acids*. J. Pure and Applied Microbiol., 7: 1567–1575.

QIN J., FAST A.W., DEANDA D., WEIDENBACH R.P. 1997. *Growth and survival of larval snakehead (Channa striatus) fed different diets*. Aquaculture, 148: 105–113.

ROSADI E., HERAWATI E.Y., SETYOHADI D., BINTORO G. 2014. *Distribution, composition, and abiotic environment of Silver Rasbora (Rasbora argyrotaenia Blkr) fish in upstream areas of Barito watershed, South Kalimantan*. J. Environ. Ecol., 5: 117–131.

SLATER M., D'ABRAMO L., ENGLE C.R. 2018. *Aquaculture research priorities for the next decade: a global perspective*. J. World Aquacult. Soc., 49: 3–6.

YOSSA R., VERDEGEM M. 2015. *Misuse of multiple comparison tests and underuse of contrast procedures in aquaculture publications*. Aquaculture, 437: 344–350, doi: 10.1016/j.aquaculture.2014.12.023.

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 38(1): 51–63, Y. 2023

DOI: 10.31648/pjns.8093

HEAVY METAL CONCENTRATIONS IN THE WATER AND FISH MUSCLES OF LAKES OGELUBE AND OJII IN SOUTHEASTERN NIGERIA

***Oma Judith Ajanwachukwu*[1], *Ikechukwu Eugene Onah*[2], *Patience Obiageli Ubachukwu*[3]**

[1] ORCID: 0009-0000-6457-9128
[2] ORCID: 0000-0002-2101-7894
[3] ORCID: 0009-0003-8268-3561
Department of Zoology and Environmental Biology
University of Nigeria Nsukka, Enugu State, Nigeria

Key words: Fish, heavy metals, pollution, lake, Nigeria.

Abstract

Heavy metal pollution is one major environmental challenge for aquatic ecosystems. Heavy metal pollution of lakes and subsequent accumulation in the tissues of fish inhabiting the lakes impair use of the lake water and constitute a public health hazard for consumers of fish from the lakes. We investigated the concentrations of Chromium (Cr), Copper (Cu), Iron (Fe), Lead (Pb), and Zinc (Zn) in the water and muscles of fish from the Ogelube and Ojii Lakes of Southeastern Nigeria using an Atomic Absorption Spectrometer (AAS). All the heavy metals tested except Cu were detected in both lakes at concentrations exceeding the permissible limits according to WHO. In the Ojii Lake concentrations of tested elements were not significantly higher than in Ogelube Lake, except for Cu and Zn, which were at similar levels in both lakes. Lead (Pb) was not detected in the fish muscles regardless of the lake. In Ogelebu Lake, the concentrations of Fe were significantly higher in *Hepsetus odoe* than *Coptodon zillii* ($p < 0.05$). In Ojii Lake, the concentrations of Cr were significantly higher in *C. zillii* than *H. odoe* while the concentrations of Fe were significantly higher in *H. odoe* than *C. zillii* ($p < 0.05$). All elements tested in fish muscles were at safe levels for human consumption according to FAO.

Introduction

Industrial revolution across the globe has heightened water pollution and good quality water bodies are scarce (EKUBO and ABOWEI 2011, TIWARI 2015). Water pollution has become a major global problem and is worse in developing nations due to lack of surface water quality protection measures and poor sanitation (LONGE and OMOLE 2008). Among water pollut-

Address: Ikechukwu Eugene Onah, University of Nigeria, Nsukka, Enugu State, Nigeria, phone: +2348064618073, e-mail: ikechukwu.onah@unn.edu.ng

ants, heavy metals are of prime concern due to their toxicity and ability to bioaccumulate in the tissues of fish (Censi et al. 2006). Heavy metals from industrial, agricultural, and domestic sources constitute serious pollutants in aquatic ecosystems and a threat to fish life globally (Diagomanolin et al. 2004, Storelli et al. 2005, Voelz et al. 2005, Akoto et al. 2008, Jibiri and Adewuyi 2008).

Fish can accumulate heavy metals in their tissues and serve as bioindicators of heavy metal contamination in aquatic ecosystems (Rashed 2001, Karadede-Akin and Unlu 2007, Sivaperumal et al. 2007, Yilmaz et al. 2007, Lasheen et al. 2012, Zhao et al. 2012, Annabi et al. 2013). Fish also form a significant proportion of the human diet (Rashed 2001, Sioen et al. 2007). In Nigeria, fish constitutes approximately 40% of the animal protein in human diets (Atta et al. 1997). However, bioaccumulation of heavy metals in fishes impair their values to human nutrition and risk the public health (Al-Khateeb and Leilah 2005, Castro-Gonzalez and Mendez-Armenta 2008, Hashim et al. 2014, Bawuro et al. 2018).

Ogelube and Ojii Lakes are tropical freshwater habitats located at Opi-Agu in the valley of the Uhere River, north-east of Nsukka, Enugu State, Nigeria. Fishes abound in Ogelube and Ojii Lakes (Onah et al. 2022) and supply the local residents with food. The lakes are surrounded by agricultural lands and important for pastoral activities and sand mining in and around the lakes (Onah et al. 2022), all of which constitute sources of heavy metals to the lakes. The lakes are the closest waterbodies to the University of Nigeria Nsukka and as a result have been a centre of research from various disciplines interested in freshwater bodies and their biotic community. Several studies have been conducted to evaluate the heavy metal contents of lakes and their fishes across Nigeria (Sambo et al. 2014, Umunnakwe and Aharanwa 2014, Jenyo-Oni and Oladele 2016, Adebayo et al. 2017, Ayoade and Nathaniel 2018, Bawuro et al. 2018, Matouke and Abdullahi 2020, Utete and Fregene 2020). However, despite the anthropogenic activities in and around Ogelube and Ojii Lakes that supply heavy metals to the lakes and the locals depending on the lakes for fish food, no research to the best of our knowledge has been carried out to determine those elements in the water and fish. These knowledge gaps threaten the public health of the locals who consume the fish from the lakes, irrigate their crops with the lake water, and the herds of cattle that regularly drink the lake water. Lack of this knowledge hampers policy advice and public health campaigns of the lakes. Therefore, the objective of this study was to determine the levels of heavy metals in the lake water and major fishes of the lakes.

Materials and Methods

Study area

Opi-Agu Community is situated north-east of the city of Nsukka in Enugu State, Nigeria, between Latitudes 6°42′–6°47′ N and Longitudes 7°28′–7°33′ E. Lake Ojii (6°42′10.7″ N, 7°32′52.9″ E) and Lake Ogelube (6°45′16.12″ N, 7°29′26.9″ E) (Figure 1) are located in the valley of the Uhere River, north-east of Nsukka, Enugu State (Ozoko 2015). The lakes do not have permanent rivers feeding into them. Sources of water to the lakes are groundwater, rainfall, and runoff from farmlands, and during the wet season the lakes overflow through the sloping northern end.

Collection of water samples

The two lakes were each divided into three stations, based on natural features of the lakes. For Lake Ojii, Station 1 was situated at the shallow end of the lake, with limited vegetation cover and runoff during heavy rainfall. Station 2 had extensive vegetation cover and was located between Station 1 and 3. Station 3 was in the middle of the lake, had no vegetation cover and was exposed to direct sunlight. For Lake Ogelube, Station 1 was situated at the southern or overflow end of the lake with good vegetation cover, shade, and runoff during heavy rainfall. Station 2 was situated in the middle of the lake with less vegetation cover and less shade than Station 1. Station 3 was situated at the northern end of the lake and had no vegetation cover or shade.

Water samples were collected from Ogelube and Ojii Lakes monthly for six months from June to December 2018. Water samples were collected from each sampling station using 150 ml plastic containers. The containers were washed with nitric acid and dried to remove any contaminants. The water samples were transported and analysed separately for heavy metals immediately at the Energy Research Centre Laboratory, University of Nigeria Nsukka. In total eighteen water samples were collected for six months from each lake.

Fish collection and identification

Two species of fish, *Coptodon zillii* and *Hepsetus odoe,* were selected for analysis of heavy metal accumulation in the muscles. *Coptodon zillii* was chosen because it is the most abundant fish in the lakes and an omnivore, while *Hepsetus odoe* was chosen because it is a carnivore and the most preferred fish by consumers, both fishes are of commercial value to the locals.

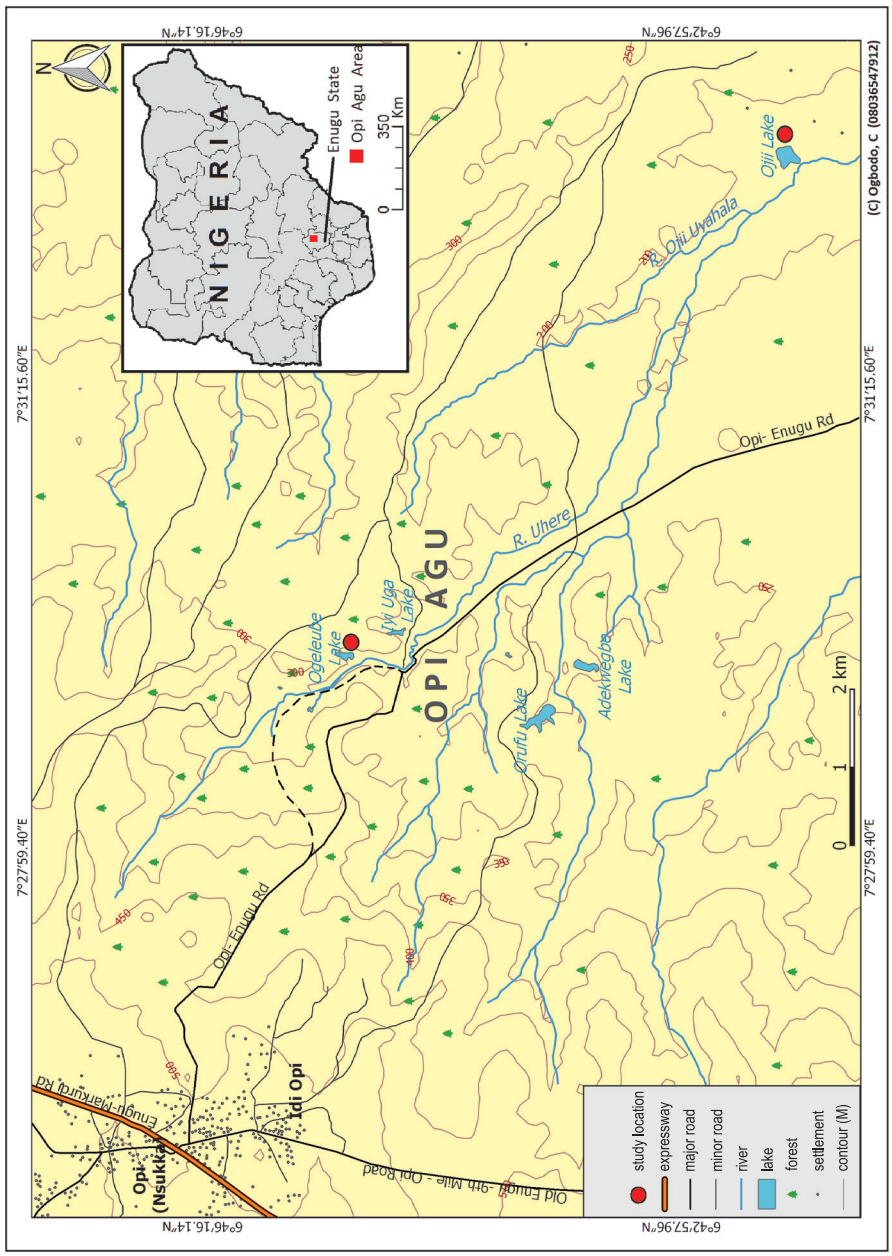

Fig. 1. Map of the Opi Agu Community, showing the study locations – Ogeleube and Ojii Lakes

Fishes were collected using a cast net with a 30–80 mm stretched mesh. For each lake, two adult fishes (1 each for *C. zillii* and *H. odoe*) were collected each month for 6 months (June – August; October – December). The mean total length (TL) of the fishes were 14.20 ±2.37 cm for *C. zillii* and 17.57 ±0.74 for *H. odoe*. Twenty-four (24) fishes were analyzed in total, 12 for each species. Thereafter, they were euthanized by severing the spinal cord, labeled, and placed in sealed plastic bags in a cooler pack containing ice and immediately taken to the Energy Research Center Laboratory, University of Nigeria Nsukka for analysis. The fishes were identified using keys by BABATUNDE and AMINU (2004) and the identities further confirmed by a fishery biologist in the Department of Zoology and Environmental Biology, University of Nigeria Nsukka.

Determination of heavy metals in the water

Heavy metals including: Chromium (Cr), Copper (Cu), Iron (Fe), Lead (Pb) and Zinc (Zn) were analyzed in this study. The concentrations of heavy metals in each water sample were determined using an Atomic Absorption Spectrometer (AAS). A sample of 1 ml from each of the water samples was measured into a digestion flask and 30 cm^3 of aqua regia added and digested in a fume-cupboard until a clear solution was obtained. Thereafter, the solution was cooled, filtered, and made up to 50 ml in a standard volumetric flask with deionized water. A blank sample was prepared to zero the AAS; an instrument used for the analysis before re-running other series of samples. Standards (2 ppm, 4 ppm, and 6 ppm) were prepared from a 1000 ppm stock solution of the metals and used to plot the calibration curve. The calibration curve was plotted automatically by the instrument using the formula:

$$C_1 \cdot V_1 = C_2 \cdot V_2,$$

where:
C_1 = 1000 ppm
C_2 = 2 ppm.

A volume of 0.2 ml was pipetted from the 1000 ppm solution, put into a 100 ml flask and diluted to the mark with deionized water. These procedures were used in the preparation of 4 ppm and 6 ppm solutions. High temperatures produced in the ignition chamber provided enhanced reduced settings for atomization of the heavy metals. Each standard was aspirated with an aspirator by nebulizer; converted into an aerosol, mixed with the glass, and converted into atomic form. All the standard solutions were analyzed, and the calibration curve plotted automatically for the metals of interest. Each metal was analyzed with the standard using adequate

wavelengths after which its concentration was generated automatically by the instrument. The heavy metal concentrations of the three sampled stations for each month were averaged and the results presented as Mean ± SE.

Determination of heavy metals in fishes

Samples of fresh fishes were washed under tap water and drained. Thereafter, the muscles of the fishes were removed using a sharp knife. The muscles were chosen for heavy metal analysis because they are the most edible part of the fish (KESKIN et al. 2007, HASHIM et al. 2014). Fleshes were dried to a constant weight in an oven at 105ºC, grounded to powder using a ceramic mortar and pestle then sieved through a 20 µm mesh according to SAMBO et al. (2014). Thereafter, 1.0 g each of the dry and ground fish samples were digested in 10 cm^3 concentrated nitric acid. The digestates were diluted with 1% HCl. Concentrations of the heavy metals were determined by AAS (APHA 1998). Heavy metal determinations were replicated 3 times for *Coptodon zillii* and *Hepsetus odoe* for each lake. The concentrations of the heavy metals in each fish species were averaged and results presented as Mean ± SE.

Statistical analysis

Data were analysed using Statistical Packages for Social Sciences (SPSS) (version 21.0, IBM Corporation, Armonk, USA) and Paleontological Statistics (PAST) software version 3.21 (HAMMER et al. 2001). Heavy metal concentrations of the water bodies were not normally distributed, so the spatiotemporal properties of the water bodies were compared using the Mann–Whitney *U*-tests and Kruskal–Wallis *H*-tests.

Results

Heavy metal concentrations in the Ogelube and Ojii Lakes

Heavy metals fluctuated in water samples across the months studied. In Ogelube Lake, the concentrations of Cu were significantly higher in July than June and October ($p = 0.0112$, $n = 54$, Table 1). Similarly, the concentrations of Fe were significantly higher in July than June, November and December ($p = 0.0045$, $n = 54$, Table 1). In Ojii Lake the concentrations of Fe in July were significantly higher than the rest of the months ($p = 0.0001$, $n = 54$, Table 2). Concentrations of heavy metals were higher in Ojii Lake than Ogelube Lake except for Cu and Zn, which were similar

in both lakes. However, detected differences in the concentrations of the studied elements were not statistically significant (Table 3).

Table 1

Monthly variations in heavy metal concentrations in Ogelube, Opi Lake [mg/l]

Metal	June	July	August	October	November	December
Cr	0.04 ±0.03^{a}	0.04 ±0.02^{a}	0.04 ±0.02^{a}	0.05 ±0.02^{a}	0.07 ±0.04^{a}	0.03 ±0.03^{a}
Cu	0.02 ±0.01^{a}	0.06 ±0.01^{b}	0.03 ±0.00^{ab}	0.01 ±0.01^{ca}	0.04 ±0.01^{ab}	0.03 ±0.01^{ab}
Fe	0.38 ±0.07^{a}	0.79 ±0.11^{b}	0.57 ±0.13^{ab}	0.69 ±0.04^{ab}	0.39 ±0.08^{a}	0.40 ±0.08^{a}
Pb	0.06 ±0.04^{a}	0.11 ±0.06^{a}	0.25 ±0.07^{a}	0.08 ±0.04^{a}	0.08 ±0.04^{a}	0.08 ±0.04^{a}
Zn	0.07 ±0.04^{a}	0.06 ±0.01^{a}	0.11 ±0.04^{a}	0.04 ±0.01^{a}	0.03 ±0.01^{a}	0.03 ±0.01^{a}

Means of heavy metals in a row across the months with different superscripts are significantly different ($p < 0.05$).

Table 2

Monthly variations in heavy metal concentrations in Ojii, Opi Lake [mg/l]

Metal	June	July	August	October	November	December
Cr	0.10 ±0.03^{a}	0.02 ±0.01^{a}	0.08 ±0.06^{a}	0.04 ±0.01^{a}	0.22 ±0.11^{a}	0.15 ±0.10^{a}
Cu	0.01 ±0.00^{a}	0.03 ±0.01^{a}	0.05 ±0.01^{a}	0.01 ±0.01^{a}	0.05 ±0.02^{a}	0.03 ±0.02^{a}
Fe	0.32 ±0.07^{a}	1.19 ±0.09^{b}	0.52 ±0.10^{a}	0.51 ±0.09^{a}	0.46 ±0.06^{a}	0.40 ±0.03^{a}
Pb	0.14 ±0.04^{a}	0.22 ±0.05^{a}	0.17 ±0.04^{a}	0.14 ±0.04^{a}	0.08 ±0.04^{a}	0.11 ±0.04^{a}
Zn	0.02 ±0.00^{a}	0.14 ±0.04^{a}	0.08 ±0.04^{a}	0.02 ±0.01^{a}	0.03 ±0.01^{a}	0.09 ±0.05^{a}

Means of heavy metals in a row across the months with different superscripts are significantly different ($p < 0.05$).

Table 3

Mean heavy metal contents in water samples of Ogelube and Ojii Lakes compared with the literature data

Lakes	Cr [mg/l]	Cu [mg/l]	Fe [mg/l]	Pb [mg/l]	Zn [mg/l]	References
Ogelube	0.05	0.03	0.54	0.11	0.06	present study
Ojii	0.10	0.03	0.56	0.14	0.06	present study
Oguta*	0.03–0.87	0.26–1.80	0.30–4.75	0.00–0.05	–	Umunnakwe and Aharanwa (2014)
Ibrahim Adamu	3.35	0.75	–	0.81	1.64	Sambo et al. (2014)
Man-made Ibadan	0.01	0.05	0.81	0.08	–	Ayoade and Nathaniel (2018)
Asejire	–	–	0.01	0.015	–	Jenyo-Oni and Oladele (2016)
Oguta	–	–	0.17	–	–	Adebayo et al. (2017)
Asejire	–	–	0.02	0.05	–	Oladele et al. (2018)
Permissible values	0.05	2.00	0.50	0.01	0.05	WHO (2011)

* Only ranges are available; (–) not analysed.

Seasonal variations in heavy metal concentrations of Ogelube and Ojii Lakes

The heavy metal contents of Ogelube and Ojii lakes varied between the seasons. In Ogelube Lake, only Zn was significantly higher in the wet season than the dry season ($p < 0.05$, Table 4) while in Ojii Lake, only Fe was significantly higher in the wet season than the dry season ($p < 0.05$, Table 4).

Table 4

Seasonal variations in heavy metal concentrations in the studied Lakes

Element	Ogelube Lake			Ojii Lake		
	wet season [mg/l]	dry season [mg/l]	*p*-value	wet season [mg/l]	dry season [mg/l]	*p*-value
Cr	0.04 ±0.01	0.05 ±0.02	0.64	0.07 ±0.02	0.14 ±0.05	0.21
Cu	0.04 ±0.01	0.03 ±0.01	0.30	0.03 ±0.01	0.03 ±0.01	1.00
Fe	0.58 ±0.07	0.50 ±0.05	0.32	0.67 ±0.09	0.45 ±0.04	**0.02***
Pb	0.14 ±0.04	0.08 ±0.02	0.20	0.18 ±0.03	0.11 ±0.02	0.07
Zn	0.08 ±0.02	0.04 ±0.00	**0.03***	0.08 ±0.02	0.05 ±0.02	0.25

* *p*-values < 0.05 indicate significant difference between wet and dry seasons.

Concentrations of heavy metals in fish muscles

In Ogelube Lake, the concentrations of Fe were significantly higher in *H. odoe* than *C. zillii* ($P < 0.05$, Table 5). In Ojii Lake, the concentrations of Cr were significantly higher in *C. zillii* than *H. odoe,* while the concentrations of Fe were significantly higher in *H. odoe* than *C. zillii* ($P < 0.05$, Table 5).

Table 5

Concentration of heavy metals in fish muscles [mg/kg dry weight]

Element	Ojii Lake			Ogelube Lake			FAO (1989)
	fish species		*p*-values	fish species		*p*-values	
	Coptodon zillii	*Hepsetus odoe*		*Coptodon zillii*	*Hepsetus odoe*		
Cr	0.23 ±0.01	0.06 ±0.00	<0.01*	0.25 ±0.06	0.25 ±0.01	1.00	0.50
Cu	0.20 ±0.01	0.19 ±0.01	0.67	0.14 ±0.01	0.17 ±0.02	0.32	30.00
Fe	0.13 ±0.01	0.24 ±0.00	<0.01*	0.17 ±0.01	0.24 ±0.02	**0.02***	–
Pb	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	0.50
Zn	0.18 ±0.00	0.28 ±0.01	0.15	0.18 ±0.00	0.22 ±0.04	0.30	30.00

***** *p*-values < 0.05 indicate significant difference between *Coptodon zellii* and *Hepsetus odoe*

Discussion

The heavy metals: chromium, copper, iron, lead, and zinc were detected in the Ogelube and Ojii Lakes. The values of 0.05 mg/l and 0.1 mg/l recorded for Cr in Ogelube and Ojii lakes respectively in this study are lower than 0.87 mg/l in Oguta Lake and 3.35 mg/l in Ibrahim Adamu Lake but higher than 0.01 mg/l in Man-made Lake in Ibadan. In the Ogelube Lake Cr was at the WHO (2011) permissible value of 0.05 mg/l, while in the Ojii Lake Cr at a value of 0.10 ± 0.03 mg/l exceeded the permissible value (Table 3). The concentrations of Pb in both lakes are higher than the values recorded in Oguta Lake, Man-made Lake in Ibadan and Lake Asejire but lower than the values recorded in Ibrahim Adamu Lake (Table 3). Both studied lakes were polluted by Fe, Pb, and Zn. The mean values recorded for these elements slightly exceeded the permissible values established by WHO (2011) – Table 3. Heavy metal pollution in the lakes may be the result of agricultural runoff and domestic wastes washed down into the lakes (SIVALINGAM et al. 2021, SOJKA et al. 2022). Runoff during the rainy season from agricultural lands into the lakes probably affected significantly higher concentrations of Fe and Zn, respectively in Lakes Ojii and Ogelube, in the wet than the dry seasons (Table 4).

Concentrations of Cr in the fish muscles ranged from 0.06 mg/kg in *H. odoe* from Ojii Lake to 0.25 mg/kg in *H. odoe* from Ogelube. The values of Cr in the fish muscles were higher than 0.0038 mg/kg recorded by JENYO-ONI and OLADELE (2016) in Lake Asejire, Oyo State, Nigeria from a related species of tilapia (*Oreochromis niloticus*). In Oguta Lake, Imo State, Nigeria, UMUNNAKWE and AHARANWA (2014) did not detect Cr in *Tilapia* sp. However, the values of Cr in the fish muscles were lower than 2.77 mg/kg recorded by SAMBO et al. (2014) in Ibrahim Adamu Lake, Jigawa State, Nigeria for tilapia species (*O. niloticus*). The fish muscles are not considered polluted with Cr because the mean values recorded are lower than the 0.05 mg/kg permissible limit determined by FAO (1989) – Table 5.

The lowest and highest concentrations of Cu were recorded in muscles of *C. zillii* and ranged from 0.14 mg/kg in Ogelube to 0.20 mg/kg in Ojii Lake. These values are visibly higher than 0.07 mg/kg recorded by UMUNNAKWE and AHARANWA (2014) for *Tilapia* sp. in Oguta Lake, Imo State, Nigeria, but lower than 0.30 mg/kg recorded by SAMBO et al. (2014) in tilapia fish (*O. niloticus*) from Ibrahim Adamu Lake, Jigawa State, Nigeria and with ranges of 0.15–11.27 mg/kg recorded by BAWURO et al. (2018) for the flesh of tilapia fish from lake Geriyo, Adamawa State, Nigeria. The values of Cu recorded in the lakes in this study are lower than the maximum permissible value of 30.00 mg/kg given by FAO (1989) – Table 5.

The concentrations of Fe in the fish muscle ranged from 0.13 mg/kg in *C. zillii* from Ojii Lake to 0.24 mg/kg in *H. odoe* from Ojii Lake. These values are lower than 21.17 mg/kg recorded by UMUNNAKWE and AHARANWA (2014) in the muscle of tilapia fish from Oguta Lake, Imo State, Nigeria, and 7.43 mg/kg recorded by JENYO-ONI and OLADELE (2016) and OLADELE et al. (2018) for tilapia fish (*O. niloticus*) from Lake Asejire, Oyo State, Nigeria.

Pb was not found in the muscles of fish from either lake. This observation agrees with the findings of UMUNNAKWE and AHARANWA (2014) who did not detect Pb in the muscles of *Tilapia* sp. from Oguta Lake, Imo State, Nigeria. However, SAMBO et al. (2014), JENYO-ONI and OLADELE (2016), BAWURO et al. (2018) and OLADELE et al. (2018) recorded Pb values of 0.27 mg/kg, 0.05 mg/kg, 0.01–3.78 mg/kg and 0.05 mg/kg, respectively in tilapia (*O. niloticus*) from lakes in different parts of Nigeria.

The concentrations of Zn in the fish muscles ranged from 0.18 mg/kg in *C. zillii* from both lakes to 0.28 mg/kg in *H. odoe* from Ojii lake. These values are lower than 0.87 mg/kg and in the range 4.48–5.06 mg/kg recorded by SAMBO et al (2014) and BAWURO et al. (2018) in the muscles of tilapia (*O. niloticus*) from Ibrahim Adamu Lake, Jigawa State, Nigeria and Lake Geriyo, Adamawa State, Nigeria, respectively. The mean values of zinc recorded in the fishes from both lakes were lower than 30.00 mg/kg, which is the permissible limit recommended by FAO (1989) – Table 5.

Conclusions

This study provides baseline data on the status of several heavy metal elements in the Ogelube and Ojii lakes and fishes inhabiting these lakes. Lakes Ogelube and Ojii are polluted with heavy metals, but the fish are safe for consumption because the concentrations of the heavy metals in the muscles of the fish are lower than the maximum permissible limit as determined by the World Health Organization. Consuming fish from these lakes does not pose a public health risk from heavy metal contamination to the local residents. However, the lakes may be a health risk to herds of cattle that constantly drink the lake water. In addition, the use of the lake water to irrigate crops in the nearby farmlands may lead to accumulation of heavy metals in cultivated crops. Activities that add heavy metals to the lakes such as use of chemical pesticides in the nearby farmlands and dumping of refuse into the lakes should be discouraged to minimize further contamination of the lakes with heavy metals.

Acknowledgements

We wish to express our profound gratitude to Dr Chika Ikele, Mr Godwin Nwosu and Mr Oscar Aguzie of the Department of Zoology and Environmental Biology, University of Nigeria Nsukka for their assistance during field and laboratory work. We are also grateful to Prof. DeMar Taylor for providing the English editing service.

Accepted for print 27.06.2023

References

ADEBAYO E.T., C-OLUWATOSIN T.J., NNEJI P.C. 2017. *Limnological assessment of heavy metals concentration in water and sediment of Oguta Lake, South-East Nigeria*. Nigeria Int. J. Innov. Stud. Aquat. Biol. Fish., 3(3): 18–22.

AKOTO O., BRUCE T.N., DARKO G. 2008. *Heavy metals pollution profiles in streams serving the Owabi reservoir*. *Afr. J. Environ. Sci. Technol*., 2(11): 354–359.

AL-KHATEEB S.A., LEILAH A.A. 2005. *Heavy metals accumulation in the natural vegetation of Eastern province of Saudi Arabia*. J. Biol. Sci., 5: 707–712.

ANNABI A., SAID K., MESSAOUDI I. 2013. *Cadmium: bioaccumulation, histopathology and detoxifying mechanisms in fish*. Am. J. Res. Commun., 1(4): 62.

APHA 1998. *Standard methods for the examination of water and wastewater 20th edition*. American Public Health Association, American Water Work Association, Water Environment Federation, Washington, DC.

ATTA M.B., ELS-EBALE L.A., NAOMAN M.A., KASSAB H. 1997. *The effect of cooking on the content of heavy metals in fish (Tilapia nilotica)*. Food Chem., 58: 1–4.

AYOADE A.A., NATHANIEL O.G. 2018. *Assessment of heavy metals contamination of surface water and sediment of a tropical manmade lake southwestern Nigeria*. International Journal of Environment and Pollution Research, 6(3): 1–16.

BABATUNDE D.O., AMINU R. 2004. *Field guide to Nigerian freshwater fishes, 2nd Edition*. Federal College of Freshwater Fisheries Technology, New Bussa, Nigeria.

BAWURO A.A., VOEGBORLO R.B., ADIMADO A.A. 2018. *Bioaccumulation of heavy metals in some tissues of fish in Lake Geriyo, Adamawa State, Nigeria*. J. Environ. Public Health, doi: 10.1155/2018/1854892.

CASTRO-GONZALEZ M.I., MENDEZ-ARMENTA M. 2008. *Heavy metals: implications associated to fish consumption*. Environ. Toxicol. Pharmacol., 26: 263–271.

CENSI P., SPOTO S.E., SAIANO F., SPROVIERI M., MAZZOLA S. 2006. *Heavy metals in coastal water system, a case study from North Western Gult of Thailand*. Chemosphere, 64(1): 1167–1176.

DIAGOMANOLIN V., FARHANG M., JAFARZADEH N. 2004. *Heavy metals in the Karoon Waterway River, Iran*. Toxicol. Lett., 15: 63–68.

EKUBO A.T., ABOWEI J.F.N. 2011. *Aspects of aquatic pollution in Nigeria*. Research Journal of Environmental and Earth Sciences., 3(6): 673–693.

FAO 1989. *Evaluation of certain food additives and the contaminants mercury, lead and cadmium;* WHO Technical Report Series No. 505.

HAMMER O., HARPER D.A.T., RYAN P.D. 2001. PAST: *Paleontological Statistics Software Package for Education and Data Analysis*. Palaeontol. Electron., 4: 9, http://palaeo-electronica.org/2001_1/past/issue1_01.htm, access: ?

HASHIM R., SONG T.H., MUSLIM N.Z.M., YEN T.P. 2014. *Determination of heavy metal levels in fishes from the lower reach of the Kelantan River, Kelantan, Malaysia*. Trop. Life Sci. Res., 25(2): 21–39.

Jenyo-Oni A., Oladele A.H. 2016. *Heavy metals assessment in water, sediments and selected aquatic organisms in Lake Asejire, Nigeria.* Eur. Sci. J., 12(24), doi: 10.19044/esj.2016.v12n24p339.

Jibiri N.N., Adewuyi G.O. 2008. *Radionuclide contents and physico-chemical characterization of solid waste and effluent samples of some selected industries in the city of Lagos, Nigeria.* Radioprotection, 43(2): 203–212, doi: 10.1051/radiopro:2007053.

Karadede-Akin H., Unlu E. 2007. *Heavy metal concentrations in water, sediment, fish and some benthic organisms from Tigris River, Turkey.* Environ. Monit. Assess., 131: 323–337.

Keskin Y., Baskaya R., Ozyaral O., Yurdun T., Luleci N., Hayran O. 2007. *Cadmium, lead, mercury and copper in fish from the Marmara Sea, Turkey.* Bull. Environ. Contam. Toxicol., 78: 258–261.

Lasheen M.R., Abdel-Gawad F.K., Alaneny A.A., El-Bary H.M.H.A. 2012. *Fish as bio indicators in aquatic environmental pollution assessment: a case study in Abu-Rawash area, Egypt.* World Appl. Sci. J., 19(2): 265–275.

Longe E.O., Omole D.O. 2008. *Analysis of pollution status of river Illo, Ota Nigeria.* Environmentalist, 2(3): 34–39.

Matouke M.M., Abdullahi K.L. 2020. *Assessment of heavy metals contamination and human health risk in Clarias gariepinus [Burchell, 1822] collected from Jabi Lake, Abuja, Nigeria.* Sci. Afr. 7 e00292, doi: 0.1016/j.sciaf.2020.e00292.

Oladele A.H., Digun-Aweto O., Jenyo-Oni A. 2018. *The relationship between iron, lead and cobalt content in water, sediments, Nile tilapia and African river prawn of lake Asejire, Nigeria.* Pol. J. Natur. Sc., 33(3): 417–432.

Onah I.E., Ajanwachukwu O.J., Ubachukwu P.O. 2022. *Comparison of physico-chemical parameters with macroinvertebrate and vertebrate fauna of Lake Ogelube and Lake Ojii, Opi-Agu, south-eastern Nigeria.* Afr. J. Aquat. Sci., doi: 10.2989/16085914.2022.2080174.

Ozoko D.C. 2015. *Hydrochemistry of lakes in Opi Agu, Enugu state, southeastern Nigeria.* International Journal of Science and Technology, 4(7): 304–311.

Rashed M.N. 2001. *Monitoring of environmental heavy metals in fish from Nasser Lake.* Environ. Int., 27: 27–33.

Sambo F., Haruna M., Idris S., Moha S., Nasir A. 2014. *Assessment of heavy metals in water and fish from Ibrahim Adamu Lake, Jigawa, Nigeria. Trends Appl. Sci. Res.*, 9(10): 603–606.

Sioen I., De Henauw S., Verdonck F., Thuyne N. van, Camp J. van. 2007. *Development of a nutrient database and distributions for use in a probabilistic risk-benefit analysis of human seafood consumption.* J. Food Compost. Anal., 20: 662–670.

Sivalingam P., Al Salah D.M., Poté J. 2021. *Sediment heavy metal contents, ostracod toxicity and risk assessment in tropical freshwater lakes, Tamil Nadu, India.* Soil Sediment. Contamin., 30(2): 231–252, doi: 10.1080/15320383.2020.1835822.

Sivaperumal P., Sankar T.V., Nair P.G.V. 2007. *Heavy metal concentrations in fish, shellfish and fish products from internal markets of India vis-a-vis international standards.* Food Chem., 102: 612–620.

Sojka M., Jaskuła J., Barabach J., Ptak M., Zhu S. 2022. *Heavy metals in lake surface sediments in protected areas in Poland: concentration, pollution, ecological risk, sources and spatial distribution.* Sci. Rep., 12: 15006, doi: 10.1038/s41598-022-19298-y.

Storelli M.M., Storelli A., D'addabbo R., Marano C., Bruno R., Marcotrigiano G.O. 2005. *Trace elements in loggerhead turtles (Caretta caretta) from the eastern Mediterranean Sea: overview and evaluation.* Environ Pollut., 35(1): 163–70, doi: 10.1016/j.envpol.2004.09.005.

Tiwari S. 2015. *Water quality parameter. A Review.* International Journal of Engineering Science Invention, Research and Development,1(9): 319–324.

Umunnakwe J.E., Aharanwa B.C. 2014. *Assessment of water quality and heavy metal levels of fish species in Oguta Lake, Imo State Nigeria.* J. Nat. Sci. Res., 4(8): 103–112.

Utete B., Fregene B.T. 2020. *Heavy metal concentrations in surface water, sediments and fish and their potential toxicity in highly degraded multipurpose periurban Eleyele Lake, Ibadan City, Oyo State, south-western Nigeria.* Cogent Environ. Sci., 6:1, 1785137, doi: 10.1080/23311843.2020.1785137.

VOELZ J.N., ZUELLIG S.S., WARD J.V. 2005. *Effects of urban areas on benthic macroinvertebrates in two Colorado Plain Rivers.* Environ. Monit. Assess.,101(4): 175–202.

WHO. 2011. *Guidelines for drinking-water quality.* Fourth Edition. chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://apps.who.int/iris/bitstream/handle/10665/44584/9789241548151_eng.pdf, access: ?

YILMAZ F., OZDEMIR N., DEMIRAK A., TUNA A.L. 2007. *Heavy metal levels in two fish species Leuciscus cephalus and Lepomis gibbosus.* Food Chem., 100: 830–835.

ZHAO S., FENG C., QUAN W., CHEN X., NIU J., SHEN Z. 2012. *Role of living environments in the accumulation characteristics of heavy metals in fishes and crabs in the Yangtze River Estuary, China.* Mar. Pollut. Bull., 64: 1163–1171.

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 38(1): 65–87, Y. 2023

DOI: 10.31648/pjns.7402

THE CONTENTS OF INORGANIC COMPONENTS IN SELECTED POLISH AND ROMANIAN HERBAL TEAS INFUSIONS

Joanna Kończyk[1], ***Rajmund Michalski***[2], ***Edward Muntean***[3], ***Wojciech Lasoń***[4], ***Jerzy Gęga***[5]

[1] ORCID: 0000-0002-9945-1420
[2] ORCID: 0000-0003-4441-7409
[3] ORCID: 0000-0003-3422-7054
[5] ORCID: 0000-0002-5366-4498
[1,4] Institute of Chemistry, Faculty of Science & Technology
Jan Dlugosz University in Częstochowa, Poland
[2] Institute of Environmental Engineering
Polish Academy of Sciences, Zabrze, Poland
[3] Food Science Department, Faculty of Food Science & Technology
University of Agricultural Sciences and Veterinary Medicine, Cluj Napoca, Romania
[5] Faculty of Production Engineering and Materials Technology
Częstochowa University of Technology, Częstochowa, Poland

Key words: anions, cations, metals, herbal teas infusion, chemical risk.

Abstract

The aim of this study was to analyze the contents of the selected inorganic ions (F^-, Cl^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, Na^+, K^+, Mg^{2+}, Ca^{2+}) and trace metals (Cr, Mn, Fe, Ni, Cu, Zn, Cd, Al, Pb, Co) in infusions prepared from popular herbal teas available in the Polish and Romanian markets. Ion chromatography with conductivity detection and microwave plasma-atomic emission spectroscopy were applied for this purpose. The experimental data were subjected to chemometric analysis to identify common traits and differences among them. Principal component analysis was employed to create a model using selected variables. The findings revealed that certain herbal teas exhibited elevated concentrations of potentially hazardous analytes, such as nitrate, cadmium, lead, and chromium. Based on these results, an assessment of the associated risk from these analytes was conducted.

Introduction

Herbal teas and medicinal plants are largely used mainly due to their health benefits and fragrance characteristics that result from the contents of the organic and inorganic compounds they contain. They are important

Address: Joanna Kończyk, Jan Dlugosz University in Częstochowa, Czestochowa, Poland, e-mail: j.konczyk@ujd.edu.pl

sources of substances for human health as they contain various biologically active compounds (i.e. polyphenols, carbohydrates, proteins, amino acids, volatile organic compounds) (Butiuk et al. 2016, Dalar and Konczak 2013, Kaczmarczyk and Lochyński 2014, Theuma and Attard 2020), as well as metals, inorganic anions and cations (Martín-Domingo et al. 2017). Herbal teas are a large group, including herbs, fruits and their mixtures (Vuong 2014); some of them are available in supermarkets, while others can be bought at pharmacies.

The condition of the environment in which the herbs plant are grown (geographical location, climate, soil type, cultivation method) has an important impact on the composition and the quality of the final products (Kojta et al. 2012, Ngure and Kinuthia 2020). For that reason, the cultivation areas should be the subject to specific strict rules and monitoring (Muntean et al. 2016). If we consider also that tea drinking is a ritual and a lifestyle in many countries, herbal teas are among the most popular beverages in the world and hold the second place in the beverage popularity ranking just after water.

However, there is a question whether using herbal teas or any other products derived from such raw materials have only positive effects, since besides beneficially biologically active constituents, medicinal plants contain also variable amounts of hazardous substances, such as heavy metals, pesticides, polyhydroxy aromatic compounds, mycotoxins, nitrates, etc. (Muntean et al. 2013, 2016, Street 2012). For most cases, anthropic pollution is the major cause, leading to unwanted side-effects of using contaminated plant remedies, many cases of liver, kidney or other organ damage being recorded (Rodriguez-Fragoso et al. 2008).

Inorganic compounds present in food (including herbal teas) are responsible for a number of body functions. They have to be delivered with food as human beings are not able to synthesize those (Abd El-Aty et al. 2014). Among the inorganic substances present in herbal teas, some metals and metalloids (Cr, Mn, Fe, Ni, Cu, Zn, Cd, Al, Pb, Co), and inorganic ions (F^-, Cl^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, Na^+, K^+, Mg^{2+} and Ca^{2+}) play important roles.

In turn, certain metals, such heavy metals which can be present in medicinal herbal teas, can have also a negative effect on human organisms. The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) highlighted several metals because of their toxicity: Cd, Pb, Hg, As, Sb, Cu, Sn, Mn, Ca, Ni, Cr, Zn, Se, and Fe (Bolan et al. 2017). Some of them play important biological roles in the living organisms and are necessary in certain concentration ranges for their normal functioning; microelements such as Cu, Zn, Co, Mo, Cr, V,

Mn and Fe belongs to this group. Nonetheless, they are harmful to the organism at high concentrations, when they become toxic (ERDEMIR 2018).

When taking into account the chemical composition of the consumed tea, its preparation and consumption methods are important (WELNA et al. 2013). Usually, herbal teas are prepared by infusion with water; hot water is poured over the prepared product portion or a tea bag and left under cover for approx. 10–20 minutes. Fresh or dried plant parts can be used, such as roots, leaves, fruits, or grains. However, more complex procedures are also used (PERRY 1996).

Various analytical methods and techniques are employed to study the chemical compositions of medicinal herbal teas, and tea infusions. They mainly include: chromatographic methods (MICHALSKI 2006a), capillary electrophoresis (HORIE and KOHATA 2000), neutron activation analysis (CHAJDUK 2009) or spectroscopic methods (FRASER et al. 2014, SZYMCZYCHA-MADEJA et al. 2012). For organic and inorganic ions, the ion chromatography is the dominant analytical method; it has been used for over 35 years as a reference method for determining anions and cations in water and wastewater (KONCZYK et al. 2019; MICHALSKI 2006b). Recently, its use largely expanded, including even nutrient analysis from seeds (YALDIZ and CAMLICA 2019) or phosphate from oils (ZHANG et al. 2013). On the other hand, microwave plasma-atomic emission spectroscopy has a very low utilization cost as it does not require expensive gases, while its detection limits are lower that those in the atomic absorption spectrometry (POIRIER et al. 2017).

The aim of the present study is to evaluate the elemental composition of 56 different herbal teas, and one green tea available on Polish and Romanian market. The contents of the main inorganic anions (F^-, Cl^-, NO_3^-, PO_4^{3-}, SO_4^{2-}) and cations (Na^+, K^+, Mg^{2+}, Ca^{2+}) as well as metals (Cr, Mn, Fe, Ni, Cu, Zn, Cd, Al, Pb, Co) were analyzed in teas infusions. Additionally, the chemometric evaluation of the studied teas was carried out to determine the common traits and differences. Based on the obtained results, an assessment of the associated risk from these analytes was conducted.

Materials and Methods

Reagents

To prepare the eluents for ion chromatography (IC) analysis, the following substances were used: Na_2CO_3, $NaHCO_3$, PDCA (pyridine-2,6-di-

carboxylic acid) and 70% nitric acid (Sigma-Aldrich, Poland). The anion and cation standards (concentrations of 1,000 ±2 mg/L) used to prepare the standard curves were from Fluka (Steinheim, Switzerland). The examined metal standards (concentrations of 1,000 ±2 mg/L) used to prepare the standard curves for the microwave plasma-atomic emission spectroscopy (MP-AES) analysis were delivered by SPEX (Montreal, Canada). The deionized water (conductivity <0.08 μS/cm) used for the tea infusions, eluents and standard solutions was obtained from a Direct-Q 3 UV Millipore system (Merck, Germany).

Teas

54 herbal teas bought from supermarkets from Poland (48) and Romania (6) were used for the tests: peppermint (*Mentha piperita* L.), chamomile (*Matricaria chamomilla* L.), melissa (*Melissa officinalis* L.), linden (*Tilia platyphollos*), sage (*Salvia officinalis* L.), horsetail (*Equisetum arvense* L.), violet tricolor (*Violae tricoloris herba*), St. John's wort (*Hypericum perforatum* L.), calendula (*Hypericum perforatum* L.), nettle (*Urtica dioica* L.), wild rose (*Rosa canina* L.), 11 herbal mixtures with various compositions and only for comparison one popular green tea with prickly pear (*Opuntia ficus-indica*). The tea names were coded taking into account the country of origin (Poland – P; Romania – R) and the base plants: mint – MI; chamomile – CH; melissa – ME; linden – L; sage – S; horsetail – H; violet tricolor – VT; St. John's wort – SJ; calendula – CA; nettle – N; wild rose – WR; herbal mixtures – HM; green tea with prickly pear – GT.

For obtaining comparable data, water infusions were prepared as follows: 1.0000 ±0.0001 g herbal tea samples was weighed in 250 mL Berzelius beakers, in which 100 mL of boiling deionized water were poured then left under cover for 10 minutes. The obtained infusions were filtered through a medium-density filter paper; after cooling, the infusions were transferred in 100 mL volumetric flask, bringing the volume to the mark with deionized water and homogenizing the filtrate. Afterwards, they were filtered through a 0.45-μm Nylon 66 syringe filter (Sigma Aldrich, Germany) in plastic containers, being analyzed with IC (inorganic ions) and MP-AES (metals). From each tea, two independent infusions were prepared, and each infusion was analyzed triplicate.

Analytical methods

The inorganic ions' content in the herbal tea infusions was determined with a 930 Compact IC Flex ion chromatograph (Metrohm, Switzerland) equipped with a conductivity detector; the separation conditions for IC

analysis are presented in Table 1. Calibration of the IC methodologies was carried out in accordance with ISO 8466-1 (POLISH NORM 2004). In order to determine the validation parameters, 6 standard solutions of anions and cations with concentration ranges given in Table 2 were prepared. The calibration solutions were analyzed in triplicate. The obtained areas of the analytes were used to calculate the standard deviation, the coefficient of variance, the limits of detection and limits of quantification, as well as the correlation coefficient. In addition, the recovery for the analyzed ions was calculated; the results are presented in Table 2. The range of concentrations in the standard solutions was matched to the expected concentrations in the real samples. The standard deviation ranges from 0.102 for PO_4^{3-} to 0.172 mg/L for SO_4^{2-}. The limits of detection (LOD) and quantification (LOQ) are also at good levels (from 0.1 for F^- and K^+ up to 0.25 mg/L for SO_4^{2-}, and from 0.27 for Na^+ up to 1.23 mg/L for PO_4^{3-}, respectively), with recoveries varied from 96.8 for PO_4^{3-} up to 104.2% for Mg^{2+}.

Table 1

IC analysis conditions

Parameter	Anions analysis	Cations analysis
Analytical column	Metrohm Metrosep Supp 2 (150 x 4.0 mm)	Metrohm Metrosep C6 (150 x 4.0 mm)
Eluent	3.6 mM Na_2CO_3 + 1 mM $NaHCO_3$	1.7 mM PDCA + 1.7 mM HNO_3
Eluent flow rate	0.7 mL/min	0.9 mL/min
Detection	suppressed conductivity	non-suppressed conductivity

Table 2

Methods validation parameters for inorganic ions

Parameter	F^-	Cl^-	NO_3^-	PO_4^{3-}	SO_4^{2-}	Na^+	K^+	Mg^{2+}	Ca^{2+}
Concentration range [mg/L]	0.1–10.0	0.5–20.0	0.5–30.0	0.2–20.0	0.5–30.0	0.2–10.0	1–100.0	0.2–10.0	0.5–20.0
Standard deviation [mg/L]	0.105	0.167	0.142	0.102	0.172	0.103	0.121	0.104	0.137
Coefficient of variance [%]	1.02	0.85	0.62	2.07	0.95	0.68	3.41	1.47	1.30
Limit of detection (LOD) [mg/L]	0.10	0.17	0.23	0.41	0.25	0.09	0.10	0.17	0.21
Limit of quantification (LOQ) [mg/L]	0.30	0.51	0.70	1.23	0.75	0.27	0.28	0.51	0.63
Correlation coefficient (r)	0.9997	0.9989	0.9997	0.9996	0.9996	0.9997	0.9994	0.9996	0.9998
Recovery [%]	103.1	99.6	101.7	96.8	101.2	97.9	102.2	104.2	99.6

The trace metal content was determined by MP-AES, using a 4200 Agilent spectrometer (Agilent Technologies, USA). The limits of quantification for the determined metals by the applied method (based on 10-fold repeats for the standard samples) and other validation parameters are given in Table 3.

Table 3

MP-AES validation parameters for determined metals

Element	λ [nm]	Adjustment of the calibration curve	Concentration range [mg/L]	Limit of detection [µg/L]	Limit of quantification [µg/L]	Correlation coefficient (r)
Cr	425.433	linear	1.0–50.0	0.34	1.04	0.9997
Mn	403.076	linear	1.0–100.0	0.08	0.25	0.9998
Fe	371.993	linear	1.0–100.0	0.56	1.69	0.9999
Ni	352.454	linear	1.0–60.0	0.36	1.08	0.9995
Cu	324.754	linear	1.0–40.0	0.18	0.54	0.9995
Zn	213.857	nonlinear	1.0–20.0	0.43	1.29	1.0000
Cd	228.802	linear	1.0–20.0	0.32	0.97	0.9998
Al	396.152	linear	1.0–60.0	0.39	1.18	0.9996
Pb	405.781	linear	1.0–60.0	0.35	1.04	0.9997
Co	340.512	linear	1.0–60.0	0.35	1.04	1.0000

The data matrices were processed in Excel (Microsoft, USA), then principal component analysis (PCA) and cluster analysis were performed using MatLab (The Mathworks, USA) after mean center preprocessing.

Results and Discussion

Content of the inorganic anions and cations

The determination results concerning the contents of the inorganic anions and cations in milligrams per product portion [mg/p.p.] in the analyzed herbal teas are presented on Figure 1 and given in Table 4 (the product portion (p.p.) relates to the mass of analyzed tea listed in Appendix 1 – Table 1.1.

The contents of inorganic ions in the examined herbal tea infusions were different and depended on the tea type, its producer and country of origin (Table 4). The fluoride contents in the studied infusions ranged from 0.11 to 5.20 mg/p.p. (max. for mint tea P-MI2); the chloride contents ranged between 0.32 and 20.78 mg/p.p. (max. for herbal mixture tea P-HM5); the

nitrate contents ranged between 0.11 and 22.26 mg/p.p. (max. for nettle tea P-N1), the phosphate contents ranged between 0.16 and 17.75 mg/p.p. (max. for wild rose tea P-WR2) and the sulphate contents ranged from 0.31 to 27.92 mg/p.p. (max. for herbal mixture tea P-HM5).

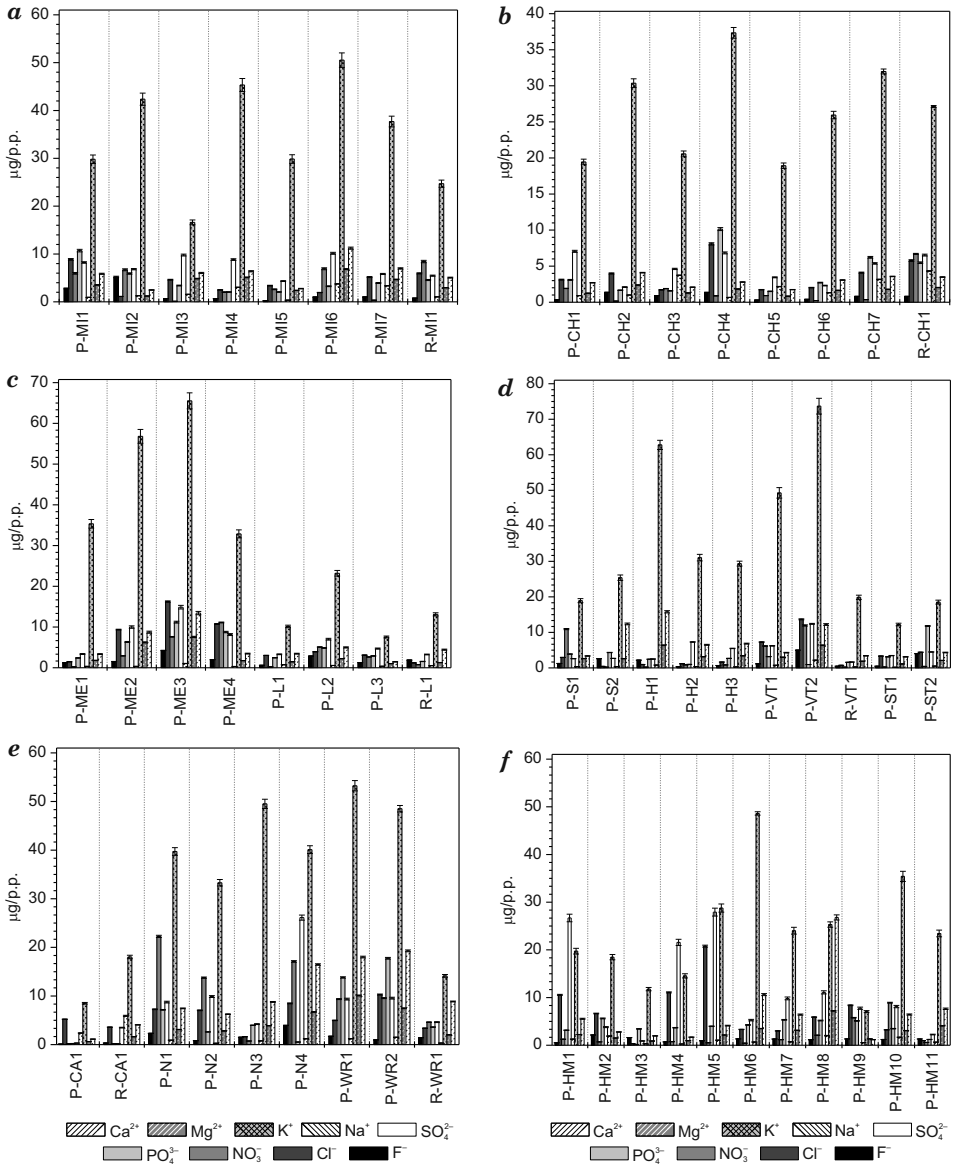

Fig. 1. Mean values of determined ions in particular tea types: *a* – mint; *b* – chamomile; *c* – melissa and linden; *d* – sage, horsetail, violet tricolor and St. John's wort; *e* – calendula, nettle and wild rose; *f* – herbal mixture

Table 4

Inorganic ions content in the studied herbal teas [mg/p.p.]

Ion	Minimum	Maximum	Median	Mean
F^-	0.11	5.2	1.23	1.47
Cl^-	0.32	20.78	3.76	5.11
NO_3^-	0.11	22.26	1.23	3.87
PO_4^{3-}	0.16	17.75	3.45	4.41
SO_4^{2-}	0.31	27.92	6.03	7.46
Na^+	0.18	5.92	0.86	1.27
K^+	7.05	73.69	27.95	30.30
Mg^{2+}	0.62	10.11	2.39	3.13
Ca^{2+}	1.13	26.8	4.72	6.51

Fluorine in the form of the F^- anions in large concentrations has a toxic effect on the human organism (BALCERZAK and JANISZEWSKA 2013); its excessive amount can disturb the absorption and metabolism of iodine, which is necessary for the normal functioning of thyroid and parathyroid glands. Tolerable upper intake level (UL) of fluoride for adults was established on value 7 mg/day. It means that drinking P-MI2 mint (5.20 mg/p/p.) provides the body with as much as 74% of UL (EFSA 2005a). Chloride is a major anion of the extracellular fluid; together with other electrolytes, it influences the water distribution in the organism and acid-base homeostasis. The Scientific Committee on Food did not establish a Population Reference Intake for chloride (EFSA 2021), but concluded that the requirements should match those for sodium (on a molar basis), i.e. 25–150 mmol/day. The US Institute of Medicine established an Adequate Intake (AI) for chloride at a level equivalent (on a molar basis to that of sodium), equaled to 2.3, 2.0 and 1.8 g/day, respectively for younger adults, older adults and the elderly (EFSA 2005b). The tested teas make a negligible contribution to achieving these AI values.

Nitrate and nitrite ions may be the cause of unfavorable changes as they are responsible for the oxidation reaction of iron in the hemoglobin to its Fe^{3+} form. As a result, methemoglobin (unable to bind oxygen) is formed. Phosphate is part of adenosine triphosphate (ATP) – a basic source of energy for the majority of living organisms. The phosphorous deficiency in the diet severely hampers human development, while its excessive amount has adverse effects because it contributes to osteoporosis and damages kidneys. At higher concentrations (up to 1,000 mg/L), sulphate can have a laxative effect and cause stomach problems. When having in mind the highest permissible levels of anions in food products, the obtained contents were not high enough to have a negative impact on human health.

However, a daily consumption of a few strong herbal tea infusions containing around 20 mg nitrate and more, phosphate or sulfate in one portion recommended by the producer may have an adverse effect. In the acidic environment of the stomach, nitrates undergo reduction to nitrites, with demonstrate toxic influence on the human organism. It ought to be remembered that humans ingest large amounts of nitrates with other foods (mainly vegetables, fruit, and meat products) every day (RACZUK et al. 2015) and any additional dose of these ions may exceed the acceptable daily intake (ADI) established at the level of 3.7 mg/kg of the body weight by the Joint FAO/WHO Expert Committee on Food Additives in 2002 (JECFA 2002). Therefore, the consumption of three P-N1 nettle tea portions in a day by a child weighing 20 kg will result in reaching the ADI level.

When comparing the mean contents of specific anions in the Polish tea infusions with the values obtained for the Romanian teas (Table 4), important differences were observed in the following cases: for fluoride in the mint and violet tricolor teas (contents two and six times higher in the Polish teas, respectively); for chloride in the linden and wild rose teas (contents over three times higher in the Polish teas) and violet tricolor tea (content nearly four times higher in the Romanian teas); for nitrates in the chamomile and mint teas (contents over seven and two times higher in the Romanian teas, respectively), and linden and wild rose teas (contents three-and-a-half and two times higher in the Polish teas, respectively); for phosphate in the wild rose and linden teas (contents four and two times higher in the Polish teas, respectively) and violet tricolor tea (content nearly three times higher in the Romanian teas) and for sulfate in the violet tricolor and wild rose teas (contents more than twice as high in the Polish teas).

The contents of the sodium, potassium, magnesium and calcium cations also varied depending on the herbal tea producers and types (Table 4). The differences observed for cations were bigger than those observed for anions in the same tea group. The following cation contents were established: for Na^+ - from 0.18 to 5.92 mg/p.p. (max. for calendula tea R-CA1), for K^+ – from 7.05 to 73.69 mg/p.p. (max. for violet tricolor tea P-VT2), for Mg^{2+} – from 0.62 to 10.11 mg/p.p. (max. for wild rose tea P-WR1) and for Ca^{2+} - from 1.13 to 26.80 mg/p.p. (max. for herbal mixture tea P-HM8).

Sodium and potassium are the basic electrolytes in the human biological system. Although no UL value has been established for these elements, it has been noted strong evidence for the contribution of sodium and potassium to high blood pressure in European populations. It is leading to the development of renal and cardiovascular diseases and stroke. The European Food Safety Authority (EFSA) Panels (EFSA 2019, 2016) point out that 2.0 g sodium/day and 3.5 g potassium/day is a safe and AI for the

general European population of adults. The sodium content in the studied teas was relatively low and did not usually exceed 2 mg/p.p., except for calendula teas and three mint teas. The Na^+ content in Romanian calendula tea was more than twice as high as that found in the Polish tea. Potassium was the major cation present in all the teas (Table 4). Its content in the Polish violet tricolor as well as in melissa and in wild rose teas exceeded 60 and 50 mg/p.p., respectively. The Romanian violet color infusion had a three times lower K^+ content in comparison with the mean content of these ions in the Polish teas (19.89 mg/p.p.). A nearly four times lower amount of the K^+ ions was also observed in the Romanian wild rose infusion (14.14 mg/p.p.).

Magnesium ions belong to many enzyme activators and participate in the metabolic processes. According to EFSA Panel (2015), AI for magnesium is set at 350 mg/day for adult men and 300 mg/day for adult women. The calcium deficiency leads to hypocalcemia whose symptoms include seizures, nervous system weakness, general weakness, muscle weakness, and skin problems (VAN DRONKELAAR et al. 2018). A UL of 2,500 mg calcium/day from all sources was proposed for adults, and for pregnant and lactating women by EFSA Panel (2012). Besides herbal mixtures, the highest magnesium and calcium contents were observed in the Polish wild rose teas: the mean contents of those ions were four and two times higher than the contents in the Romanian teas, respectively. However, these values do not significantly affect the total intake of these elements in the daily diet. When comparing the mean contents of specific analytes in the Polish tea infusions with the values obtained for the Romanian teas, important differences were observed in few cases. They concern e.g. fluorides in the mint and violet tricolor teas, nitrates in the chamomile and mint teas. The differences observed for cations were bigger than those discerned for anions in the same tea group.

There are few studies on the metal content in herbal infusions (ALTINTIG et al. 2014, JURANOVIĆ et al. 2013, ÖZCAN et al. 2008, SCHUNK et al. 2016).

The content of sodium, potassium, calcium and magnesium in infusions of herbal teas from Polish producers was investigated by PYTLAKOWSKA et al. (2012): the highest content of Na (389 μg/g) they have found in the peppermint infusion, K (2650 μg/g – in melissa), Ca (114 μg/g) – in the infusion with nettle, and Mg (271 μg/g) – in sage herbal tea. These values are much lower than those obtained in this work, which may indicate the effect on the result of the analysis caused by the method of brewing, the origin of herbs, environmental conditions, etc. However, so far no one has examined and compared the content of these metals in Polish and Romanian herbal tea infusions.

Trace metal content

Figure 2 displays the mean concentrations of selected metals in the studied herbal teas; additional statistical information regarding the metal concentrations is summarized in Table 5.

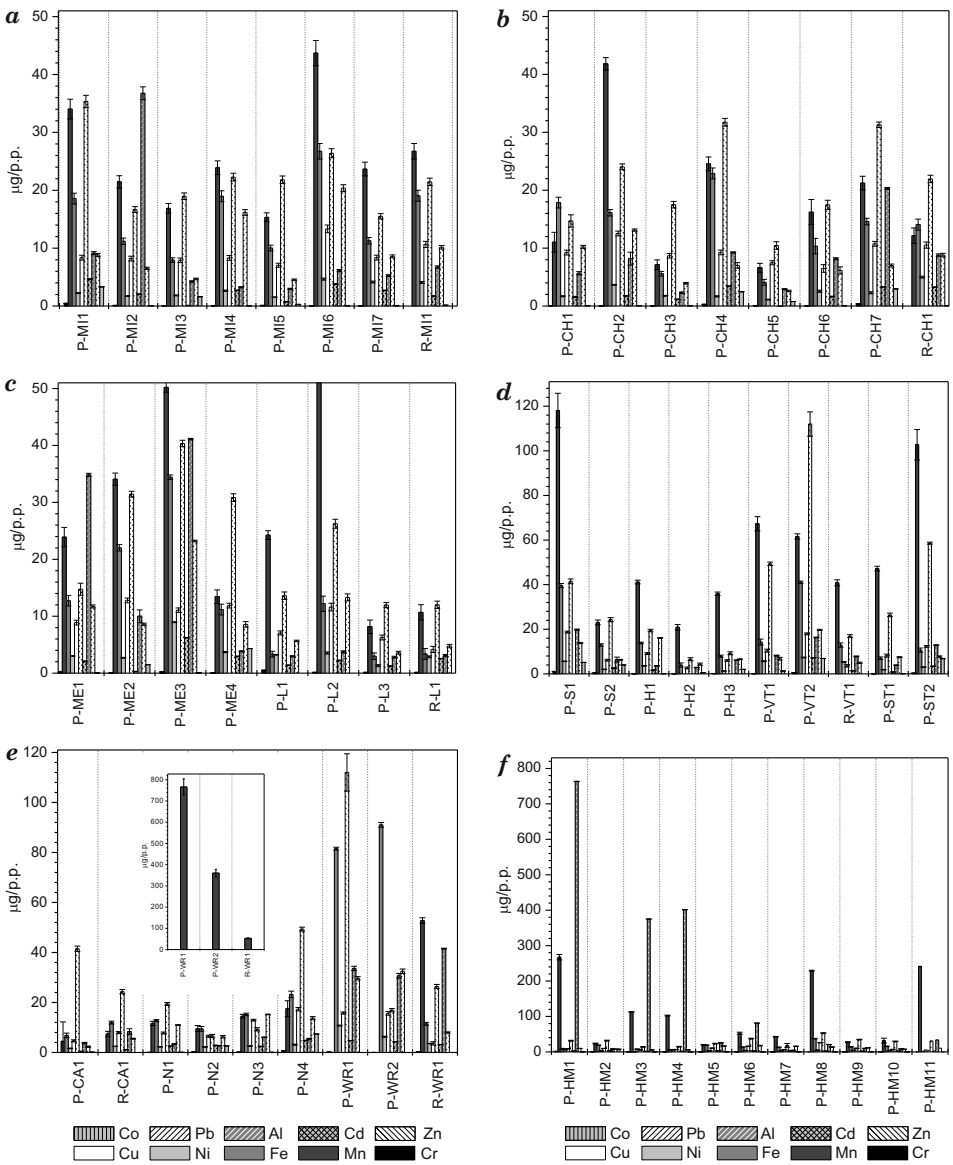

Fig. 2. Mean values of determined metals in particular tea types: *a* – mint; *b* – chamomile; *c* – melissa and linden; *d* – sage, horsetail, violet tricolor and St. John's wort; *e* – calendula, nettle and wild rose; *f* – herbal mixture

Table 5

Trace metal content in the studied herbal teas [µg/p.p.]

Metal	Minimum	Maximum	Median	Mean
Cr	< LOQ	1.38	0.13	0.22
Mn	4.51	765.9	29.71	74.04
Fe	0.47	91.04	12.84	17.01
Ni	< LOQ	10.76	3.23	3.86
Cu	2.64	25.99	8.9	9.66
Zn	6.57	112.0	28.12	31.57
Cd	< LOQ	7.20	2.34	2.34
Al	2.30	763.35	8.30	47.94
Pb	2.32	32.49	7.87	10.34
Co	< LOQ	7.42	0.06	1.32

Cadmium is a highly toxic metal that belongs to the so-called *priority substances* with mutagenic and carcinogenic effects; it can be responsible for anemia and hypertension. Lead is usually transported into the human and animal organisms through consumption of contaminated plants. Zinc is a metal participating in the protein metabolism; its deficiency disturbs the functioning of the immune, nervous and reproductive systems. Copper is among elements necessary in the hemoglobin synthesis and in the iron absorption by the living organisms; the clinical research showed that the organism is more susceptible to diseases and infections if the copper deficiency is severe (SCHULZKI et al. 2017). From the toxicological point of view, chromium (VI) has a harmful influence on the organism; it is quickly absorbed and demonstrates carcinogenic and mutagenic characteristics (POHL et al. 2016). The nickel compounds have low toxicity, but they cause allergic symptoms if the organism is at a constant exposure. Iron is counted among microelements that have an important influence on the functioning of the living organisms. The soluble aluminum compounds have a negative impact on the human nervous system; it may be one of the factors influencing the development of the Alzheimer's and Parkinson's diseases (MALIK et al. 2013).

The contents of metals in the studied infusions were largely diverse. Among 10 tested metals, manganese and zinc demonstrated the highest share of the total contents of this group of elements in most infusions (Table 5). The analysis of 11 herbal mixtures also demonstrated large differences in the aluminum and manganese contents in the prepared infusions: the aluminum and manganese content were between 4.47 and 763.35 µg/p.p. and

between 20.02 and 267.27 µg/p.p., respectively, with definitely lower differences in the contents of the remaining metals. EFSA Panel (EFSA 2013) proposed an AI value of 3 mg Mn/day for adults, including pregnant and lactating women, whilst Regulation No. 1169/2011 of the European Parliament and of the Council (REGULATION (EU) NO 1169... 2011) indicates 2 mg of Mn as a reference value for beverages which means that not more than 150 µg/p.p. may be present in 100 mL of the beverage. The manganese contents in the studied Polish wild rose teas, and three herbal mixtures exceeded this value. High manganese content observed in the infusions of Polish wild rose teas (765.92 and 360.27 µg/p.p., respectively for P-WR1 and P-WR2) can be due to the fact that the manufacturer added hibiscus to these teas. 1 g of rose hips of the Rosa canin variety contains an average of 35 to 67 µg/g of manganese (LEVENT et al. 2010) while 1 g of hibiscus contains about 390 µg of this metal (WRÓBEL et al. 2000). In turn, high content of manganese in herbal mixtures may be due to the presence of green tea (P-HM1), hibiscus (P-HM8) or birch leaves (P-HM11) in them. Both the results obtained in this paper for green teas (977 µg/g) and those presented in the literature (WRÓBEL et al. 2000) confirmed the high content of manganese and aluminum in this type of tea, and REIMANN et al. (2007) stated high content of Mn in birch leaves. The average Al concentration of 976.66 µg/p.p. for the studied infusions is in the concentration range obtained for Brazilian herbal teas (SCHUNK et al. 2016). In the case of chamomile infusion ca. 5 times lower Al content was found in the Polish herbs (11.29 µg/g; this paper); 10.9–15.0 µg/g (PYTLAKOWSKA et al. 2012) in comparison to the Brazilian herbs (51.62 µg/g).

The chromium content in nearly half the cases and cobalt content in 16 cases were lower than LOQ (0.10 µg/p.p.). According to the REGULATION (EU) NO 1169... 2011, not more than 7.5% of the daily reference intake for chromium (40 µg, i.e. max. 3 µg/p.p.) should be present in 100 mL of the beverage. None of the studied infusions demonstrated a value higher than 1.40 µg Cr/p.p., which means that the chromium doses ingested with infusions are safe for the human organism. Cr concentration in Polish chamomile infusion is the same as in the case of the infusion prepared on the base the Brazilian chamomile (ca. 0.1 µg/g) but lower than that coming from Spain (0.61 µg/g) (MARTÍN-DOMINGO et al. 2017) or Turkey (1.22 µg/g) (BAŞGEL and ERDEMOĞLU 2005).

The iron contents ranged from below the LOQ level (<0.17, P-HM3, P-HM11) to 91.04 µg/p.p. (P-WR2). For zinc, it was from 6.57 (P-H2) to 112.02 µg/p.p. (P-VT2). The nickel contents ranged between below the LOQ value (0.11; P-H2) and 10.76 µg/p.p. (P-WR1); for copper, the values ranged from 2.64 (P-H2) to 25.99 µg/p.p. (P-HM8). Regulation (EU)

No 1169... 2011 defines daily reference intake values not only for manganese and chromium, but also for iron, zinc and copper at the levels of 14 mg (1050 µg/100 mL of the beverage), 10 mg (750 µg/100 mL of the beverage) and 1 mg (75 µg/100 mL of the beverage), respectively. When relating the reference values to the contents of particular elements in the studied infusions, it may be said that no recommended standards were exceeded in any case. However, the increased lead contents observed for the Polish wild rose tea infusions may be distressing from the health point of view. The values were approx. four times higher than the ones observed for the Romanian tea (Table 5). These differences may result, as in the case of manganese and aluminum, from the presence of hibiscus in infusions of Polish teas. Cadmium content varied between the below LOQ level (<0.10 µg/p.p; 9 infusions) and 7.20 µg/p.p (P-VT2), whereas the lead content ranged from 2.32 (P-CA1) to 32.49 µg/p.p. (P-WR2). Both of these metals are characterized by the highest toxicity among the tested metals. EFSA Panel on Contaminants in the Food Chain established tolerable weekly intake (TWI) of 2.5 µg/kg body weight to ensure sufficient protection of all consumers (EFSA 2012). In turn, adult exposure for lead was estimated at 0.50 µg/kg body weight per day (EFSA 2012).

The obtained mean concentrations of selected heavy metals were compared with the data obtained by other authors for sage infusions and presented in Table 6.

Table 6

Comparison of the obtained and literature data for selected elements in sage infusions

Reference	Pytlakowska et al. (2012)	Özcan et al. (2008)	Martín-Domingo et al. (2017)	Juranović Cindrić, et al (2013)	This work
Element	the average content of the element [mg/g]				
Cr	–	20	2.02	< LOD	0.40
Mn	4.31	44	38.3	0.369	39.35
Fe	4.81	668	934	0.009	14.23
Ni	–	3	–	0.006	2.06
Cu	1.14	17	5.88	0.082	6.74
Zn	8.71	53	19.8	0.585	17.07
Cd	–	–	0.02	0.4	0.52
Al	35.5	14	–	< LOD	7.11
Pb	–	0.28	1.50	0.150	5.32
Co	–	2	–	–	2.31

The studied medicinal plants revealed diverse ionic and elemental profiles that can provide evidences for uses for certain purposes. It was established that in some instances, several herbal teas exhibit higher concentrations of hazardous analytes, such as cadmium, lead, or chromium; in such cases, the expected therapeutic effect can suffer a drawback due to the unwanted contaminants, with potentially negative effects. The variability of the contaminants' contents from the studied medicinal herbal teas can be due to the factors like differences between the plants species, geographical areas and exposure to different pollution sources or conditions during drying process. To assure the quality of the medicinal plants, good manufacturing practice shall provide a proper control of the raw material, with a special emphasis on a strict survey of the harvest areas and on processing in order to maintain the heavy metals' content at the lowest possible value. Since the medicinal plants and their extracts are used in traditional medicine, there is a possible hazard of heavy metal poisoning, if they originate from polluted areas; therefore, these raw materials should be collected from unpolluted regions and they should be analyzed for heavy metal content in order to avoid their cumulative toxicities in long-term use. Due to their hazard, the content of heavy metals has to be one of the main criteria for the use of medicinal plants as raw materials in the production of traditional remedies; hence it is essential to have a proper quality control to ensure safety and efficacy of herbal products.

Chemometric data analysis

Principal component analysis (PCA) of the full data set led to a model built on nine variables (the concentrations of the major studied ions), which reveals a fuzzy data structure, the model explaining only 54.9% of the variance (Figure 3a); the corresponding loading plot reveals a close correlation for the concentrations of chloride and sulfate/fluoride, nitrate and phosphate/potassium, calcium and magnesium (Figure 3b).

More suggestive is the PCA for anions' data set; the model corresponding for the scores' plot from Figure 4a and dendrogram from Figure 4b was built using five variables (the concentrations of the major studied anions), explains 77.22% variance and reveals three clusters: one containing the outliers P-HM4, P-HM1 and P-HM5 (with the highest concentrations of sulfate and chloride), another corresponding to the highest concentrations of fluoride, nitrate and phosphate and a major one, including most of the samples. This model emphasizes the herbal teas that can pose a risk on human health due the presence of hazardous nitrate anion.

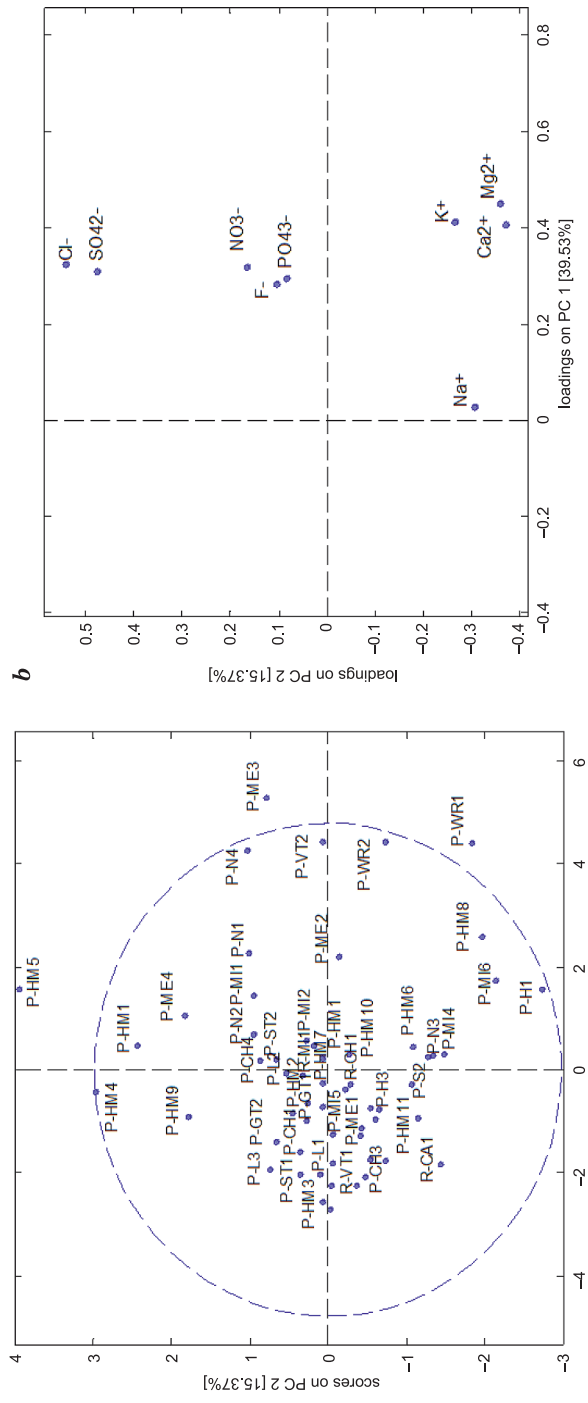

Fig. 3. The scores' plot (a) and the loadings' plot (b) for the full dataset

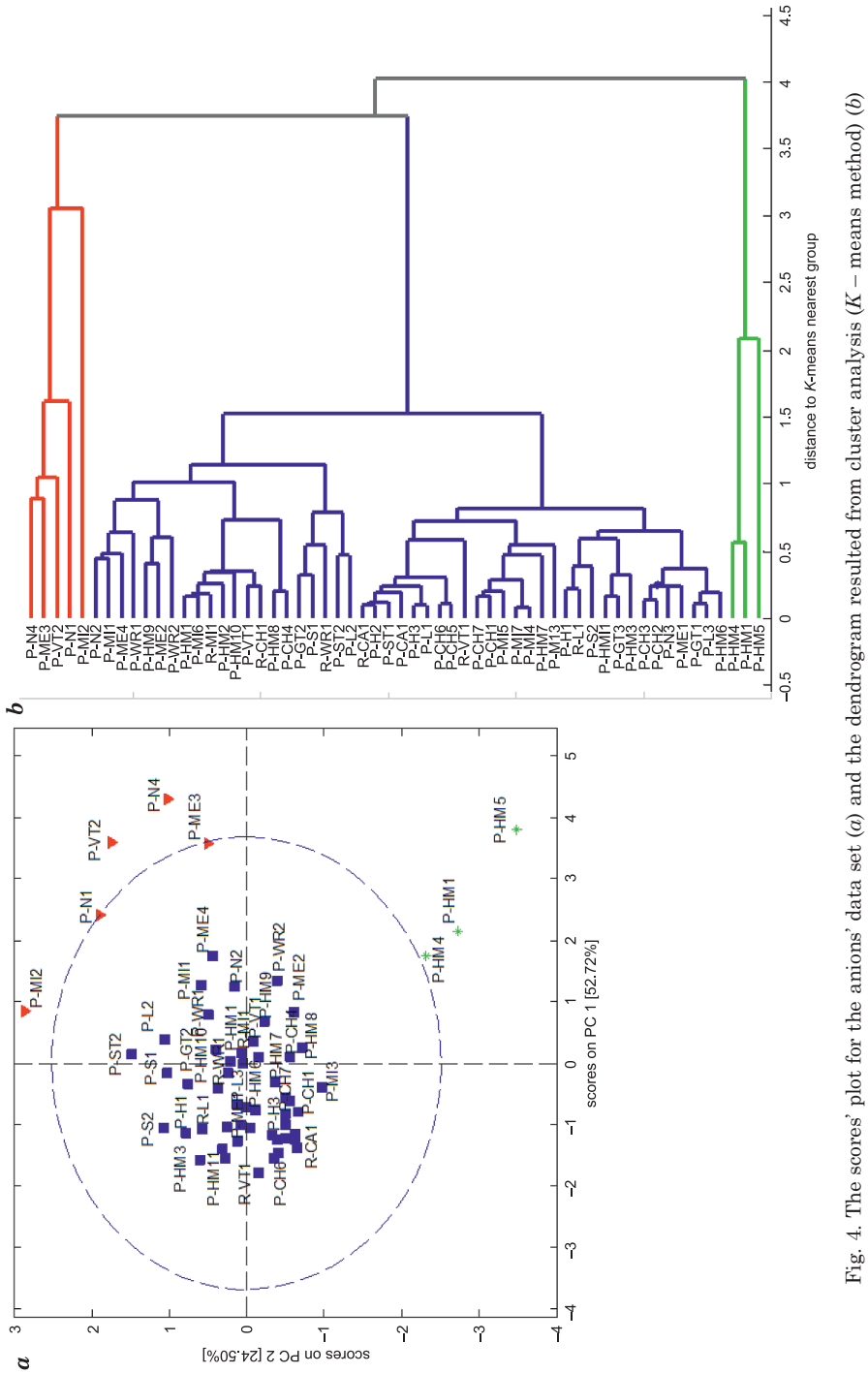

Fig. 4. The scores' plot for the anions' data set (*a*) and the dendrogram resulted from cluster analysis (K – means method) (*b*)

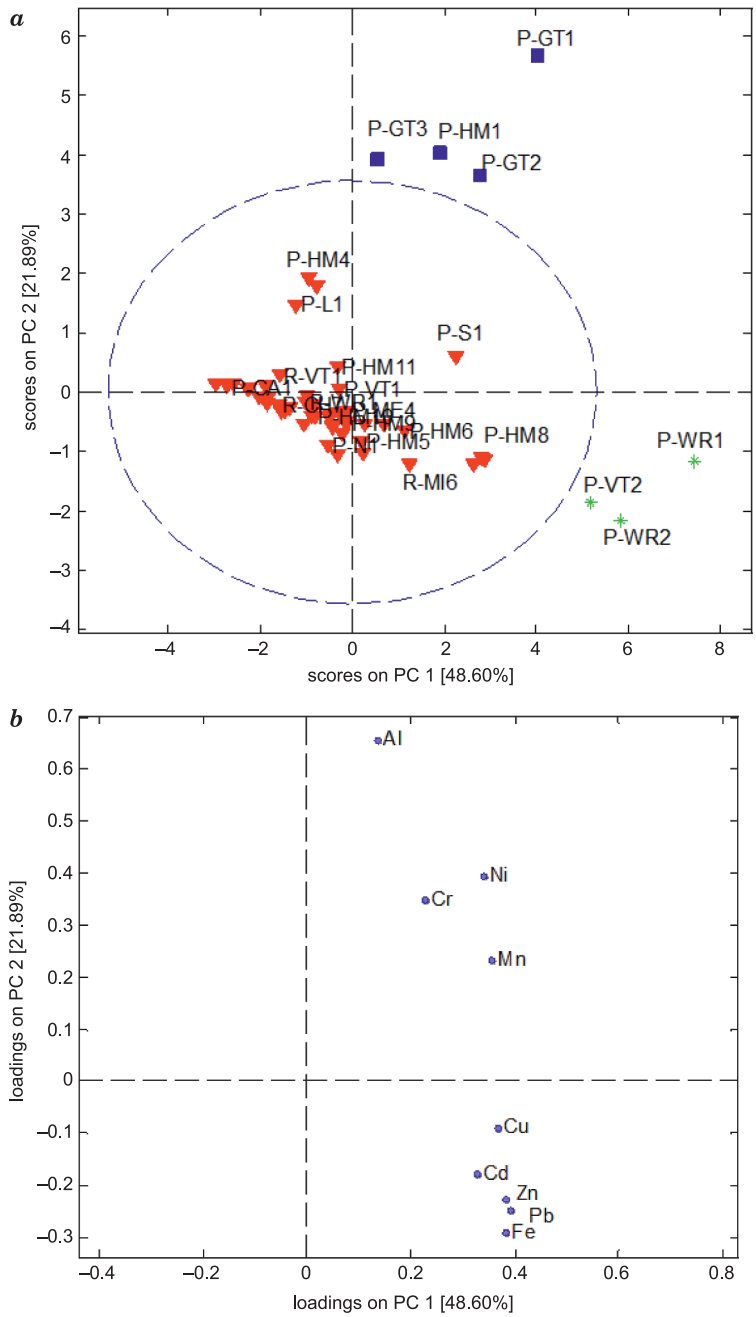

Fig.5. The scores' plot (*a*) and the loadings' plot (*b*) for the metal dataset

For the studied metals, PCA of the full dataset led to a model built on ten variables (the concentrations of the studied metals), which reveals four clusters, the model explaining 70.49% of the variance (Figure 5); the corresponding loading plot reveals a close correlation for the content of copper, cadmium, zinc, lead and iron (Figure 5b). Two from those three clusters contain the outliers green teas (P-GT) and PHM1 (in dark blue, with the highest concentrations of aluminum, chromium and nickel), another containing the samples with the highest concentrations of iron (PWR1, PWR2 and PVT2), a third one containing the samples high in nickel and copper (P-HM8, P-S1, P-N4, PME3) and a major one, corresponding to the most of the samples. This model emphasizes also several herbal teas that can pose a risk on human health due the presence of heavy metals of concern.

Conclusions

When comparing the mean contents of specific analytes in the Polish tea infusions with the values obtained for the Romanian teas, important differences were observed in few cases. These may have resulted from the country of origin, herb cultivation and harvest methods, as well as its processing, including additions of other herbs, aroma substances or spices that may have had an important impact on the effectiveness of extraction of particular ions into water in the herb steeping/brewing process. This may be crucial in terms of the health risks associated with drinking such teas. Consumers should be accurately informed about these parameters, given the popularity and easy access to herbal teas.

Translated by Wiktoria Mroczek (www.usatlumacz.pl)

Accepted for print 24.02.2023

References

Abd El-Aty A.M., Choi J.H., Rahman Md.M., Kim S.W., Tosun A., Shim J.H. 2014. *Residues and contaminants in tea and tea infusions: a review*. *Food* Addit. Contam., *Part* A, 31: 1794–1804.

Andishe Tadbir A., Pourshahidi S., Ebrahimi H., Hajipour Z., Memarzade M.R., Shirazian S. 2015. *The effect of Matricaria chamomilla (chamomile) extract in Orabase on minor aphthous stomatitis, a randomized clinical trial*. J. Herb. Med., 5: 71–76.

Altintig E., Altundag H., Tuzen M. 2014. *Determination of multi element levels in leaves and herbal teas from Turkey by ICP-OES*. Bull. Chem. Soc. Ethiop., 28: 9–16.

Balcerzak M., Janiszewska J. 2013. *Fluorides in tea products and analytical problems with their determination*. Crit. Rev. Anal. Chem., 43: 138–147.

Başgel S., Erdemoğlu S.B. 2005. *Determination of mineral and trace elements in some medicinal herbs and their infusions consumed in Turkey*. Sci. Total Environ., 359: 82–89.

Butiuk A.P., Martos M.A., Adachic O., Hoursa R.A. 2016. *Study of the chlorogenic acid content in yerba mate (Ilex paraguariensis St. Hil.): Effect of plant fraction, processing step and harvesting season*. J. App. Res. Med. Arom. Plant., 3: 27–33.

Bolan S., Kunhikrishnan A., Seshadri B., Choppala G., Naidu R., Bolan N.S., Ok Y.S., Zhang M., Li C.G., Li F., Noller B., Kirkham M.B. 2017. *Sources, distribution, bioavailability, toxicity, and risk assessment of heavy metal(loid)s in complementary medicines*. Environ. Int., 108: 103–118.

Chajduk E. 2009. *Elemental analysis of black and green tea leaves by instrumental neutron activation analysis*. Chem. Anal.-Warsaw, 54: 841–855.

Chen Z.M., Lin Z. 2015. *Tea and human health: biomedical functions of tea active components and current issues*. J. Zhejiang Univ.-Sci. B, 16: 87–102.

Dalar A., Konczak I. 2012. *Botanicals from Eastern Anatolia Region of Turkey: antioxidant capacity and phenolic constituents of endemic herbal medicines*. J. Herb. Med. 2: 126–135.

De Oliveira L.M., Das S., Da Silva E.B., Gao P., Gress J., Liu Y.G., Ma L.Q. 2018. *Metal concentrations in traditional and herbal teas and their potential risks to human health*. Sci. Total Environ., 633: 649–657.

EFSA (The European Food Safety Authority). 2005a. *Opinion of the scientific panel on dietetic products, nutrition and allergies on a request from the commission related to the tolerable upper intake level of fluoride*. EFSA J., 192: 1–65.

EFSA (The European Food Safety Authority). 2005b. *Opinion of the scientific panel on dietetic products, nutrition and allergies on a request from the commission related to the tolerable upper intake level of chloride*. EFSA J., 210: 1–9.

EFSA (The European Food Safety Authority). 2012. *Scientific Opinion on the Tolerable Upper Intake Level of calcium*. EFSA J. 10(7): 2814.

EFSA (The European Food Safety Authority). 2013. *Scientific Opinion on dietary reference values for manganese*. EFSA J., 11(11): 3419.

EFSA (The European Food Safety Authority). 2015. *Scientific opinion on dietary reference values for magnesium*. EFSA J.,13(7): 4186.

EFSA (The European Food Safety Authority). 2016. *Scientific opinion on dietary reference values for potassium*. EFSA J., 14(10): 4592.

EFSA (The European Food Safety Authority). 2019. *Scientific opinion on the dietary reference values for sodium*. EFSA J., 17(9): 5778.

EFSA (The European Food Safety Authority). 2021. Scientific Committee, *Statement on the derivation of health-based guidance values (HBGVs) for regulated products that are also nutrients*. EFSA J., 19(3): 6479.

Erdemir U.S. 2018. *Contribution of tea (Camellia sinensis* L.*) to recommended daily intake of Mg, Mn, and Fe: An in vitro bioaccessibility assessment*. J. Food Compos. Anal., 69: 71–77.

Fraser K., Harrison S.J., Lane G.A., Otter D.E., Hemar Y., Quek S.Y., Rasmussen S. 2014. *Analysis of low molecular weight metabolites in tea using mass spectrometry-based analytical methods*. Crit. Rev. Food Sci. Nutri., 54: 924–937.

Gallaher R.N., Gallaher K., Marshall A.J., Marshall A.C. 2006. *Mineral analysis of ten types of commercially available tea*. J. Food Compos. Anal., 19: Suppl. 53–57.

Horie H., Kohata K., 2000. *Analysis of tea components by high-performance liquid chromatography and high-performance capillary electrophoresis*. J. Chromatogr., A 881: 425–438.

JECFA 2002. Joint FAO/WHO Expert Committee on Food Additives. *Evaluation of certain food additives and contaminants*. Fifty-ninth report of JECFA. Technical Reports, 913: 20–32.

Juranović Cindrić I., Zeier M., Glamizuna E., Stingeder G. 2013. *Elemental characterisation of the medical herbs Salvia officinalis L. and Teucrium montanum L. grown in Croatia*. Microchem. J., 107: 185–9.

Kaczmarczyk D., Lochyński S. 2014. *Products of biotransformation of tea infusion – properties and application*. Pol. J. Natur. Sc., 29(4): 381–392.

Karak T., Kutu F.R., Nath J.R., Sonar I., Paul R.K., Boruah R.K., Sanyal S., Sabhapondit S., Dutta A.K. 2017. *Micronutrients (B, Co, Cu, Fe, Mn, Mo, and Zn) content in made tea (Camel-*

lia sinensis L.*) and tea infusion with health prospect: A critical review*. Crit. Rev. Food Sci. Nutri., 57: 2996–3034.

KOJTA A. JARZYŃSKA G., FALANDYSZ J. 2012. *Mineral composition and heavy metal accumulation capacity of Bay Bolete (Xerocomus badius) fruiting bodies collected near a former gold and copper mining area*. J. Geochem. Explor., 121: 76–82.

KONCZYK J., MUNTEAN E., GEGA J., FRYMUS A., MICHALSKI R. 2019. *Major inorganic anions and cations in selected European bottled waters*. J. Geochem. Explor., 197: 27–36.

LEVENT A., ALP Ş., EKIN S., KARAGÖZ S. *Trace heavy metal contents and mineral of Rosa canina* L. *fruits from van region of Eastern Anatolia, Turkey*, Rev. Analyt. Chem., 29(1): 13–24.

MALIK J., FRANKOVA A., DRABEK O., SZAKOVA J., ASH C., KOKOSKA L. 2013. *Aluminum and other elements in selected herbal tea plant species and their infusions*. Food Chem., 139: 728–734.

MARTÍN-DOMINGO M.C., PLA A., HERNÁNDEZ A.F., OLMEDO P., GIL F. 2017. *Determination of metalloid, metallic and mineral elements in herbal teas. Risk assessment for the consumers*. J. Food Compos. Anal., 60: 81–89.

MICHALSKI R. 2006a. *Simultaneous determination of common inorganic anions in black and herbal tea by suppressed ion chromatography*. J. Food Qual., 29: 607–616.

MICHALSKI R. 2006b. *Ion chromatography as a reference method for the determination of inorganic ions in water and wastewater*. Crit. Rev. Anal. Chem., 36: 107–127.

MUNTEAN N., MUNTEAN E., CRETA C., DUDA M. 2013. *Heavy metals in some commercial herbal teas*. ProEnvironment/ ProMediu, 6: 591–594.

MUNTEAN E., MICHALSKI R., MUNTEAN N., DUDA M. 2016. *Chemical risk due to heavy metal contamination of medicinal plants*. Hop Med. Plant., XXIII, 1–2: 71–78.

NGURE V., KINUTHIA G., 2020. *Health risk implications of lead, cadmium, zinc, and nickel for consumers of food items in Migori Gold mines, Kenya*. J. Geochem. Explor., 209: 106430.

ÖZCAN M.M., ÜNVER A., UÇAR T., ARSLAN D. 2008. *Mineral content of some herbs and herbal teas by infusion and decoction*. Food Chem., 106: 1120–1127.

PERRY S. 1996. *The book of herbal teas: a guide to gathering, brewing, and drinking*. CA: Chronicle Books, San Francisco.

POHL P., DZIMITROWICZ A., JEDRYCZKO D., SZYMCZYCHA-MADEJA A., WELNA M., JAMROZ P., 2016. *The determination of elements in herbal teas and medicinal plant formulations and their tisanes*. J. Pharm. Biomed. Anal., 130 SI: 326–335.

POLISH NORM PN-ISO 8466-1:2004. *Jakość wody. Kalibracja i ocena metod analitycznych oraz szacowanie ich charakterystyk. Część 1: Statystyczna ocena liniowej funkcji kalibracji* (in Polish).

POIRIER L., NELSON J., GILLELAND G., WALL S., BERHANE L., LOPEZ-LINARES F. 2017. *Comparison of preparation methods for the determination of metals in petroleum fractions (1000 degrees F+) by microwave plasma atomic emission spectroscopy*. Energ. Fuel., 31: 7809–7815.

PYTLAKOWSKA K., KITA A., JANOSKA P., POŁOWNIAK M., KOZIK V. 2012. *Multi-element analysis of mineral and trace elements in medicinal herbs and their infusions*. Food Chem., 135: 494–501.

RACZUK J., WADAS W., GŁOZAK K. 2015. *Nitrates and nitrites in selected vegetables purchased AT supermarkets in Siedlce, Poland*. Rocz. Panst. Zakl. Hig. 65: 15–20.

REGULATION, 2011. Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers.

RODRIGUEZ-FRAGOSO L., REYES-ESPARZA J., BURCHIEL S.W., HERRERA-RUIZ D., TORRES E. 2008. *Risks and benefits of commonly used herbal medicines in Mexico*. Toxicol. Appl. Pharmacol., 227: 125–135.

SCHULZKI G., NUESSLEIN B., SIEVERS H. 2017. *Transition rates of selected metals determined in various types of teas (Camellia sinensis L. Kuntze) and herbal/fruit infusions*. Food Chem., 215: 22–30.

SCHUNK P.F.T., KALIL I.C., PIMENTEL-SCHMITT E.F., LENZ D., DE ANDRADE D.U., RIBEIRO J.S., ENDRINGER D.C. 2016. *ICP-OES and micronucleus test to evaluate heavy metal contamination in commercially available Brazilian herbal teas*. Biol. Trace Elem. Res., 172: 258–265.

SHALAYEL M.H.F., ASAAD A.M., QURESHI M.A., ELHUSSEIN A. 2017. *Anti-bacterial activity of peppermint (Mentha piperita) extracts against some emerging multi-drug resistant human bacterial pathogens*. J. Herb. Med., 7: 27–30.

SPYCHALSKI G. 2013. *Determinants of growing herbs in Polish agriculture*. Herba Pol., 59: 5–18.
STREET R.A. 2012. *Heavy metals in medicinal plant products – an African perspective*. S. Afr. J. Bot., 82: 67–74.
SZYMCZYCHA-MADEJA A., WELNA M., POHL P. 2012. *Elemental analysis of teas and their infusions by spectrometric methods*. Trend Anal. Chem., 35: 165–181.
THEUMA M., ATTARD E. 2020. *From herbal substance to infusion: The fate of polyphenols and trace elements*. J. Herb. Med., 21:100347.
TUPEC M., HÝSKOVÁ V., BĚLONOŽNÍKOVÁ K., HRANÍČEK J., ČERVENÝ V., RYŠLAVÁ H. 2017. *Characterization of some potential medicinal plants from Central Europe by their antioxidant capacity and the presence of metal elements*. Food Biosci., 20: 43–50.
WRÓBEL K., WRÓBEL K., COLUNGA URBINA E.M., *Determination of total aluminum, chromium, copper, iron, manganese, and nickel and their fractions leached to the infusions of black tea, green tea, Hibiscus sabdariffa, and Ilex paraguariensis (Mate) by ETA-AAS,* Biol. Trace Element Res., 78: 271–279.
VAN DRONKELAAR C., VELZEN A. VAN, ABDELRAZEK M., STEEN A. VAN DER, WEIJS P.J.M., TIELAND M., 2018. *Minerals and sarcopenia; the role of calcium, iron, magnesium, phosphorus, potassium, selenium, sodium, and zinc on muscle mass, muscle strength, and physical performance in older adults: a systematic review*. J. Am. Med. Dir. Assoc., 19: 6–18.
VUONG Q.V. 2014. *Epidemiological evidence linking tea consumption to human health: a review*. Crit. Rev. Food Sci. Nutr., 54: 523–536.
WANG CH.Z., MEHENDALE S.R., YUAN CH.S. 2007. *Commonly used antioxidant botanicals: active constituents and their potential role in cardiovascular illness*. Am. J. Chin. Med., 35: 543–558.
WELNA M., SZYMCZYCHA-MADEJA A., POHL P. 2013. *A comparison of samples preparation strategies in the multi-elemental analysis of tea by spectrometric methods*. Food Res. Int., 53: 922–930.
YALDIZ G., CAMLICA M., 2019. *Variation in the fruit phytochemical and mineral composition, and phenolic content and antioxidant activity of the fruit extracts of different fennel (Foeniculum vulgare* L.*) genotypes*. Ind. Crop Prod., 142: 111852.
ZHANG Y., THEPSITHAR P., JIANG X., TAY J.H. 2013. *Direct determination of phosphate in raw Jatropha curcas oil by ion chromatography*. Ind. Crop Prod., 44: 459–464.

Appendix 1

Table 1.1

Preparation method recommended by the manufacturer, according to the information provided on the product label

Code	Amount of tea [g] (p.p.)	Amount of water [mL]	Code	Amount of tea [g] (p.p)	Amount of water [mL]	Code	Amount of tea [g] (p.p.)	Amount of water [mL]
P-MI1	1 tea bag (2.3)	200	P-ME3	1 tea bag (1.7)	200	P-N1	1 tea bag (2.1)	200
P-MI2	1 tea bag (1.6)	200	P-ME4	2 teaspoons (2.1)	150	P-N2	1 tea bag (1.7)	200
P-MI3	1 teaspoon (1.6)	150	P-L1	1 tea bag (1.5)	150	P-N3	1 tea bag (1.9)	200
P-MI4	1 tea bag (2.2)	200	P-L2	1 tea bag (2.0)	200	P-N4	2 tea bags (3.3)	200
P-MI5	1 teaspoon (1.1)	200	P-L3	1 teaspoon (1.3)	150	P-WR1	1 tea bag (4.1)	150
P-MI6	1 tea bag (2.5)	150	R-L1	1 teaspoon (1.1)	200	P-WR2	1 tea bag (3.6)	150
P-MI7	1 tea bag (2.2)	150	P-S1	1 tea bag (1.7)	150	R-WR1	1 teaspoon (2.3)	150
R-MI1	1 teaspoon (2.1)	200	P-S2	1.5 teaspoons (2.5)	100	P-HM1	1 tea bag (2.1)	200
P-CH1	1 tea bag (1.5)	200	P-H1	1 tea bag (2.5)	100	P-HM2	1 tea bag (2.2)	200
P-CH2	1 tea bag (2.0)	200	P-H2	1 teaspoon (1.0)	250	P-HM3	1 teaspoon (1.8)	200
P-CH3	1-2 teaspoons (2.1)	150	P-H3	1 teaspoon (2.0)	250	P-HM4	1 teaspoon (1.7)	200
P-CH4	2 tea bags (1.7)	200	P-VT1	2 teaspoons (3.0)	150	P-HM5	2 tea bags (2.2)	200
P-CH5	2 teaspoons (1.7)	150	P-VT2	1 tea bag (4.4)	200	P-HM6	1 tea bag (2.2)	200
P-CH6	1 tea bag (1.6)	150	R-VT1	1 teaspoon (1.0)	200	P-HM7	1 tea bag (2.2)	200
P-CH7	1 tea bag (1.8)	200	P-ST1	1 teaspoon (1.1)	200	P-HM8	1 tea bag (2.7)	200
R-CH1	1 teaspoon (1.4)	200	P-ST2	1 tea bag (2.3)	200	P-HM9	2 tea bags (2.2)	200
P-ME1	1 teaspoon (2.0)	200	P-CA1	2 teaspoons (1.0)	200	P-HM10	1 tea bag (2.0)	200
P-ME2	1 tea bag (1.4)	200	R-CA1	1 teaspoon (0.5)	200	P-HM11	2 tea bags (2.0)	200

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 38(1): 89–101, Y. 2023

DOI: 10.31648/pjns.8689

EFFECT OF *L*-TRYPTOPHAN ON THE MORPHO-FUNCTIONAL CHANGES OF WHITE ADIPOSE TISSUE AN INDUCED VISCERAL OBESITY RAT MODEL*

***Roman Yanko*[1], *Elena Chaka*[2], *Sergey Safonov*[3], *Mikhail Levashov*[4]**

[1] ORCID: 0000-0002-0397-7517
[2] ORCID: 0000-0001-7425-2751
[3] ORCID: 0000-0002-4785-0315
[4] ORCID: 0000-0003-1354-2047
Department of Clinical Physiology of Connective Tissue
Bogomoletz Institute of Physiology, National Academy of Sciences of Ukraine, Kiev, Ukraine

Key words: *L*-tryptophan, white adipose tissue, induced visceral obesity rat model.

Abstract

Visceral obesity (VO) is a widespread issue that contributes to the development of various diseases. Consequently, there is a need to identify effective methods for preventing VO. One of the ways to prevent VO can be the use of the amino acid tryptophan. This study aimed to investigate the impact of *L*-tryptophan on morpho-functional changes in white adipose tissue (WAT) in rats with VO and assess its potential for disease prevention. Male Wistar rats were involved in the study. Control animals (Group I) followed a standard diet, while Group II rats were fed a high-fat and high-carbohydrate diet for 12 weeks. Group III animals received a high-calorie diet supplemented with *L*-tryptophan (80 mg/kg). Blood and tissue samples of WAT were collected for standardized biochemical, histological, and biophysical evaluations. The inclusion of *L*-tryptophan in the high-calorie diet led to inhibition of visceral fat accumulation and reduced levels of total lipids, cholesterol, and triglycerides in the blood serum. Rats in Group III exhibited smaller adipocyte sizes, larger cell nucleus areas, and a decreased relative area of connective tissue in the WAT compared to Group II animals. Tryptophan also mitigated disturbances in WAT bioimpedance among obese rats. These findings suggest that *L*-tryptophan can attenuate the manifestations of VO and reduce fat accumulation in WAT, thus holding promise for disease prevention.

Address: Roman Yanko, Bogomoletz Institute of Physiology, Bogomoletz street 4, Kiev, Ukraine, 01024, e-mail address: biolag@ukr.net

* The work was performed as part of the state assignment “Bogomoletz Institute of Physiology of NAS of Ukraine” (No. 0119U103965).

Introduction

Visceral obesity (VO) is a chronic, multifactorial disease characterized by the excessive accumulation of abdominal fat, primarily triglycerides (KONG et al. 2022). The primary cause of VO is overeating and the consumption of high-calorie foods. This condition contributes to various metabolic disorders, hormonal imbalances, increased insulin resistance, hypertension, type II diabetes, cardiovascular diseases, and cancer (APARECIDA et al. 2020, YANKO and LEVASHOV 2022). These complications significantly impact quality of life, leading to long-term disability and premature death (TCHERNOF and DESPRES 2013). The increasing prevalence of VO, along with its asymptomatic nature, limited understanding of its pathogenesis, and insufficient knowledge about prevention and treatment strategies, emphasizes the importance of addressing this medical and social concern (SHUSTER et al. 2012). The etiology of obesity involves complex physiological and biochemical mechanisms that are not yet fully understood. Consequently, there is a growing need to study the pathogenesis of VO and develop effective prevention and treatment methods. One potential approach for preventing VO is the use of the amino acid tryptophan.

Tryptophan is known to participate in the regulation of energy metabolism, food consumption, and has a direct impact on adipose tissue (LISCHKA et al. 2022). Administration of tryptophan to rats on a high-calorie diet has been shown to normalize body weight by reducing visceral fat (SHIPELIN et al. 2021). Furthermore, studies have indicated that tryptophan levels decrease in the blood with obesity (BRANDACHER et al. 2006). However, despite the available literature, there is a lack of sufficient research on the use of tryptophan for obesity prevention, particularly regarding its direct influence on biochemical indicators of lipid metabolism, histomorphological changes, and biophysical properties of visceral white adipose tissue (WAT) in VO.

Notably, the process of obesity involves not only an increase in the amount of WAT but also changes in its quality. These morpho-functional, biochemical, and biophysical alterations in WAT can significantly impact the efficacy of drugs used for obesity treatment and prevention. However, this aspect of the problem remains poorly investigated.

Moreover, the use of different doses of *L*-tryptophan, variations in the duration of research, differences in experimental models of obesity, and varying ages of animals have resulted in ambiguous results. Therefore, comprehensive studies that evaluate the role of tryptophan and its mechanisms of influence on WAT with existing signs of VO are necessary.

The aim of this study was to examine the effect of *L*-tryptophan on morpho-functional changes in WAT in rats with VO and determine its potential for disease prevention. By elucidating the impact of tryptophan on VO, we can contribute to a better understanding of its therapeutic potential and facilitate the development of effective preventive strategies.

Materials and Methods

Research object and experiment design

The study was conducted on 30 male Wistar rats (10 animals in each group), which were taken into the experiment at the age of 3 months. Rats were divided into 3 groups: I – control; II – rats that received a high-calorie diet (HCD) for 12 weeks; III – animals that were on the HCD and additionally received *L*-tryptophan ("Ajinomoto Eurolysine S.A.S", France) in a dose of 80 mg/kg of body weight. VO in rats was induced by feeding them a high-calorie diet containing fat (45% from the mass of the ration) and easily assimilable carbohydrates (31% from the mass of the ration) for 12 weeks. Each rat received daily: 6 g of specially prepared granulated feed (70% standard compound feed with the addition of 30% pork lard); 6.8 g of pork lard; 3.6 g of white breadcrumbs; 3.6 g of sunflower seeds. The total calorie content of the daily diet was 116 kcal. Experimental animals received feed *ad libitum* under daily monitoring of the completeness of its consumption. A day later, instead of water, experimental rats received a 10% fructose solution (YANKO et al. 2021, YANKO et al. 2022). A rat of the control group consumed 20 g of standard compound feed daily, the calorie content of which was 66 kcal. At the end of the experiment, visceral fat was obtained from the abdominal cavity by a mechanical separation method. The weight of visceral fat was determined by the gravimetric method. The degree of obesity was judged by the weight of isolated visceral fat.

Rats were euthanized the day after the last dose of *L*-tryptophan. The work with rats was carried out in compliance with the recommendations of the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes (Strasbourg, 1986). The study was approved by the biomedical ethics committee of the Bogomoletz Institute of Physiology National Academy of Sciences of Ukraine (protocol No. 5 dated 11/31/19).

Histological studies

For histo-morphological studies WAT samples were randomly selected. The histological preparations were made according to the standard method: fixed in Buen's fluid, dehydrated in alcohols of increasing concentration and dioxane. The obtained samples were embedded in paraffin. Paraffin sections (6 μm thick) were made on a sled microtome. Staining of the obtained sections was carried out with Bemer's hematoxylin and eosin and according to Van Gieson (REHFELD et al. 2017, YANKO et al. 2021). Hematoxylin and eosin staining allows for the visualization of the general morphological structure of WAT, while Van Gieson staining facilitates the identification of connective tissue elements. The micropreparations were photographed on a microscope "Nikon Eclipse E100" (Japan) with the use of a digital camera. Morphometry was performed using the computer program "ImageJ 1.34p".

Several parameters were measured on the WAT micrographs, including the relative area of the parenchyma, connective tissue, and blood vessels. The average diameter and cross-sectional area of adipocytes, as well as the area of the adipocyte nucleus, were determined. The number and density of adipocyte placement per unit area were also assessed. Distribution of adipocytes by size (<50 μm, 50–100 μm, >100 μm) was carried out. The stromal-parenchymal index (the ratio of the relative area of vessels and connective tissue to the area of the parenchyma) and the trophic index (the ratio of the relative area of the vessels to the area of the parenchyma) were determined (COSTA et al. 2011, MILJKOVIC et al. 2022).

Biochemical studies

The concentration of lipids, cholesterol, triglycerides, and high-density lipoproteins in the blood serum of rats was determined by the photometric method using standard sets of reagents ("Filisit-Diagnostika", Ukraine) on a biochemical analyzer ("Sinnowa", China). Standardized protocols were used to determine these indicators in blood serum. Normal values were established as follows: total lipids – 2.50 ±0.15 mmol/L, triglycerides – 94.9 ±3.9 mg/dL, cholesterol – 1.70 ±0.08 mmol/L, and high-density lipoproteins – 1.70 ±0.14 mmol/L.

Biophysical studies

The method of multifrequency bioelectrical impedance analysis (BIA) was used to assess the biophysical properties of WAT (SHCHELYKALINA et al. 2021). The BIA method is increasingly used in experimental and

clinical research as one of the highly informative methods for assessing the viability of biological tissues, their functional and metabolic activity, as well as for tissue histological verification (KHALIL et al. 2014). BIA testing of preparations of freshly removed WAT was carried out *ex tempore* on "LCR – meter Quad Tech 1920" (USA) in the mode of operation of the device with a parallel equivalent circuit. Absolute values of electrical parameters were determined at frequencies of 1000 Hz – 1 MHz. Measurements were made using 2 flat silver electrodes with an area of 25 mm^2. The impedance values obtained at the maximum (10^4 Hz) and minimum (10^6 Hz) polarization frequencies of the object were used for the analysis. The impedance dispersion coefficient was calculated from the obtained results as the ratio of its values measured at low and high frequencies ($D_Z = Z_{10}{}^4/Z_{10}{}^6$).

Data analysis

The obtained data were processed by the methods of variational statistics using the software "Statistica 6.0 for Windows" (StatSoft, USA) and "Excel 2010" (Microsoft, USA). Data are reported as the mean ± SD when normally distributed. Groups were analyzed by one-way analysis of variance followed by Bonferroni *t*-tests with a significance level of 0.05.

Results

Rats that received HCD for 12 weeks (Group II) showed signs of VO development. The absolute and relative weights of visceral fat were higher than those of control animals by 145% and 122%, respectively ($p < 0.05$). In rats from the Group III, which received HCD along with *L*-tryptophan, the absolute and relative weights of visceral fat were 38% and 23% lower ($p < 0.05$), respectively, compared to rats in the Group II (Table 1).

Table 1

Visceral fat weight (Mean ± SD)

Indicators	Absolute weight of visceral fat [g]	Relative weight of visceral fat [%]
Control (Group I)	19.0 ±1.4	0.046 ±0.005
High-calorie diet (Group II)	46.6 ±2.6*	0.102 ±0.010*
High-calorie diet + *L*-tryptophan (Group III)	28.7 ±1.3*#	0.079 ±0.006*#

* Different from Group I at $P < 0.05$, #Different from Group II at $P < 0.05$ (One way ANOVA followed by Bonferroni post hoc test, $N = 10$ rats/group)

Histologically, the WAT in control and experimental rats consisted of parenchymal and stromal components. Adipocytes, representing the parenchymal component, appeared optically empty with a narrow cytoplasmic rim and a flattened nucleus displaced to the cell's edge due to fat accumulation. Adipocytes were closely packed, but significant differences in size and shape were observed between control and obese rats. Control animals had smaller, predominantly round-oval-shaped cells, while obese rats showed hypertrophied adipocytes with irregular shapes (Figure 1). Thus, in the WAT of the animals that received HCD, the adipocytes were larger in size: diameter – by 36% ($p < 0.05$), area – by 50% ($p < 0.05$) compared to the control. Additionally, the number of adipocytes and their density per unit area were 19% ($p < 0.05$) lower in the Group II (Table 2).

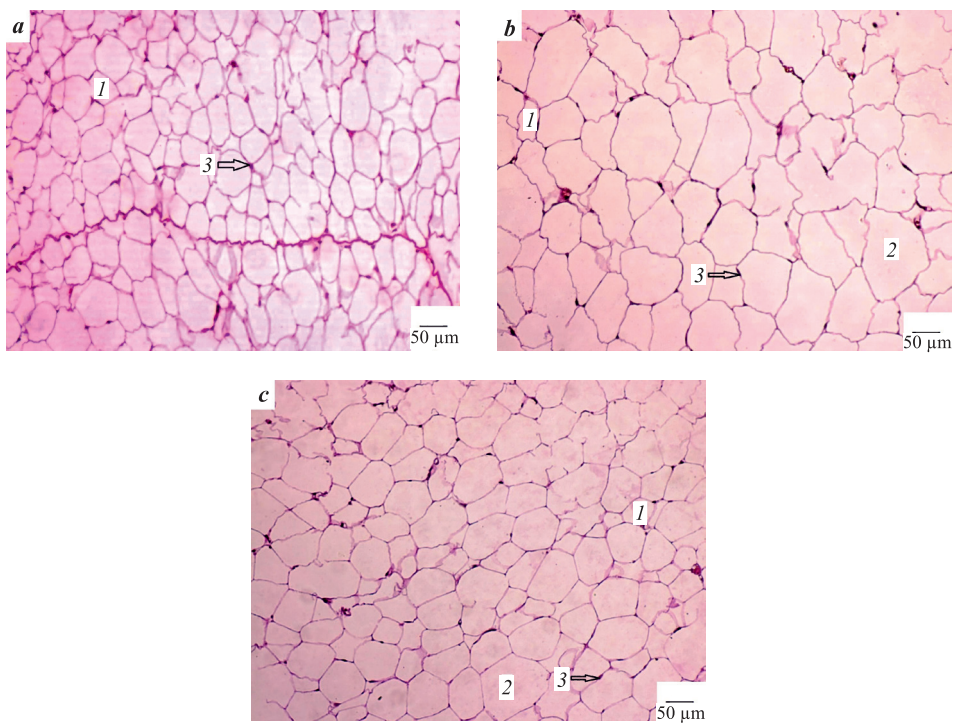

Fig. 1. Micrograph of the visceral white adipose tissue in control rat (*a*), after exposure to a high-calorie diet (*b*) and simultaneous exposure to a high-calorie diet and *L*-tryptophan (*c*). Van Gieson staining, ×200

Note: *1* – adipocyte in diameter <50 µm; *2* – adipocyte in diameter >100 µm; *3* – adipocyte nucleus

Table 2

Morphometry of white adipose tissue (Mean ±SD)

Indicators	Control (Group I)	High-calorie diet (Group II)	High-calorie diet + *L*-tryptophan (Group III)
Mean diameter of adipocyte [μm]	49.7 ±1.3	67.7 ±1.1*	52.2 ±1.8$^{\#}$
Area of adipocyte [μm^2]	2408 ±124	3608 ±164*	2765 ±150*$^{\#}$
Area of adipocyte nucleus [μm^2]	17.5 ±1.1	19.1 ±1.0	25.9 ±1.4*$^{\#}$
Number of adipocytes [pcs] (on an area of 0.35 mm^2)	100.5 ±6.9	81.7 ±12.4*	87.4 ±2.6*
Density of placement of adipocytes [pcs./mm^2]	287 ±19	233 ±35*	250 ±25*
Distribution of adipocytes by diameter [%]			
<50 μm	64.2 ±2.1	46.3 ±1.7*	51.6 ±1.7*
50–100 μm	34.7 ±1.1	45.0 ±1.5*	45.5 ±1.4*
>100 μm	1.1 ±0.4	8.7 ±0.6*	2.9 ±0.6*$^{\#}$
Relative area [%]:			
– parenchyma	91.3 ±1.4	90.7 ±1.8	91.8 ±0.7
– connective tissue	4.4 ±0.8	5.6 ±0.9*	4.5 ±0.3$^{\#}$
– vessels	4.3 ±0.5	3.7 ±0.3*	3.7 ±0.7*
Stromal-parenchymal index	0.095 ±0.017	0.10 ±0.01	0.090 ±0.009
Trophic index	0.047 ±0.007	0.041 ±0.007*	0.040 ±0.006*

* Different from Group I at $P < 0.05$, $^{\#}$Different from Group II at $P < 0.05$ (One way ANOVA followed by Bonferroni post hoc test, $N = 10$ rats/group)

Rats in the Group II had a lower number of adipocytes with a diameter of <50 μm (by 28%, $p < 0.05$), a higher number of cells with a size of 50–100 μm (by 30%, $p < 0.05$), and a number of adipocytes with a diameter of >100 μm (by 690%, $p < 0.05$) compared to controls (Table 2).

The stromal component of WAT, including microcirculatory vessels, lymphatic capillaries, nerve fibers, and connective tissue fibers, was present in both control and obese rats. However, the relative area of connective tissue was 27% higher ($p < 0.05$), and the relative area of vessels and the trophic index were 14% and 13% lower ($p < 0.05$), respectively, in the WAT of obese rats from the Group II (Table 2). These changes in the stromal component of WAT in obese rats indicate the deterioration blood supply to adipocytes.

In WAT of rats that received HCD and *L*-tryptophan (Group III) the area of adipocytes, the number of adipocytes with diameter >100 μm and the relative area of connective tissue were 23%, 97% and 20% ($p < 0.05$)

smaller, respectively, and the nuclear area was 36% ($p < 0.05$) larger than in rats of Group II (Table 2).

In the blood serum of rats in the Group II, the concentrations of lipids, triglycerides, and cholesterol were higher ($p < 0.05$) by 54%, 72%, and 29%, respectively, compared to control animals. Conversely, the concentration of high-density lipoproteins was 54% lower ($p < 0.05$) in the II group. Rats in the Group III showed smaller concentrations of triglycerides, cholesterol, and lipids (by 40%, 21%, and 43%, respectively, $p < 0.05$) in their blood serum compared to the Group II. Moreover, the concentration of high-density lipoproteins was 112% larger ($p < 0.05$) in the Group III compared to rats that received only HCD (Figure 2).

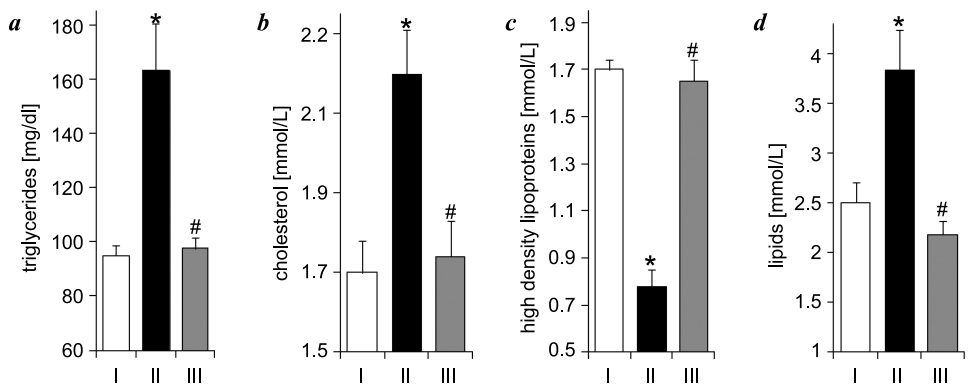

Fig. 2. Effect of *L*-tryptophan on lipid metabolism in blood serum: *L*-tryptophan reduced the concentration triglycerides (*a*); cholesterol (*b*); and total lipid (*d*) in rat's blood serum accumulated due to HCD. *Different from Group I at $P < 0.05$; #different from Group II at $P < 0.05$ (One way ANOVA followed by Bonferroni post hoc test, $N = 10$ rats/group)

Significant changes in BIA indicators were observed in rats with induced VO. The impedance values increased at both low and high frequencies of the testing current. At a frequency of 10^4 Hz, the impedance was 67% higher ($p < 0.05$) than the control, while at a frequency of 10^6 Hz, the increase was 70% ($p < 0.05$). Consequently, rats with VO exhibited a decrease in the impedance dispersion coefficient of WAT to 1.27 units compared to the control's value of 1.34 units. Rats which received HCD and *L*-tryptophan, showed lower impedance values at frequencies of 10^4 Hz and 10^6 Hz by 26% and 28% ($p < 0.05$), respectively, compared to the Group II (Figure 3).

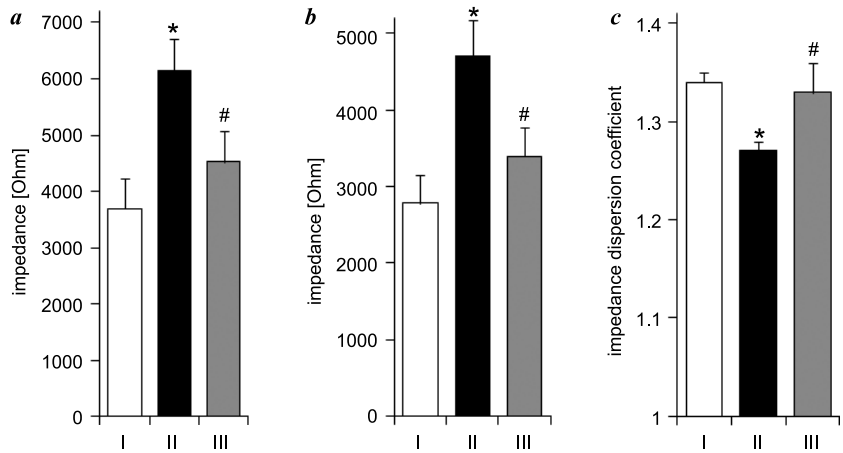

Fig. 3. Effects of *L*-tryptophan on bioimpedancemetry indicators: *L*-tryptophan reduced the impedance on 10^4 Hz (*a*) and 10^6 Hz (*b*) in rat's visceral fat increased due to HCD *Different from Group I at $P < 0.05$; #different from Group II at $P < 0.05$ (One way ANOVA followed by Bonferroni post hoc test, $N = 10$ rats/group)

Discussion

The diagnosis and prevention of VO are critical tasks in modern medicine. Clinicians place special emphasis on detecting and treating the disease in its early stages, where morpho-functional changes in visceral adipose tissue of varying severity are observed. During the initial stages of VO, there are no significant disruptions to the morpho-functional state of WAT that are typically associated with the development of inflammatory processes. Consequently, the effectiveness of treatment measures is highest during this phase. Therefore, it is crucial to identify reliable methods for preventing the progression of VO.

Tryptophan, an essential amino acid, plays a role in numerous metabolic functions. It serves as a unique building block for proteins (RICHARD et al. 2009). Tryptophan is also a precursor to essential endogenous indolamines such as serotonin and melatonin, which function as neurotransmitters, neuromodulators, and neurohormones (TORDJMAN et al. 2017). Increased synthesis of these signaling molecules can enhance overall health and quality of life (POEGGELER et al. 2022). Tryptophan is employed in the treatment of various conditions, including depression, sleep disorders, cognitive impairments, anxiety, and neurodegenerative diseases (KALUZNA-CZAPLINSKA et al. 2019). Conversely, decreased tryptophan secretion has been associated with obesity, anorexia, bulimia nervosa, and other diseases (SHIPELIN et al. 2021).

Our studies confirmed a significant increase in both absolute and relative visceral fat weight in an induced visceral obesity rat model. This increase was primarily due to hypertrophy of WAT adipocytes. Additionally, a decrease in blood saturation of adipose tissue was observed. The concentration of total lipids, triglycerides, and cholesterol increased in the blood serum of these rats.

In rats that simultaneously received HCD and *L*-tryptophan morphological, biochemical, and biophysical changes of WAT were manifested to a much lesser extent, and in some parameters did not differ from the control. The weight gain of visceral fat was less pronounced, and the adipocytes were smaller compared to the experimental group that did not receive *L*-tryptophan. Additionally, the lipid metabolism parameters were similar to the control values. These findings suggest that *L*-tryptophan reduces the manifestations of VO and attenuates fat accumulation in WAT.

The bioelectrical impedance analysis (BIA) method has been shown to be highly informative for assessing body composition and diagnosing the degree of obesity (BRUNANI et al. 2021). Our studies using the BIA method revealed significant changes in the bioelectrical properties of WAT in experimental rats that developed VO due to the HCD. These changes were attributed to the histologically confirmed hypertrophy of adipocytes and the significant accumulation of free lipids within them. Lipids are known to have low hydration levels and high electrical resistance. Furthermore, an increase in connective tissue elements and a decrease in blood supply to adipose tissue further contributed to these effects.

The increase in electrical impedance at both low and high frequencies of the test current, along with a decrease in the coefficient of frequency dispersion of impedance, indicates a decline in the polarization processes, functional activity, and metabolic activity of adipose tissue. However, it's important to note that changes in bioelectrical parameters can be influenced not only by increased fat content in adipocytes but also by alterations in its physical and chemical properties. Previous research by other authors has indicated a connection between BIA indicators and the properties and structure of adipose tissue (YUKEN et al. 2004).

In rats that received *L*-tryptophan, the degree of changes in impedance and the coefficient of frequency dispersion were significantly lower, suggesting a reduction in the manifestation of VO. This indicates that tryptophan has protective properties and contributes to the preservation of normal bioelectrical properties of WAT in the case of VO.

The lipolytic effect of tryptophan has also been observed by other researchers. For example, in rats on an HCD, the concentration of triglycerides in the blood serum increased. However, when the rats received

tryptophan (at a dose of 250 mg/kg), the triglyceride levels approached the control values (SHIPELIN et al. 2021). In experiments on mice with alimentary and genetic obesity, orally administered *L*-tryptophan at a dose of 1 mg/ml of water led to a decrease in body and adipose tissue weight, and serum cholesterol levels. These changes were accompanied by a reduction in inflammation markers (SIVAPRAKASAM et al. 2021). Similarly, in piglets receiving tryptophan with drinking water at concentrations of 0.4% and 0.8%, researchers observed a decrease in food intake, triglyceride levels, hepatic lipogenesis, gluconeogenesis, and an increase in glycolysis and lipolysis intensity (GOODARZI et al. 2021).

The positive effects of tryptophan on the state of WAT in VO can be attributed to the influence of its metabolites, such as serotonin and melatonin (MANGGE et al. 2014, NAMKUNG et al. 2015). Increased levels of serotonin in the central nervous system have been shown to contribute to a reduction in food consumption and body weight, as well as an increase in energy expenditure through the activation of brown adipose tissue by the sympathetic nervous system. Serotonin also regulates the metabolism of carbohydrates and fats and helps alleviate stress, which further affects calorie consumption (BLUNDELL and LAWTON 1995, BUWALDA et al. 2001). The researchers investigated the association between increased visceral fat weight and elevated tryptophan catabolism through the kynurenine pathway, resulting in reduced serotonin production in individuals with severe obesity. Increased levels of kynurenine, as a consequence, may contribute to metabolic disorders in obesity (GELPI et al. 2022, LISCKA et al. 2022). Furthermore, melatonin exerts significant effects on WAT by promoting the formation of beige adipocytes, enhancing mitochondrial function, and reducing oxidative stress (JIMENÉZ-ARANDA et al. 2014).

Conclusions

Our study using an induced visceral obesity rat model revealed that obesity development is accompanied by an increase in the weight of visceral adipose tissue and significant morphological, biochemical, and biophysical changes.

The administration of *L*-tryptophan at a dose of 80 mg/kg, in conjunction with a high-calorie diet, mitigated the accumulation of visceral fat and improved lipid metabolism by reducing the elevated levels of total lipids, cholesterol, and triglycerides in the blood serum.

Furthermore, *L*-tryptophan attenuated the severity of morpho-functional and bioimpedance changes in white adipose tissue caused by obesity.

These findings have both theoretical and practical implications, particularly in the clinical application of tryptophan for comprehensive prevention of visceral obesity development. Future research should focus on elucidating the mechanisms by which tryptophan and its metabolites contribute to the correction of visceral obesity.

Translated by Roman Yanko and Mikhail Levashov

Accepted for print 28.07.2023

References

Aparecida S.E., Vaseghi G., De Carvalho S., Kliemann N., Masoudkabir F., Noll M., Mohammadifard N., Sarrafzadegan N., De Oliveira C. 2020. *Visceral obesity and its shared role in cancer and cardiovascular disease: a scoping review of the pathophysiology and pharmacological treatments*. Int. J. Mol. Sci., 21(23): 9042, doi: 10.3390/ijms21239042.

Blundell J.E., Lawton C.L. 1995. *Serotonin and dietary fat intake: effects of dexfenfluramine*. Metabolism, 44: 33–37.

Brandacher G., Winkler C., Aigner F., Schwelberger H., Schrotcksnadel K., Margreiter R., Fuchs D., Weiss H.G. 2006. *Bariatric surgery cannot prevent tryptophan depletion due to chronic immune activation in morbidly obese patients*. Obes Surg., 16(5): 541–548, doi: 10.1381/096089206776945066.

Brunani A., Perna S., Soranna D., Rondanelli M., Zambon A., Bertoli S., Vinci C., Capodaglio P., Lukaski H., Cancello R. 2021. *Body composition assessment using bioelectrical impedance analysis (BIA) in a wide cohort of patients affected with mild to severe obesity*. Clinical Nutrition, 40(6): 3973–3981, doi: 10.1016/j.clnu.2021.04.033.

Buwalda B., Blom W.A., Koolhaas J.M., van Dijk G. 2001. *Behavioral and physiological responses to stress are affected by high-fat feeding in male rats*. Physiol. Behav., 73: 371–377.

Costa S.S., Blotta R.M., Meurer L., Edelweiss M.I. 2011. *Adipocyte morphometric evaluation and angiogenesis in the omentum transposed to the breast: a preliminary study*. Clinics (Sao Paulo), 66(2): 307–312, doi: 10.1590/s1807-59322011000200021.

Gelpi M., Vestand B., Raju S.C., Hansen S., Hogh G., Mildttun O., Ueland P.M., Ueland T., Benfield T., Kofoed K.F., How J.R., Troseid M., Nielsen S.D. 2022. *Association of the kynurenine pathway of tryptophan metabolism with human immunodeficiency virus-related gut microbiota alterations and visceral adipose tissue accumulation*. The Journal of Infectious Diseases, 225(11): 1948–1954, doi: 10.1093/infdis/jiac018.

Goodarzi P., Habibi M., Roberts K., Sutton J., Shili C.N., Lin D., Pezeshki A. 2021. *Dietary tryptophan supplementation alters fat and glucose metabolism in a low-birthweight piglet model*. Nutrients, 13(8): 2561, doi: 10.3390/nu13082561.

Jimenez-Aranda A., Fernandez-Vazquez G., Mohammad-Serrano M., Reiter R., Agil A. 2014. *Melatonin improves mitochondrial function in inguinal white adipose tissue of Zücker diabetic fatty rats*. J. Pineal Res.,57: 103–109, doi: 10.1111/jpi.12147.

Kaluzna-Czaplinska J., Gatarek P., Chirumbolo S., Chartrand M.S., Bjorklund G. 2019. *How important is tryptophan in human health?* Crit Rev Food Sci Nutr., 59(1): 72–88, doi: 10.1080/10408398.2017.1357534.

Khalil S.F., Mohktar M.S., Ibrahim F. 2014. *The theory and fundamentals of bioimpedance analysis in clinical status monitoring and diagnosis of diseases*. Sensors (Basel), 14(6): 10895–10928, doi: 10.3390/s140610895.

Kong M., Xu M., Zhou Y., Geng N., Lin N., Song W., Li S., Piao Y., Han Z., Guo R., Yang C., Luo N., Wang Z., Ma L., Xu Q., Wang L., Qiu W., Li J., Shi D., Cheung E.C., Li R., Chen Y., Duan Z. 2022. *Assessing visceral obesity and abdominal adipose tissue distribution in healthy*

populations based on computed tomography: a large multicenter cross-sectional study. Front. Nutr., 9: 871697, doi: 10.3389/fnut.2022.871697.

Lischka J., Schanzer A., Baumgartner M., De Gier C., Greber-Platzer S., Zeyda M. 2022. *Tryptophan metabolism is associated with BMI and adipose tissue mass and linked to metabolic disease in pediatric obesity*. Nutrients, 14(2): 286, doi: 10.3390/nu14020286.

Mangge H., Summers K.L., Meinitzer A., Zelzer S., Almer G., Prassl R., Schnedl W.J., Reininghaus E., Paulmichl K., Weghuber D., Fuchs D. 2014. *Obesity-related dysregulation of the tryptophan-kynurenine metabolism: role of age and parameters of the metabolic syndrome*. Obesity (Silver Spring), 22(1): 195–201, doi: 10.1002/oby.20491.

Miljkovic D., Drljaca J., Lovrenski A., Gajic M. 2022. *A comprehensive morphometric study of visceral and subcutaneous adipose tissue depots in mice, hamsters and rats*. Int. J. Morphol., 40(5): 1219–1227.

Namkung J., Kim H., Park S. 2015. *Peripheral serotonin: a new player in systemic energy homeostasis*. Mol. Cells., 38(12): 1023–1028.

Poeggeler B., Singh S.K., Pappolia M.A. 2022. *Tryptophan in nutrition and health*. Int. J. Mol. Sci., 23(10): 5455, doi: 10.3390/ijms23105455.

Rehfeld A., Nylander M., Karnov K. 2017. *Histological methods. in: compendium of histology*. Springer, Cham, doi: 10.1007/978-3-319-41873-5_2.

Richard D.M., Dawes M.A., Mathias C.W., Acheson A., Hill-Kapturczak N., Dougherty D.M. 2009. *L-tryptophan: basic metabolic functions, behavioral research and therapeutic indications*. Int. J. Tryptophan Res., 23(2): 45–60, doi: 10.4137/ijtr.s2129.

Shchelykalina S.P., Nikolaev D.V., Kolesnikov V.A., Korostylev K.A., Starunova O.A. 2021. *Technology of two-dimensional bioimpedance analysis of the human body composition*. Journal of Electrical Bioimpedance, 12(1): 17–25, doi: 10.2478/joeb-2021-0004.

Shipelin V.A., Trusov N.V., Apryatin S.A., Shumakova A.A., Balakina A.S., Riger N.A., Gmoshinski I.V., Nikityuk D.B. 2021. *Effects of tyrosine and tryptophan in rats with diet-induced obesity*. Int. J. Mol. Sci., 22: 24–29, doi: 10.3390/ijms22052429.

Shuster A., Patlas M., Pinthus J.H., Mourtzakis M. 2012. *The clinical importance of visceral adiposity: a critical review of methods for visceral adipose tissue analysis*. Br. J. Radiol., 85(1009): 1–10, doi: 10.1259/bjr/38447238.

Sivaprakasam S., Ramachandran S., Sikder M., Bhutia Y.D., Wachtel M.W., Ganapathy V. 2021. *α-Methyl-l-tryptophan as a weight-loss agent in multiple models of obesity in mice*. Biochem. J., 478(7): 1347–1358, doi: 10.1042/BCJ20210100.

Tchernof A., Despres J.P. 2013. *Pathophysiology of human visceral obesity: an update*. Physiol. Rev., 93: 359–404, doi: 10.1152/physrev.00033.2011.

Tordjman S., Chokron S., Delorme R., Charrier A., Bellissant E., Jaafari N., Fougeron C. 2017. *Melatonin: pharmacology, functions and therapeutic benefits*. Curr. Neuropharmacol., 15(3): 434–43, doi: 10.2174/1570159X14666161228122115.

Yanko R., Chaka E., Levashov M. 2021. *Effect of methionine on the lungs respiratory part in rats of different ages*. Pol. J. Natural. Sc., 36(4): 447–456.

Yanko R.V., Chaka E.G., Zinchenko A.S., Safonov S.L., Levashov M.I. 2021. *Features of modeling fatty liver disease in rats of different ages based on a high-calorie diet*. Obesity and metabolism., 18(4): 387–397, doi: 10.14341/omet12789.

Yanko R., Levashov M. 2022. *Histomorphological features of the thyroid gland in rats of different ages with alimentary-induced obesity*. Enviromental and Experimental Biology, 20(3): 171–177, doi: 10.22364/eeb.20.16.

Yanko R., Zinchenko A., Chaka O., Levashov M. *The method of modeling alimentary fatty hepatosis in laboratory rats*. Utility model patent No. 150511. 02/23/2022.

Yuken O., Tadaoki M., Yohsuke K., Tadamitsu I., Mieko T., Mari H. 2004. *Basic study of new diagnostic modality according to noninvasive measurement of the electrical conductivity of tissues*. J. Med. Invest., 51(3–4): 218–225, doi: 10.2152/jmi.51.218.