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Original Research

Domestic cat – the unspoken threat to wildlife in the sub-urban location of Maribor

Larisa Bedrač¹, Nina Šajna^{1*}

Abstract

The monitoring of domestic cats' prey brought home was conducted between the 13th of March and the 1st of May 2023 in two residential areas near Maribor, Slovenia. This study aimed to provide information about the amount, diversity, and frequency of prey that the cats captured to identify the predatory impact of domestic cats in peri-urban areas. We present the results of the prey return, whether the prey was brought alive or not, and whether it was eaten or not. We further provide information about the predation frequency of domestic cats concerning circadian activity according to weather conditions and air temperature. The frequencies of various taxonomic groups of prey captured were described in relation to land use in a location and species' biology. Our results include a total of 50 records of 12 species (3 species of birds and 9 of mammals). Domestic cats were more active during the mornings and on days without rain, and the frequency of bringing home prey increased with higher temperatures.

Keywords

domestic cats, cat predation, wildlife, indoor-outdoor cat, survey

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Domača mačka – tiha grožnja prostoživečim vrstam živali v primestni lokaciji Maribora

Izvleček

Med 13. marcem in 1. majem 2023 smo v dveh stanovanjskih naseljih v bližini Maribora v Sloveniji izvajali popis plena domačih mačk, prinesenega domov. Namen te študije je bil pridobiti informacije o količini, raznolikosti in pogostosti plena, ki so ga mačke uplenile, ter s tem ovrednotiti plenilski vpliv domačih mačk v primestnih območjih. Predstavljamo rezultate o prinesenem plenu ter ali je bil plen prinesen lastnikom živ ali ne in ali je bil konzumiran ali ne. Nadalje podajamo informacije o plenilskih aktivnostih domačih mačk čez dan, glede na vremenske razmere in temperaturo zraka. Opisane so bile pogostnosti različnih taksonomskih skupin ulovljenega plena glede na rabo tal na lokaciji in biologijo vrste. Naši rezultati kažejo, da je bilo uplenjenih 50 živali, ki so pripadale 12-im vrstam (3 ptice in 9 sesalcev). Domače mačke so bile bolj aktivne zjutraj, v dneh brez dežja, pogostost prinašanja plena pa se je z višjimi temperaturami povečala.

Ključne besede

domača mačka, plenilska aktivnost mačk, prostoživeče živali, notranje-zunanja mačka

Introduction

Domestic cats (*Felis catus* Linnaeus, 1758) are the most widely embraced pets across the globe. In the United States, one-third of households include feline companions, and over 600 million cats share their world with humanity (Driscoll et al., 2009). As they are beloved and widely cherished animal companions, bringing substantial joy to their human caretakers, we tend to overlook their primal nature as carnivorous mesopredators and the threat they pose to wildlife. Even though owned cats are fed, their hunting instinct is not lost, and they often kill wild animals. What is more, because cat owners provide care for domestic cats, their populations are not regulated by natural selection (e.g. diseases, parasites, food, shelter). Even though a domestic cat is a species that has been present in Central Europe since the expansion of the Roman empire (Krajcarz et al., 2022), its population has never in our history been higher (Lepczyk et al., 2010). Domestic cats in our society assume unique and strange roles. On the one hand, we regard them as domesticated pet animals; however, they are mostly capable of surviving in the wild on their own. This is why many owners allow their cats to fully or partially freely live outside of their home. It is often regarded as proper care and an ideal lifestyle for these pets if they have the opportunity to roam outdoors freely. However, as

commented by Lepczyk et al. (2010), these norms do not apply to any other domestic animal species, such as dogs, ferrets, livestock, or others.

Owned domestic cats that are granted outdoor access represent an anthropogenic threat to smaller wild animals from different taxa of vertebrates (Blancher, 2013; Loss et al., 2013; Loss & Marra, 2017) and invertebrates (Medina & García, 2007; Eisenhauer, 2018; Li et al., 2021). Loss et al. (2013) estimated in their study that 1.3–4.0 billion birds and 6.3–22.3 billion mammals are annually lost due to predation by free-roaming domestic cats in the United States alone. Li et al. (2021) estimated that the predation by all free-roaming cats in China is at least 2.69–5.52 billion birds and 3.61–9.80 billion mammals, but also 1.61–3.58 billion fishes, 1.13–3.82 billion amphibians, 1.48–4.31 billion reptiles, and 1.61–4.95 billion invertebrates annually. In areas where free-roaming domestic cats are an introduced species and the local native prey is naïve, the predation rates can have a more devastating impact, even causing extinctions (Medina & García, 2007; Nogales & Medina, 2009). Unrestrained cats living on islands have played a role in causing or contributing to 33 (14%) of the recent extinctions of birds, mammals, and reptiles documented on the International Union for Conservation of Nature (IUCN) Red List (Medina et al., 2011). Besides predation, free-roaming domestic cats impact biodiversity by causing disturbances and indirect

fear effects (Loss & Marra, 2017), representing a competing species for wild mesopredators, acting as a vector for diseases and parasites (Loss & Marra, 2017), and hybridization with wildcats *Felis silvestris* (Todesco et al., 2016).

While meaningful controls are being pursued for feral and stray cat populations in various countries, hardly any strategies were designed for free-roaming domestic cats (Trouwborst et al., 2020). As a response, we can observe the rise of animal welfare organizations and conservation scientists in recent years, raising appeals to owners and governments to implement changes to prevent the biodiversity impacts of free-roaming domestic cats. The most desired policy seems to be restricting the outdoor access of owned cats (Trouwborst et al., 2020). In countries where domestic cats are listed as an invasive alien species (IAS), many national and international laws on IAS apply to owned domestic cats. In the European Union, some international legal obligations indirectly address the threats of free-roaming domestic cats to wildlife (e.g. The EU Birds and Habitats Directive). However, in the Slovenian legislature - Animal protection law (Zakon o zaščiti živali, 1999), there are restrictions on having owned animals in public, only for dogs – use of leashes in public spaces, and owned cats are addressed only within welfare factors (feeding, adequate shelter, health care, freedom of movement, socialization).

Given the considerable success of domestic cats as predators, characterized by significant variations in catch rates across different locations and a discernible reliance on environmental and landscape factors, there is an imperative need for more comprehensive and refined local assessments. This necessity is particularly underscored by the ongoing initiatives for state-level biodiversity protection. It is crucial to avoid formulating legislation based on broad generalizations and instead prioritize the integration of specific, localized data. By conducting meticulous and site-specific evaluations, we can ensure that regulatory measures are grounded in a nuanced understanding of the intricate interactions between domestic cats and local ecosystems, thus fostering more effective and targeted conservation efforts.

As such evaluation has not been conducted in our area, this study sought to offer insights into the quantity, variety, and occurrence frequency of prey captured by cats to discern the predatory influence of domestic cats in peri-urban regions. Because this is a very crowd-splitting theme, we wanted to put forth for owners who allow their

cats to roam outside an alternative to keeping them inside for the sake of wildlife conservation. Therefore, our prey return survey also included the need for abiotic factors to be noted, such as the temperature, the time of the day the prey was brought, and what type of weather was on that day. With this, we wanted to find the appropriate time of the day when owners could let their cats roam free with the least negative impact on wildlife. With spatial analysis, we also wanted to identify land uses and connect them to the preferred habitat of prey species. This would enable us to predict what prey is present in each land use, which could be a foundation for establishing outdoor cat-free zones if vulnerable species are present in that area.

Materials and Methods

The research participants were selected based on location and the knowledge that individual households have cats that were allowed to go out freely. Cat owners were requested to meticulously document the prey items that each of their cats brought home.

The monitoring took place in the spring of 2023 for seven weeks, starting on the 13th of March. This timeframe was chosen because that is when the reproductive period begins for most vertebrates and marks the return of migratory species to the observed area.

The household in location 1 is located outside the housing community on the border between cultivated areas and natural forest – showing more rural habitats (Fig.1a). The household in the 2nd location is on the outer edge of a compacted community bordering on arable land, characterizing this as more urban habitats (Fig.1b). Both locations have surface watercourses nearby. In location 2, where two cats resided in the same household, it was not always feasible to attribute the items brought home to a particular cat. For that reason, we assumed that the frequency of cat predation is similar; therefore, the number of prey brought home was divided between both cats.

We compiled a survey form in such a way that the cat owners could document the days at which the prey was brought home. Prey items were recorded, a photograph was taken, and we identified preyed animals to the highest taxonomic level possible allowed by the condition of the prey remains. We used the Key for determining the vertebrates of Slovenia (Janžekovič et al., 1999) for prey identification. Cat owners recorded information about the

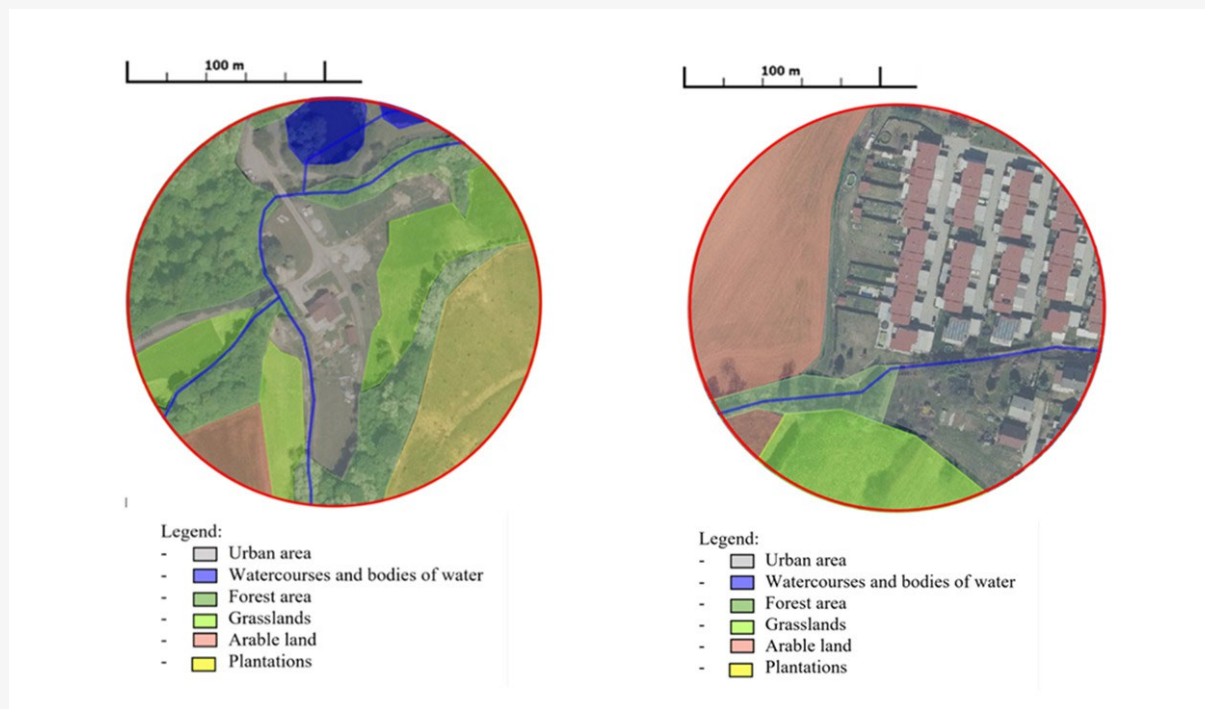


Figure 1. a) land use at location 1 – rural area; b) land use at location 2 – urban area.

Slika 1. a) Raba tal na lokaciji 1 – ruralno okolje; b) raba tal na lokaciji 2 – urbano okolje.

prey – whether the animals brought by the cats were alive or dead, and for the latter, if there was any consumption (marked as complete or partial or not consumed). Additionally, cat owners recorded the weather conditions according to four categories proposed: 1 - clear weather, 2 - partially cloudy, 3 - cloudy, and 4 – rainy; and the time of the predation according to three categories: 1 - morning, 2 - midday and 3 - evening. For more details on the weather, we obtained average daily air temperature data available freely at the government online archives of the Environmental Agency of the Republic of Slovenia (ARSO, 2023). This would enable us to examine if the probability of the prey being brought back is dependent on the temperature.

We also studied the habitats in which cats move. We summarized the results for area size from Pirie et al., 2022 where their results showed that the outdoor cats have a mean home range of 3.42 ha. We utilized the obtained result to visually depict the spatial distribution of areas of locations 1 and 2 and assessed land use in each location. We classified different land use types: urban areas, watercourses and bodies of water, forest areas, grasslands,

arable land, and orchards. From the base map, we were able to calculate percentages for each category and compare them between the locations.

Statistical analysis

We employed various methods to assess biodiversity and investigate correlations between environmental variables and prey return frequencies. For alpha diversity, we calculated Shannon and Simpson diversity indexes using an online calculator, Omniculator (<https://www.omniculator.com/>). This analysis provided insights into the within-sample diversity of our study area. To explore the potential correlation between the average temperature over a 5-day period and prey return frequency, we conducted Pearson's correlation analysis using the software PAST (Version 4.14, 64-bit Windows) (Hammer et al., 2001).

Furthermore, we employed chi-square tests to analyze variations in prey return frequencies across different parts of the day and under different weather conditions. For this analysis, we utilized an online calculator, GraphPad (<https://www.graphpad.com/quickcalcs/chisquared1.Chi-square/>).

Results

Prey diversity

The results of the survey give us information on the frequency and consumption patterns of prey return in two locations, each with different environmental characteristics, with location 1 having more rural habitats and location 2 having more urban habitats. A total of 50 prey items were recorded, with 24 in the household on location 1 and 26 in the household on location 2. Notably, location 2 had two cats, while location 1 had a single cat. Overall, 12 species were identified, 9 in rural and 9 in urban (Table 1).

The species distribution varied between the locations, with notable differences in the preyed-upon species returned to the households (Table 1). In rural locations, the yellow-necked mouse (*Apodemus flavicollis* Malchior, 1834) and the wood mouse (*Apodemus sylvaticus* Linnaeus, 1758) were prominent, representing 67% of prey return in location 1. In contrast, urban locations saw higher numbers of the wood mouse (*Apodemus sylvaticus*) and the common shrew (*Sorex araneus* Linnaeus, 1758), representing 65% of total prey return to that household. The three most preyed-upon species—*A. sylvaticus*, *A. flavicollis*, and *S. araneus*—comprised 66% of the overall prey return.

Species were categorized taxonomically, revealing that three species belong to the order Passeriformes, one

belongs to the order Eulipotyphla, and eight belong to the order Rodentia (Fig. 2). Thus, we can see that the most represented order of prey is Rodentia, as it has not only the highest species diversity but also the highest frequencies of brought prey, which amounts to 74%.

We compared the α -diversity of prey return for each location and calculated β -diversity between our two sampling locations (Table 2). Only identified species were included in the calculations (Table). The Shannon Diversity Index for location 1 (rural) was calculated to be 1.49, indicating a moderate level of species diversity. The Simpson Diversity Index for the same location was found to be 0.26, suggesting a certain degree of dominance by a few species.

Location 2 (urban) exhibited a higher Shannon Diversity Index at 1.75, reflecting a marginally more even distribution of species. The Simpson Diversity Index for location 2 was 0.20, indicative of a relatively low dominance of specific species.

These indices collectively underscore moderate diversity within both locations, with location 2 showcasing a somewhat more even distribution of prey species returned.

The Sørensen-Dice Index is 0.375 and signifies a moderate degree of similarity in species composition between the two samples (Table 2). Approximately 33% of species were found to be shared between the samples, while 25% were unique for rural and 42 for urban samples.

Table 1. Frequency of prey return species by location and total number of prey.

Tabela 1. Število osebkov prinesenih vrst plena glede na lokacijo in skupno število plena

Prey species	Rural (1 cat)	Urban (2 cats)	Rural + urban
<i>Apodemus sylvaticus</i>	6	9	15
<i>Apodemus flavicollis</i>	10	0	10
<i>Mus musculus</i>	0	2	2
<i>Rattus norvegicus</i>	0	1	1
<i>Arvicola amphibius</i>	1	1	2
<i>Microtus arvalis</i>	1	2	3
<i>Microtus agrestis</i>	2	0	2
<i>Microtus subterraneus</i>	1	0	1
Rodentia sp.	1	0	1
<i>Sorex araneus</i>	0	8	8
Sorex sp.	1	0	1
<i>Parus major</i>	1	1	2
<i>Poecile palustris</i>	0	1	1
<i>Phoenicurus ochruros</i>	0	1	1
Total number of prey	24	26	50

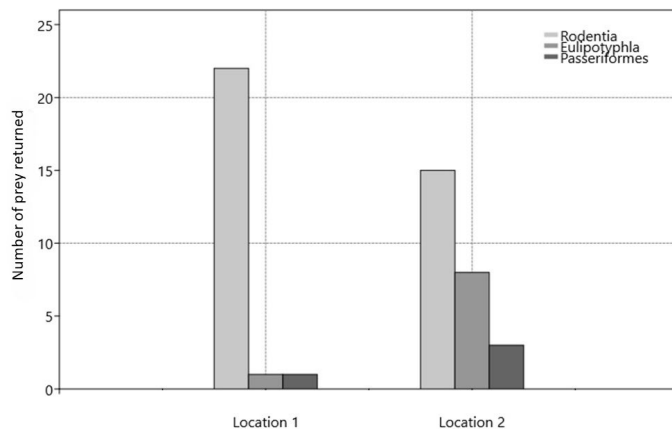


Figure 2. Frequencies of prey return according to taxonomic orders and location (location 1 = rural, location 2 = urban).

Slika 2. Število osebkov prinesenega plena glede na taksonomski red in lokacijo (lokacija 1 = ruralna, lokacija 2 = urbana).

Table 1. Frequency of prey return species by location and total number of prey.

Tabela 1. Število osebkov prinesenih vrst plena glede na lokacijo in skupno število plena

Location	Evenness	Shannon's Index	Simpson's Index	Sørensen-Dice Index
1	0.767	1.49	0.26	0.375
2	0.797	1.75	0.20	

Prey consumption

Owners were asked to note prey consumption, revealing that in rural locations, three prey items brought home were consumed, two were partially consumed, and 19 were not consumed. In urban locations, three were consumed, five were partially consumed, and 18 were not consumed. Therefore, leading to an overall consumption of 12%, partial consumption of 14%, and no consumption of 74%. Notably, in rural locations, all 24 prey items were brought home dead, while in urban locations, four were brought alive.

Temperature and predation

The results of the correlation analysis between the average temperature over five days in 10 intervals and the frequency of brought prey (Fig. 3) yielded a statistically significant positive correlation ($r(8) = 0.79$, $p = 0.007$). The p-value associated with the correlation coefficient indicates that the observed correlation is unlikely to have occurred by random chance. This positive correlation suggests a

strong positive linear relationship between the average temperature and prey return frequency. In other words, as the temperature increases, there is a tendency for an increased prey return.

Weather and predation

Out of 49 surveyed days, 17 had clear weather, seven were partially cloudy, 16 were cloudy, and 9 had rainy conditions. The frequency of brought prey was influenced by distinct weather conditions (Fig. 4). The highest frequency of prey return was observed in clear and cloudy weather, and the lowest was in rainy conditions.

On days without precipitation, the total abundance of prey return was 44, indicating that the cats brought in 44 prey items over 40 days without precipitation. Out of 9 days with precipitation, only six prey items were brought home, thus indicating a reduced frequency of brought prey.

Similarly, when we looked at the abundance of the three most commonly preyed upon species, contributing 67% of data (*Apodemus sylvaticus*, *Apodemus flavicollis*,

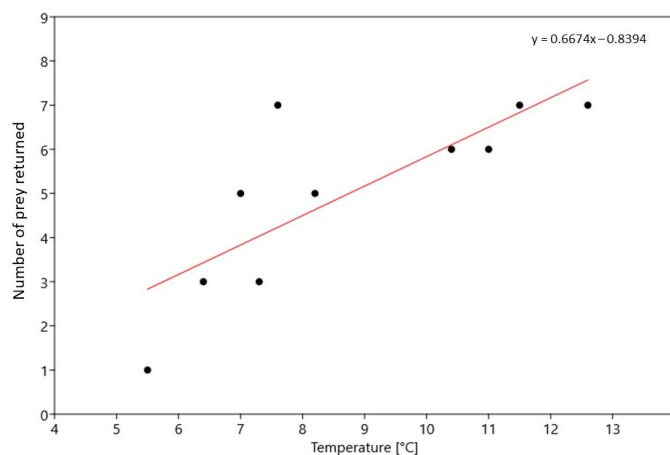


Figure 3. Correlation between 5-day average temperature [°C] and prey return frequency ($r(8) = 0.79$, $p = 0.007$).

Slika 3. Korelacija med 5-dnevnim povprečjem temperature [°C] in pogostost prinesenega plena ($r(8) = 0.79$, $p = 0.007$).

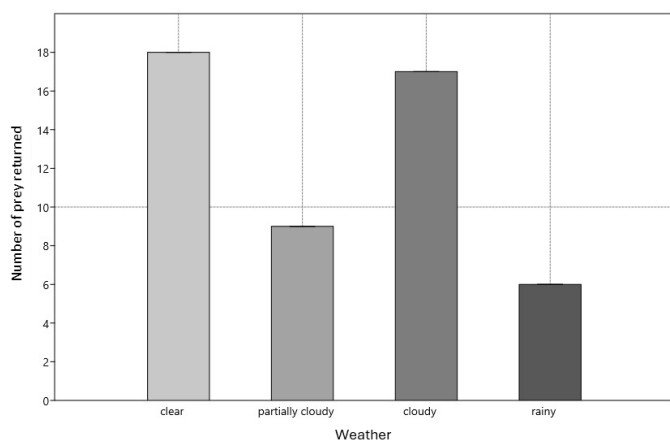


Figure 4. The frequency of prey return depends on the type of weather.

Slika 4. Število prinesenega plena glede na tip vremena.

and *Sorex araneus*), the result showed a similar pattern as the overall abundance (Fig. 5).

The chi-square test for goodness of fit was conducted to determine whether the proportion of prey return was equal between all four types of weather. Observed frequencies for prey abundance were clear weather 18, partially cloudy 9, cloudy 17, and rainy 6. The chi-square value was calculated $\chi^2(3, N = 50) = 8.4$, $p = 0.038$, indicating that the weather type may influence prey return.

Predation by time of the day

A distinct pattern in prey frequencies emerged when examining in which part of the day the prey was brought, showing noteworthy variations in prey return numbers (Fig. 6). During the morning hours, a substantial combined total of 29 brought prey items (location 1 and location 2) was observed, reflecting heightened predator activity and successful hunts during this period. However, as the day progressed into midday, the overall total abundance

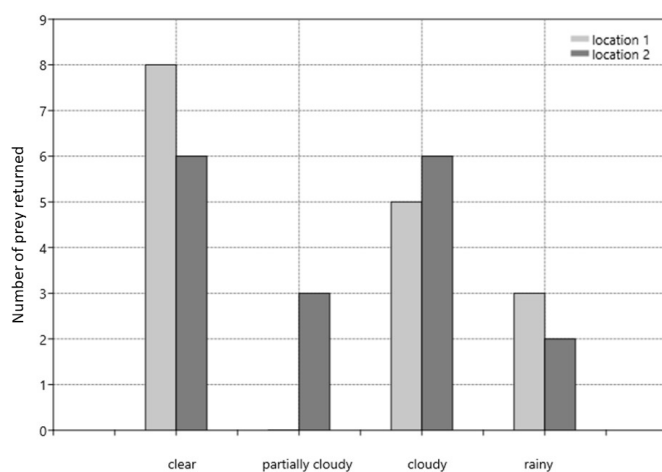


Figure 5. Abundance of 3 most frequently brought prey species: *Apodemus sylvaticus*, *Apodemus flavicollis*, and *Sorex araneus* combined depending on weather type in rural location (location 1) and urban location (location 2).

Slika 5. Abundanca 3 najpogostejše prinesenih vrst: *Apodemus sylvaticus*, *Apodemus flavicollis* in *Sorex araneus* sešteto glede na vrsto vremena na ruralni (lokacija 1) in urbani lokaciji (lokacija 2).

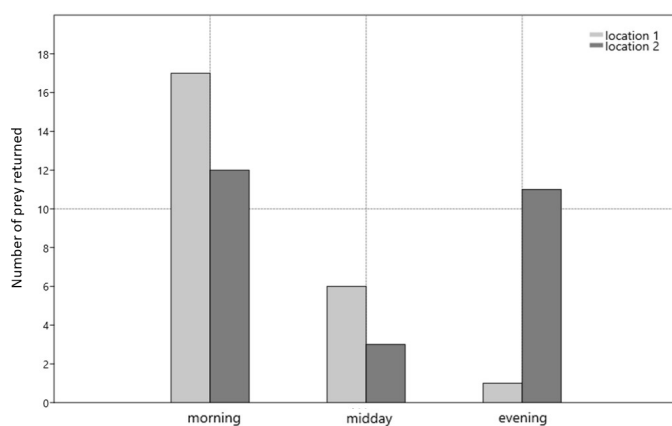


Figure 6. The frequency of prey brought depends on the part of the day the prey was caught in a rural location (location 1) and urban location (location 2).

Slika 6. Pogostost prinesenega plena glede na del dneva, ko je bil plen ulovljen na ruralni (lokacija 1) in urbani lokaciji (lokacija 2).

sharply decreased to 9 prey return items, suggesting potential changes in predator behaviour or prey availability during these hours.

The chi-square test for goodness of fit was applied to examine if the distribution of brought prey frequency across three groups of times of the day is equal. Observed frequencies were as follows: 29 in the morning, nine at midday, and 12 in the evening. The chi-square value was

computed as $\chi^2 (2, N = 50) = 16.7$, $p < 0.001$, indicating uneven prey return throughout the day.

As we looked at the frequency of brought prey in different parts of the day and included the weather factor, a similar pattern appeared (Fig. 7). Prey brought in the morning was the most abundant throughout all weather types, while prey brought in the midday was the least abundant, recorded only in 3 out of 4 weather types.

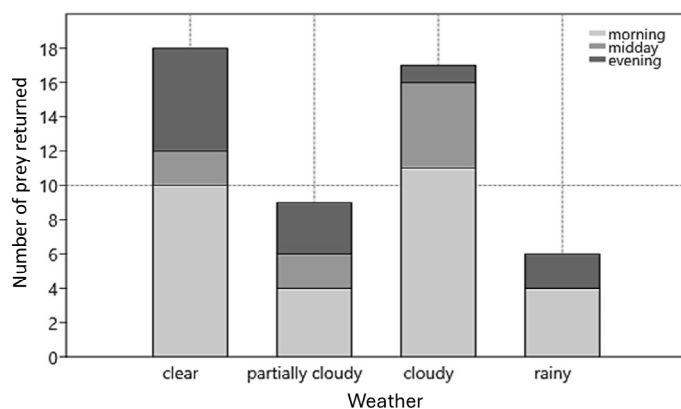


Figure 7. The frequency of prey brought depends on the combination of the part of the day and the weather.

Slika 7. Število osebkov prinesenega plena glede na kombinacijo dela dneva in vremena.

Discussion

Although our study included a small sample of studied cats and considering that only a small portion of captured prey is brought to households (Loyd et al., 2013), our results offer a brief insight into domestic cats' predation effect on wildlife in Slovenia. Our investigation into the predatory habits of domestic cats in peri-urban areas has unveiled nuanced patterns that have significant implications for wildlife conservation and pet ownership practices. The detailed examination of prey diversity, climatic influences, and temporal dynamics contributes to a more comprehensive understanding of the complex interplay between domestic cats and local ecosystems.

One of the earliest research projects that surveyed a large sample of prey return by outdoor cats that were owned was done by Churcher and Lawton (1987). In their study, 70 cats were included. Though a one-year period, 1090 items of prey were noted, with an average of about 14 items per cat. They especially noted the impact of cat predation on house sparrow (*Passer domesticus* Linnaeus, 1758) since their results, based on initial counts of house sparrows in the village at the beginning of the breeding season and the documented number of sparrows caught by cats, showed that it was evident that a minimum of 30% of sparrow fatalities in the village could be attributed to cat predation. They find that domestic cats emerge as

significant predators in their study area. In later years, further researches were conducted including more factors that could affect cat predation such as if the cats were equipped with collar bells and if they were kept indoors at night (Woods et al., 2003), estimating cats' hunting area and comparing different rates of predation by where the cat's home is located – in inner suburbs or the edge of suburban area (Pirie et al., 2022) and even if the owners' characterization of their cats' personalities could correlate to number of returned prey (Cecchetti et al., 2022).

Observing the variations in prey return species composition between rural and urban settings provides a glimpse into the intricate dynamics between domestic cats and their local habitats. In our case, specific species' prevalence in each location reflects the influence of habitat characteristics on feline prey return. For instance, rural areas exhibit a preference for species indigenous to natural or semi-natural landscapes, such as the yellow-necked mouse and wood mouse, while urban environments see the influence of human settlements, evident in the presence of the wood mouse and common shrew. However, we must acknowledge that these species are common and found in various habitats. While yellow-necked mouse prefers forests and closed habitats, wood mouse often uses habitats within their ranges at random, including cropped areas (Tattersall et al., 2001). The common shrew can be found in habitats like grasslands, woodlands, arable land, and hedges (Wang and Grimm, 2007).

The alpha diversity indices, encompassing the Shannon and Simpson Diversity Index, offer a quantitative lens through which to view the distribution of prey return species within each location. The moderately diverse nature of both settings indicates that, despite differences in habitat, both areas support a varied array of prey species. This information is pivotal for devising conservation strategies that are not only effective but also tailored to the inherent biodiversity of each specific locality.

The observed correlation between average temperature and prey abundance introduces a compelling dimension to our understanding of cat predation. The statistically significant positive correlation suggests that temperature acts as a key determinant of predatory activities, providing potential insights into seasonal variations. The interpretation of the results implies that temperature may play a significant role in influencing prey return frequency; however, our study was limited to early spring and under the influence of prey activities. The positive correlation may suggest that higher temperatures are associated with higher prey abundance, possibly due to increased activity, breeding, or availability of food sources for the prey species.

The examination of the frequency of prey return under different weather conditions provides additional depth to our understanding. The higher number of prey that is brought in during clear weather and reduced numbers during precipitation align with established behavioural patterns of domestic cats (Geary et al., 2022). The aversion to hunting in adverse weather conditions, as evidenced by the lower frequency of prey return during rainy periods, reflects the adaptive nature of cat behaviour. In our study, we employed the chi-square test for goodness of fit to investigate potential disparities in prey return frequencies across distinct weather types. With the null hypothesis assuming a random distribution of prey return across weather types, the p-value (0,038) suggests that there is statistical evidence to reject the null hypothesis. Therefore, we conclude that the distribution of prey return is not uniform across the different weather types. The observed frequencies of prey return in each weather category are significantly different from what would be expected if the prey return were randomly distributed. The results indicate that weather types have a significant impact on the distribution of prey return, supporting the notion that cats may exhibit different (hunting) behaviours under varying weather conditions. This information is particularly relevant for owners and policymakers alike, as it suggests that

weather conditions can influence the ecological impact of outdoor cats.

The distinct temporal patterns in predation, with heightened activity during the night until morning hours when the prey was found, present valuable insights into the diurnal dynamics of cat behaviour. The statistical significance of these temporal variations, confirmed by the chi-square test, underscores the importance of considering daily activity patterns when evaluating the impact of domestic cats on local prey populations. Interpreting the results, we rejected the null hypothesis, indicating that the observed distribution of prey abundance across different times of the day is significantly different from what would be expected if prey return were evenly distributed. These findings highlight the influence of temporal factors on prey capture, with mornings being particularly prolific in terms of abundance.

While our study has provided valuable insights into the predatory behaviours of domestic cats in peri-urban areas, it is crucial to acknowledge certain limitations that warrant consideration for future research. The current investigation was conducted with a specific focus on two locations and a relatively short sampling period of 7 weeks. To enhance the robustness and generalizability of our findings, it is recommended that future research endeavours encompass a larger and more diverse sample of owned cats across various geographical locations. Additionally, extending the sampling period over a more prolonged duration could yield a more comprehensive understanding of seasonal variations and potential changes in predation patterns over time. For a better understanding of the entire ecological picture, future studies should also aim to gather information on the total population of cats in the studied areas and the proportion of those cats that are owned and allowed outside. Although some studies suggest that around 50%–80% of owned cats are allowed outdoors (Loss et al., 2018; Loss et al., 2013; Loyd et al., 2013), further research should be conducted for specific areas. Studies confirm that typically, only a fraction of hunted prey is brought back to the house or the farm, for instance, 23% (Loyd et al., 2013) or 10% (Krauze-Gryz et al., 2019). A more extensive and prolonged study is currently ongoing and was launched as an MSc work of the first author, coupled with a broader demographic perspective, which will not only contribute to the refinement of our current insights but also facilitate a more nuanced comprehension of the intricate dynamics between domestic cats and their surrounding ecosystems.

Author Contributions

The authors confirm their contribution to the paper as follows: study conception and design: LB, NŠ; data collection: LB; analysis and interpretation of results: LB, NŠ; draft manuscript preparation: LB, NŠ. Both authors reviewed the results and approved the final version of the manuscript.

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Data Availability

All raw or analyzed data are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

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Original Research

Exploring the trichloroacetic acid-induced toxicity on the hepato-renal system and intervention by virgin coconut oil-rich diet

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Abstract

Virgin coconut oil (VCO) is known for many beneficial health effects associated with its phenolic acids and flavonoid contents. We investigated the mechanisms underlying the antioxidative, anti-inflammatory, and anti-apoptotic mechanisms of Virgin Coconut oil-rich diet in treating trichloroacetic acid (TCA)-induced hepatic and renal damage in rats. Rats received TCA (250 mg/Kg b.wt, p.o) for ten days, followed by 5%, 10% or 15% VCO per gram feed for twenty-one days. Serum liver enzymes, urea, creatinine, tissue oxidative stress parameters, and inflammatory and apoptotic markers were then evaluated along with histological examination. TCA raised serum transaminases (ALT, AST), alkaline phosphatase (ALP), total bilirubin, urea and creatinine levels, which were abrogated by a VCO-rich diet dose-dependently. The activity of superoxide dismutase, catalase, glutathione peroxidase and nuclear factor erythroid 2-related factor 2 in the liver and kidney were enhanced, while malondialdehyde, tumour necrosis factor- α , interleukin-1 β , nuclear factor-kB level hitherto increased by TCA were quashed by the VCO- rich diet ($p < 0.05$). Similarly, the augmented level of Caspase-3 in the organs exposed to TCA was downregulated in favour of significantly increased BCL-2. Further, histomorphometry data validated the biochemical findings observed for the anti-inflammatory and anti-apoptotic potentials of VCO. Hepatocyte ballooning, pleomorphism and vascular congestion in the liver, loss of tubular architecture, tubular congestion and leukocyte infiltration in the kidney, all occasioned by TCA-intoxication, were evidently mitigated. Virgin coconut oil-rich diet could ameliorate liver and renal injury associated with trichloroacetic acid exposure via antioxidative, anti-inflammatory and anti-apoptotic mechanisms.

Keywords

Virgin coconut oil, diet, oxidative stress, anti-inflammation, anti-apoptosis

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Raziskovanje toksičnosti, ki jo povzroča trikloroocetna kislina na hepato-renalni sistem, in posredovanje prehrane, bogate z deviškim kokosovim oljem

Izvleček

Deviško kokosovo olje (VCO) je znano po številnih blagodejnih učinkih na zdravje, povezanih z vsebnostjo fenolnih kislin in flavonoidov. Raziskali smo mehanizme, na katerih temeljijo antioksidativni, protivnetni in anti-apoptotični mehanizmi prehrane, bogate z deviškim kokosovim oljem, pri zdravljenju s trikloroocetno kislino (TCA) povzročene poškodbe jeter in ledvic pri podganah. Podgane so prejemale TCA (250 mg/kg telesne teže, p.o.) deset dni, čemur je sledil 5 %, 10 % ali 15 % VCO na gram krme enaindvajset dni. Nato smo skupaj s histološkim pregledom ocenili serumske jetrne encime, sečnino, kreatinin, parametre tkivnega oksidativnega stresa, vnetne in apoptotične markerje. TCA je zvišala serumske transaminaze (ALT, AST), alkalno fosfatazo (ALP), skupni bilirubin, sečnino in raven kreatinina, ki jih je prehrana, bogata z VCO, odpravila odvisno od odmerka. Aktivnost superoksid dismutaze, katalaze, glutation peroksidaze in faktorja 2, povezanega z jedrnim faktorjem eritroid 2, v jetrih in ledvicah je bila okrepljena, medtem ko so bili malondialdehid, faktor tumorske nekroze- α , interleukin-1 β , raven jedrnega faktorja-kB, ki je bila doslej povečana s TCA, zmanjšani s prehrano, bogato z VCO ($p < 0,05$). Podobno je bila povečana raven kaspaze-3 v organih, izpostavljenih TCA, znižana v korist znatno povečanega Bcl-2. Poleg tega so histomorfometrijski podatki potrdili biokemične ugotovitve, opažene za protivnetni in anti-apoptotični potencial VCO. Baloniranje hepatocitov, pleomorfizem in vaskularna kongestija v jetrih ter izguba tubularne arhitekture, tubularna kongestija in infiltracija levkocitov v ledvicah, ki so bili posledica zastrupitve s TCA, so bili očitno omilili. Prehrana, bogata z deviškim kokosovim oljem, bi lahko izboljšala poškodbe jeter in ledvic, povezane z izpostavljenostjo trikloroocetni kislini, prek antioksidativnih, protivnetnih in anti-apoptotičnih mehanizmov.

Ključne besede

deviško kokosovo olje, prehrana, oksidativni stres, proti vnetju, proti apoptozi

Introduction

Natural products have always been used for the treatment and prevention of various diseases and illnesses. Coconut (*Cocos nucifera*) oil is another type of natural product derived from coconut, which is an edible plant with a wide range of benefits due to its components (Ibrahim et al., 2020). It can also serve as a food source (Kabara, 2000). Unlike conventional coconut oil, virgin coconut oil (VCO) is an unrefined, unbleached and undeodorized oil (Raghavendra and Raghavarao, 2010; Ibrahim et al., 2020). It is rich in medium-chain fatty acid (MCFA), vitamins (like riboflavin, niacin, vitamin E), carbohydrates, and etcetera (Marina et al., 2009). Researchers have shown that VCO has more nutritional value/benefit than the coconut oil produced conventionally (Buderwitz 2013). Several studies have expressed the anti-inflammatory and anti-nociceptive (Zakaria et al., 2011), anti-hypercholesterolemic (Zakaria

et al., 2010), anti-stress (Yeap et al., 2015), antimicrobial, immunomodulatory and even antiviral effects of VCO (Hartono et al., 2022).

The liver is an important organ in the body due to its numerous functions, which include red blood cell (RBC) formation, digestion, clearance/detoxification, etc. These hepatocytes remain connected by adherents and desmosomes, intercellular tightness, and gap junctions (Spray et al., 2013). Disruptions to these cells destroy their integrity and affect the normal functioning of the organ and system (Lala et al., 2022). Likewise, the renal system is important in filtering and eliminating waste products, and it is the primary course of elimination of toxicants like TCA (Schultz, 1999; Yu et al., 2000). Both the liver and kidney are important organs of metabolism, detoxification, storage and excretion of xenobiotics and their metabolites, and these functions are challenged by a wide array of toxicants due to environmental exposure, including trichloroacetic acid.

Trichloroacetic acid ($\text{C}_2\text{HCl}_3\text{O}_2$), a deliquescent crystallized substance with a relative molecular mass of 163.39 with a slight characteristic odour (O'Neil et al., 2006), dissolves in water and most organic solvents like acetone, benzene, methanol, and o-xylene (Morris and Bost, 2002). Its spectroscopy data was earlier reported by West and Astle (1985). It is classified as a haloacetic acid and was introduced as a selective herbicide in the late 1940s (Zhang et al., 2019). It is a chemical substance that can be found in many things; hence, its inconspicuous ingestion is high. In dermatology, TCA is used as a photoaging treatment (Sitohang et al., 2021). It acts as a moderate-deep chemical peel, rejuvenating the skin (Fischer et al., 2010). Moreover, trichloroacetic acid is used in analytical methods. Its use is effective in preparing samples by denaturing proteins in samples (Huang et al., 2014). It is also seen as a convenient catalyst for some chemical reactions (Karimi-Jaberi and Moaddeli, 2012).

Like other haloacetic acids, TCA is a by-product of water chlorination (Singer et al., 1995), a significant metabolite for trichloroethylene and perchloroethylene (El Arem et al., 2013) and a wide-spectrum pesticide for agricultural practices (Culloch, 2002). Its toxicity damages the structural and functional aspects of organs in the body, which include but are not limited to the kidneys and the liver, have all been well enunciated (Acharya et al., 1997; Pereira et al., 2001; El Arem et al., 2013). Unfortunately, injury to one or more organs in the body can lead to alteration in the body's functions. TCA is hematotoxic (Celik et al., 2009), neurotoxic and immunotoxic (Celik et al., 2010). Goldberg et al., 1990 reported in a human study an association between gestational exposure to TCA and increased risk for congenital heart defects (CHD) in offspring. Similar cardiac malformations were observed during subsequent studies performed on rat embryos exposed to TCA (Johnson et al., 2003). Likewise, pre-neoplastic lesions and hepatocyte ballooning with extensive vacuolation have equally been associated with TCA-induced experimental hepatocarcinogenesis (Mokhamer et al., 2022).

Further epidemiological findings have shown that there is a link between ingestion of water containing 'disinfection by-products' (like TCA) and a high rate of abortions, birth defects, and cancers (Aslani et al., 2019). US Environmental Protection Agency has equally considered TCA to be a human carcinogen (EPA, 2011), even though most of the studies that alluded to the carcinogenic property of TCA are in mice and rats.

The underlying mechanisms of TCA intoxication include an increase in oxidative stress and inflammation (Abdel-Hamid et al., 2011), DNA hypomethylation, peroxisome proliferation, oncogene activation, cell proliferation, and inhibition of intercellular communication (Harmon et al., 2011).

This study investigated the mechanisms by which Virgin coconut oil-rich diet (with varied percentages) institutes its antioxidant, anti-inflammatory, and anti-apoptotic potentials against the toxic effect of trichloroacetic acid on the liver and kidney of the experimental animals.

Methods

Drugs, Chemicals and Virgin Coconut Oil

Cold-pressed Virgin coconut oil was procured from Rutzjah Oil, a local manufacturer duly registered by Nigeria's National Agency for Food and Drug Administration and Control (NAFDAC). Trichloroacetic acid was purchased from Sigma Aldrich®, St Louis, MI, USA. All other laboratory chemicals and reagents used are of analytical grade and procured from the UK.

Experimental Animals and Design

Thirty-five (35) male rats were purchased and acclimatized for seven days. These animals were randomly grouped into five groups ($n=7$), and a negative control group was treated with normal saline for the experimental period. Group I: 1mL/kg of normal saline while Groups II-V were treated with TCA (250mg/kg, p.o.) by gavage for ten days, then fed with varying doses of virgin coconut oil diet for another 21 days ad libitum. Group III: TCA+5% of VCO/g of feed; Group IV: TCA+10% of VCO/g of feed; and Group V: TCA+15% of VCO/g of feed. The negative (Group I) and positive (Group II) control groups received normal saline and normal rat feed for a total of 31 days. Studies on animal experimentation were done following the Current Animal Care Regulations and Standards approved by (ILAR, 2011) and protocols approved by the Animal Ethics Committee of the College of Medicine, Federal University, Oye-Ekiti, Nigeria (ID: FUOYE/AECCM/PHS/2022/016).

The preparation of the 5%, 10% and 15% virgin coconut diet was achieved by mixing and mashing the 50g, 100g and 150g of virgin coconut oil with 950g, 900g and 850g of normal rat chow, respectively until there was homogeneity.

Biochemical Analysis

Blood was collected by cardiac puncture, then serum bilirubin levels (T.Bil), liver and renal function biomarkers, ALT, AST, ALP, urea, and creatinine were estimated using commercial kits obtained from Randox Laboratories Ltd. (Crumlin, UK). The serum (0.1 mL) was mixed with 0.5 mL of phosphate buffer (L-alanine) and (L-aspartate) for ALT and AST, respectively. The mixture was incubated for 30 min at 37 °C, and then 0.5 mL of 2,4-dinitrophenylhydrazine was added and vortexed. The mixture was allowed to stand for 20 min at room temperature, then 5 mL of 0.4 mol/L sodium hydroxide was added, and the absorbance of the solution was read after 5 min at a wavelength of 546 nm. For the ALP activity estimation, serum was directly mixed with 2-amino, 2-methyl, 1-propanol (AMP) buffer at pH 10.5, followed by estimation of absorbance of the resultant yellow colour solution at 405 nm.

Oxidative stress and inflammatory cytokines assay

Following sacrifice by cervical dislocation, the kidneys and whole liver were immediately fetched and cut in between the right and left lobes. The right lobe and right kidney were sliced into small pieces, washed with PBS and prepared to spin for 10 minutes in a homogenizer. MDA concentration as an index of lipid peroxidation was quantified according to the method described by Varshney and Kale (1990). Briefly, 50 µL of the homogenate was dispensed into a clean test tube, 100 µL of Trichloroacetic acid (TCA)/ Thiobarbituric acid (TBA) working solution was added, followed by 1.85 mL of distilled water. The mixture was placed in a boiling water bath for 15 minutes and allowed to cool thereafter. The mixture was then centrifuged, and the absorbance was read at 535 nm using a microplate reader (BIOBASE, China BK-EL10A). Superoxide dismutase (SOD) assay was carried out by the method of Misra and Fridovich (1972) but with slight modification. Superoxide dismutase (SOD) is an enzyme whose level corresponds to cellular bioprotection, and the activity is determined by the kinetic method. The ability of the enzyme to prevent epinephrine autooxidation in a basic medium was spectrophotometrically determined when 0.2 mL homogenate was added to 2.5 mL phosphate buffer (50 mM) at pH 10.4 and the reaction initiated by the addition of 0.3 mL Adrenaline (Sigma-Aldrich). The absorbance was later read at 420 nm. Calculation of the

enzyme activity was in terms of nanomoles of unoxidized adrenaline per minute of protein using a molar extinction coefficient of 4.02×10^3 per M/cm. Catalase (CAT) activity using H₂O₂ as substrate was measured by the method of Claiborne (1995). H₂O₂ decomposition rate was measured in the mixture of 0.019 M H₂O₂, 50 µL of homogenate and 1.95 mL of 0.05 M phosphate buffer (pH 7.0), and absorbance was determined spectrophotometrically at 240 nm, and at 0 sec, 20 sec, 40 sec, 60 sec, and 80 sec for each sample as the nmol H₂O₂ consumed/min/mg protein at 240 nm. Tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), Nuclear factor-kappa B (NF-κB), Nuclear factor erythroid 2-related factor 2 (Nrf2), Caspase-3 and Bcl-2 levels were also analyzed with an ELISA kit (ElabScience, USA) following the manufacturer's protocol.

Histopathology and histomorphometry

The left lobe liver and left kidney were preserved in 10% formalin solution for 24 h and washed with 70% ethanol. Tissues were then prepared and embedded in paraffin blocks. The paraffin blocks were sectioned at 6- micrometres, distributed onto glass slides and then dried. Slides were observed under a light microscope after being stained with hematoxylin and eosin (H&E). Three independent histopathologists assessed the level of liver and kidney damage and graded it accordingly, as described by Gisder et al. (2022) (Table 1) and Toprak et al. (2020) (Table 2).

The samples were evaluated, and measurements were performed with an Olympus CX43 microscope with a colour digital camera connected to a computerized image analysis system (Image Pro, USA).

Statistical Analysis

Data were presented as Mean ± standard error of the mean (SEM) and analyzed by one-way analysis of variance (ANOVA) using GraphPad prism (version 5.0). The Student's t-test was used to compare the differences between the groups, followed by the Bonferroni post hoc test. Statistical differences were reported significant at *p < 0.05.

Table 1. Histological Scoring system for TCA-induced liver damage

Tabela 1. Ocenjevalni sistem za histologijo poškodb jeter pod vplivom TCA

Grade	Grade indication	Morphological criteria	Score
0	Normal	Hepatocytes with round euchromatic nuclei and prominent nucleoli, radiating sinusoids, and intact hepatic cords	0
I	Well-differentiated	Minimal atypia, Fatty change is frequent	1
II	Moderately differentiated	Abundant eosinophilic cytoplasm, round nuclei with distinct nucleoli, Bile or proteinaceous fluid within acini	2
III	Poor differentiated	Moderate to marked pleomorphism, Absence of sinusoid-like blood spaces, Larger, more hyperchromatic nuclei but less acidophilic cytoplasm	3
IV	Undifferentiated	Little / scanty cytoplasm with fewer granules, spindle, or round-shaped cells, Highly hyperchromatic nuclei	4

Table 2. EGTI histological scoring system for TCA-induced renal damage

Tabela 2. EGTI ocenjevalni sistem za histologijo poškodb ledvic pod vplivom TCA

Tissue type	Damage	Score
Tubular	No damage	0
	Loss of Brush Border (BB) in less than 25% of tubular cells. Integrity of basal membrane	1
	Loss of BB in more than 25% of tubular cells, Thickened basal membrane	2
	Inflammation, cast formation, necrosis up to 60% of tubular cells	3
	Necrosis in more than 60% of tubular cells	4
Endothelial	No damage	0
	Endothelial swelling	1
	Endothelial disruption	2
	Endothelial loss	3
Glomerular	No damage	0
	Thickening of Bowman Capsule	1
	Retraction of glomerular tuft	2
	Glomerular fibrosis	3
	No damage	0
Tubulo/Interstitial	Inflammation, haemorrhage in less than 25% of tissue	1
	Necrosis in less than 25% of tissue	2
	Necrosis up to 60%	3
	Necrosis of more than 60%	4

Results

Serum Biochemical Analysis

TCA caused an increase ($P < 0.05$ Student t-test) in serum ALT, AST, ALP, T.Bil, urea and creatinine when compared with the negative control of the normal saline group (Table 3) by 50%, 197%, 263%, 118%, 70%, 137% respectively. However, treatment with 5% VCO, 10% VCO and 15% VCO diet reversed the increases significantly.

Lipid peroxidation and antioxidant enzymes

TCA caused oxidative damage in both liver and kidney tissues through significant elevation of MDA by 272% but a reduction in the activities of SOD, CAT, GPx and Nrf2 by 56.6%, 61%, 68.9%, and 56.4%, respectively. Meanwhile, a VCO-rich diet not only quashed the TCA-induced lipid peroxidation but also enhanced the activities of the antioxidant system (Table 4).

Inflammation and Apoptosis

TCA caused a significant elevation in the level of the inflammatory cytokine in both liver and kidney tissues. We

observed an increase in TNF- α and IL-1 β by 141%, 135% and 133%, 120% for liver and kidney, respectively. Similarly, NF- κ B, an important transcription factor in the inflammatory response, also increased by 160% in the liver but 100% in the kidney (Figure 1). Caspase-3 and Bcl-2 serve as the pro-and anti-apoptotic markers employed in this study. While there was a significant increase in the Caspase-3 level in both liver (114%) and kidney (150%) homogenate, the Bcl-2 level decreased (250%, 220%) in the TCA-treated rats without virgin coconut oil (Figure 2). Upon treatment with a VCO-rich diet, the inflammatory response was lessened, and the anti-apoptotic marker was favoured, thereby ameliorating the toxic effects of TCA.

Histopathology

Thorough histological evaluation of the slides revealed many cytoarchitectural alterations, including hepatocyte ballooning, pleomorphism and vascular congestion in the liver, and loss of tubular architecture, tubular congestion and leukocyte infiltration in the kidney, all occasioned by TCA-intoxication (Figure 4,5). Further, the histomorphological assessment using a standard scoring system (Table 1, 2) revealed the significant mitigation of all the TCA-induced cyto-architectural alteration by the VCO-rich diet (Figure 3).

Table 3. Effects of Virgin coconut oil-rich diet on the hepatic and renal function markers in rats treated with trichloroacetic acid

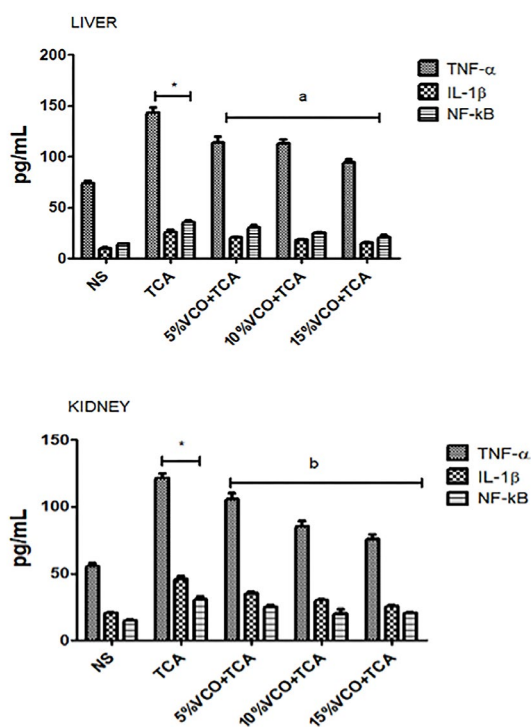
Tabela 3. Vpliv diete bogate z deviškimi kokosovim oljem na markerje jetrnih in ledvičnih funkcij pri podgani tretiranih s trikloroocetno kislino

	ALT (U/L)	AST (U/L)	ALP (U/L)	Bil. mg/dL	Urea mg/dL	Creatinine mg/dL
NS (1mL/Kg)	12.2 $\pm$ 2.0	3.85 $\pm$ 0.5	0.8 $\pm$ 0.05	7.6 $\pm$ 2.5	30.55 $\pm$ 5.1	0.40 $\pm$ 0.07
TCA (250 mg/kg)	18.5 $\pm$ 1.5*	9.9 $\pm$ 0.9*	2.9 $\pm$ 0.1*	16.5 $\pm$ 3.0*	51.30 $\pm$ 4.5*	0.95 $\pm$ 0.1*
5%VCO Diet + TCA (250 mg/kg)	13 $\pm$ 1.7 ^a	6.2 $\pm$ 0.4 ^a	1.9 $\pm$ 0.15 ^a	14 $\pm$ 1.7 ^a	45.65 $\pm$ 4.0 ^a	0.80 $\pm$ 0.05 ^a
10%VCO Diet + TCA (250 mg/kg)	12.2 $\pm$ 1.5 ^b	6.1 $\pm$ 1 ^b	1.45 $\pm$ 0.2 ^b	13.5 $\pm$ 1.5 ^b	40.25 $\pm$ 5.0 ^b	0.70 $\pm$ 0.09 ^b
15%VCO Diet +TCA (250 mg/kg)	10.5 $\pm$ 1.9 ^c	4.5 $\pm$ 0.5 ^c	1.25 $\pm$ 0.3 ^c	11.5 $\pm$ 1.0 ^c	35.70 $\pm$ 3.5 ^c	0.55 $\pm$ 0.1 ^c

* $p < 0.05$ Student t-test, TCA vs NS; ^{a,b,c} $p < 0.05$ Student t-test, test groups vs TCA. NS=Normal saline, TCA=Trichloroacetic acid (250 mg/kg/day)

Table 4. Antioxidative effects of Virgin coconut oil-rich diet on the liver and kidney of rats treated with trichloroacetic acid**Tabela 4.** Antioksidativni učinek diete bogate z deviškim kokosovim oljem na jetra in ledvice podgan tretiranih s trikloroacetno kislino

	MDA (μ M/g)	SOD (u/mg)	CAT (u/mg)	GPx (u/mg)	NrF2 (pg/mL)
Liver					
NS (1mL/Kg)	1.18 $\pm$ 0.1	15.5 $\pm$ 1.3	10.5 $\pm$ 1.5	2.25 $\pm$ 0.5	19.5 $\pm$ 2.5
TCA (250 mg/kg)	4.4 $\pm$ 0.15*	7.5 $\pm$ 0.55*	4.1 $\pm$ 0.9*	0.7 $\pm$ 0.1*	8.5 $\pm$ 1.9*
5%VCO Diet + TCA (250 mg/kg)	4.29 $\pm$ 0.2	8.5 $\pm$ 0.9	5.6 $\pm$ 0.5 ^a	1.1 $\pm$ 0.12 ^a	9.0 $\pm$ 1.5 ^a
10%VCO Diet + TCA (250 mg/kg)	3.62 $\pm$ 0.25 ^b	10.8 $\pm$ 1.85 ^b	6.5 $\pm$ 1.0 ^b	1.5 $\pm$ 0.2 ^b	12.7 $\pm$ 2.0 ^b
15%VCO Diet +TCA (250 mg/kg)	3.24 $\pm$ 0.15 ^c	12.5 $\pm$ 1.2 ^c	7.5 $\pm$ 1.2 ^c	2.0 $\pm$ 0.55 ^c	13.5 $\pm$ 2.5 ^c
Kidney					
NS (1mL/Kg)	0.6 $\pm$ 0.1	12.2 $\pm$ 2.8	14 $\pm$ 2.0	1.1 $\pm$ 0.2	29.4 $\pm$ 5.5
TCA (250 mg/kg)	3.5 $\pm$ 0.2*	4.9 $\pm$ 1.2*	7.4 $\pm$ 1.8*	0.5 $\pm$ 0.1*	12.9 $\pm$ 2.5*
5%VCO Diet + TCA (250 mg/kg)	3.0 $\pm$ 0.2	5.8 $\pm$ 1.1 ^a	9.1 $\pm$ 1.5 ^a	0.6 $\pm$ 0.2	17.7 $\pm$ 2.0 ^a
10%VCO Diet + TCA (250 mg/kg)	3.1 $\pm$ 0.25	6.0 $\pm$ 1.4 ^b	10.5 $\pm$ 1.8 ^b	0.7 $\pm$ 0.1 ^b	18.3 $\pm$ 2.5 ^b
15%VCO Diet +TCA (250 mg/kg)	2.5 $\pm$ 0.15 ^c	8.5 $\pm$ 2.0 ^c	12.0 $\pm$ 2.5 ^c	0.9 $\pm$ 0.2 ^c	21.0 $\pm$ 3.0 ^c

*p<0.05 Student t-test, TCA vs NS; ^{a,b,c}p<0.05 Student t-test, test groups vs TCA. NS=Normal saline, TCA=Trichloroacetic acid (250 mg/kg/day)**Figure 1.** Effect of Virgin coconut oil-rich diet on liver and kidney inflammatory markers in trichloroacetic treated rats. *p<0.05 Student t test, TCA vs NS; a,bp<0.05 Student t test, test groups vs TCA. NS=Normal saline, TCA=Trichloroacetic acid (250 mg/kg/day)

Slika 1. Učinek prehrane, bogate z deviškim kokosovim oljem, na označevalce vnetja jeter in ledvic pri podganah, zdravljenih s trikloroacetno kislino. *p<0,05 Student t test, TCA proti NS; a,bp<0,05 Student t test, testne skupine proti TCA. NS=običajna fiziološka raztopina, TCA=trikloroocetna kislina (250 mg/kg/dan)

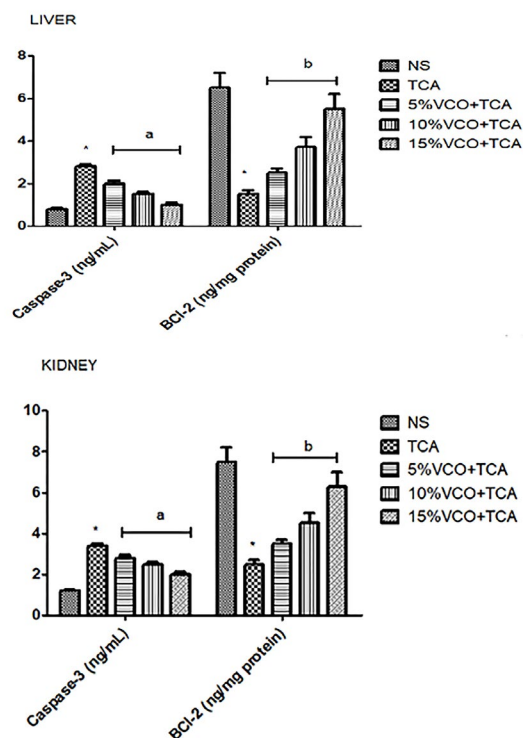


Figure 2. Effect of Virgin coconut oil-rich diet on liver and kidney apoptotic markers in trichloroacetic treated rats. * $p < 0.05$ Student t test, TCA vs NS; a,b $p < 0.05$ Student t test, test groups vs TCA. NS=Normal saline, TCA=Trichloroacetic acid (250 mg/kg/day)

Slika 2. Vpliv prehrane, bogate z deviškimi kokosovim oljem, na apoptotične označevalce jeter in ledvic pri podganah, zdravljenih s trikloroocetom. * $p < 0,05$ Student t test, TCA proti NS; a,b $p < 0,05$ Student t test, testne skupine proti TCA. NS=običajna fiziološka raztopina, TCA=trikloroocetna kislina (250 mg/kg/dan)

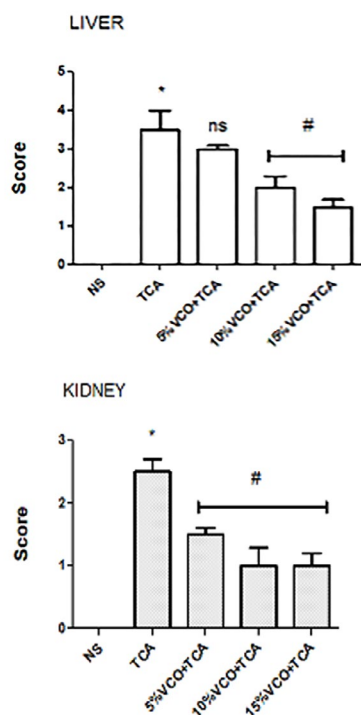


Figure 3. Liver and Kidney Histomorphological Assessment of TCA-induced toxicity upon treatment with Virgin Coconut Oil-rich diet: Grading of hepatic and renal cytoarchitectural changes. * $p < 0.05$ Student t test, TCA vs NS; # $p < 0.05$ Student t test, test groups vs TCA. NS=Normal saline, TCA=Trichloroacetic acid (250 mg/kg/day)

Slika 3. Histomorfomološka ocena jeter in ledvic toksičnosti, ki jo povzroči TCA, po zdravljenju z dieto, bogato z deviškimi kokosovim oljem: Razvrščanje jetrnih in ledvičnih citoarhitekturnih sprememb. * $p < 0,05$ Student t test, TCA proti NS; # $p < 0,05$ Student t test, testne skupine proti TCA. NS=običajna fiziološka raztopina, TCA=trikloroocetna kislina (250 mg/kg/dan)

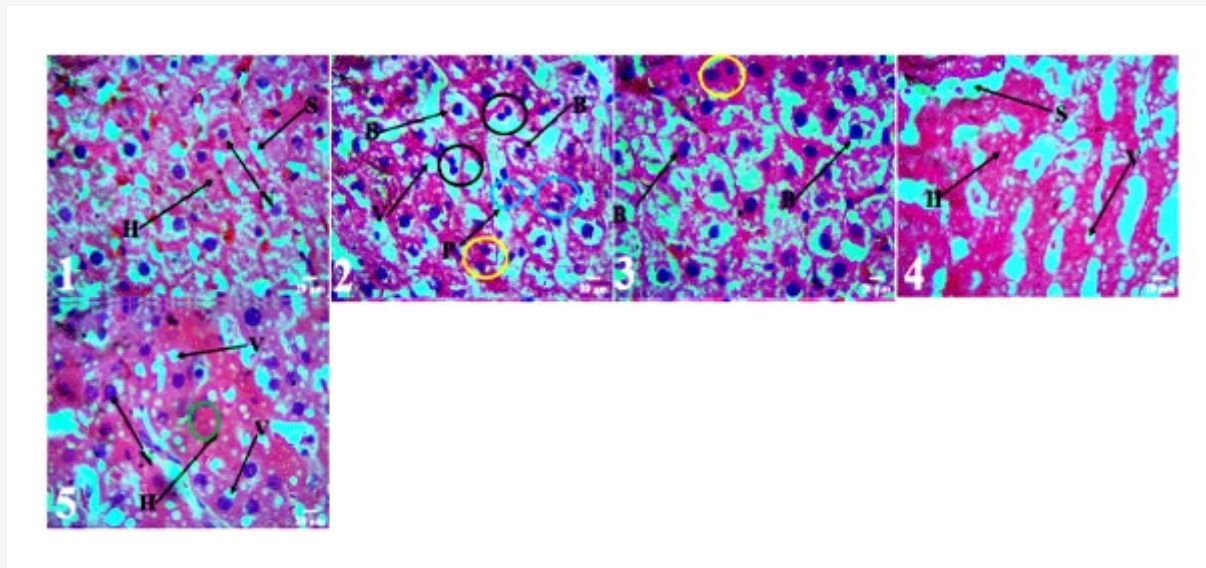


Figure 4. Representative fields of TCA-intoxicated liver sections were treated with a virgin coconut oil-rich diet. (1): Cords of hepatocytes (H) with prominent basophilic round nuclei (N) and radiating sinusoid (S). (2): Hydropic changes (ballooning of hepatocytes) with darkly-stained nuclei (B) and small-droplet steatosis (V). Phagocytic Kupffer cells (black circle) are numerous, and varying degrees of cellular and nuclear pleomorphism were also seen (blue circle). Also, note the binucleated cell (yellow circle). (3): Ballooned hepatocytes with Mallory-Denk body (B), with darkly-stained nuclei and small-droplet steatosis (V). Also note the binucleated cell (yellow circle). (4): Anucleated hepatocytes (H), small-droplet steatosis (V), near-normal cords of hepatocytes (H) (5): Small-droplet steatosis (V), Karyolysis - faded basophilia of the chromatin (green circle). Scale bars - 20μm, light microscopy, captured field – midzone, H and E – Hematoxylin and Eosin.

Slika 4. Reprezentativna polja delov jeter, zastrupljenih s TCA, zdravljenih z dieto, bogato z deviškimi kokosovim oljem. (1): Niti hepatocitov (H) z izrazitimi bazofilnimi okroglimi jedri (N) in sevajočim sinusoidom (S). (2): Hidropične spremembe (baloniranje hepatocitov) s temno obarvanimi jedri (B) in drobnokapljčno steatozo (V). Fagocitne Kupfferjeve celice (črni krog) so številne, opažene pa so bile tudi različne stopnje celičnega in jedrnega pleomorfizma (modri krog). Upoštevajte tudi dvojedno celico (rumeni krog). (3): Balonirani hepatociti z Mallory-Denkovim telesom (B), s temno obarvanimi jedri in drobnokapljčno steatozo (V). Upoštevajte tudi dvojedno celico (rumeni krog). (4): jedrni hepatociti (H), drobnokapljčna steatoza (V), skoraj normalne vrvice hepatocitov (H) (5): drobnokapljčna steatoza (V), karioliza - zbledela bazofilija kromatina (zelen krog). Merilne lestvice - 20 μm, svetlobna mikroskopija, zajeto polje – srednji del, H in E – hematoksilin in eozin.

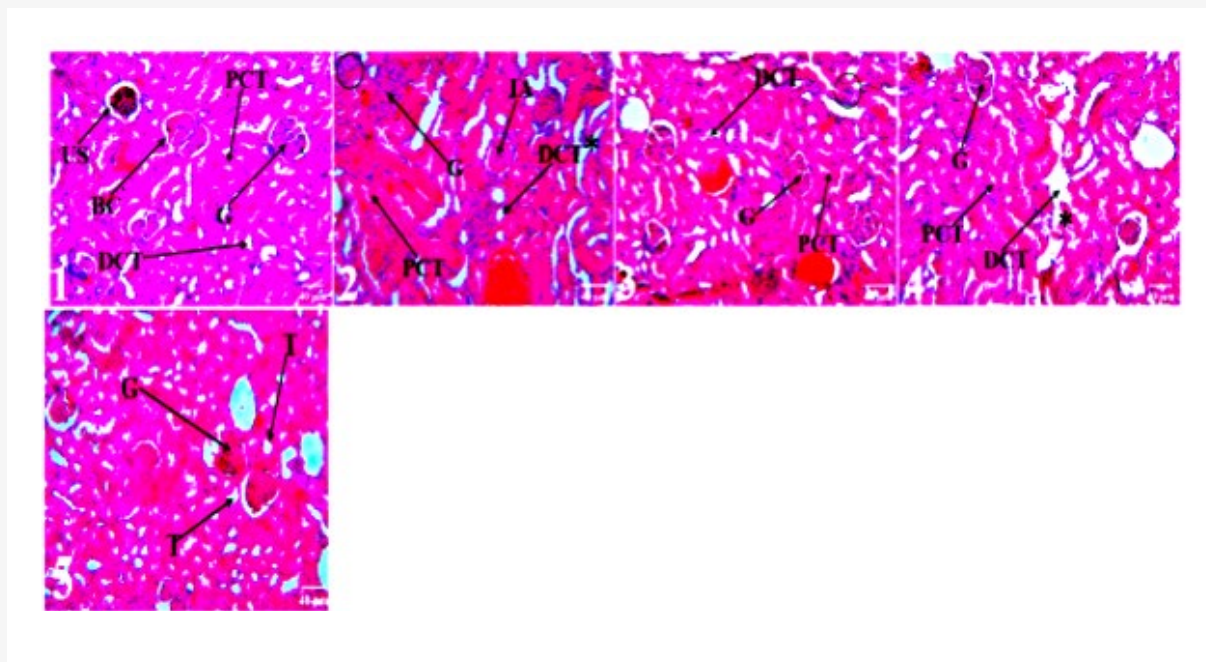


Figure 5. Representative fields of TCA-intoxicated kidney sections treated with a virgin coconut oil-rich diet.

(1): the intact architectural appearance of the renal cortex with a normal outline of the glomerular tuft (G), urinary space (US), and simple cuboidal epithelium of the tubular cells (PCT, DCT). (2, 3): some adhesion of the glomerular basement membrane to Bowman's capsule (upper left and centre glomeruli in 2, centre and lower right glomeruli in 3), thickening of the interlobular artery and obliteration of vascular lumen in 2 (IA), leukocyte infiltration (black circle), loss of tubular architecture as evidenced by interruptions in the otherwise continuous epithelial wall in 2 (right tubules - asterisk). Tubular congestion in 3. (4): Wrinkled glomerular capillary loops (upper and lower right glomeruli), loss of tubular architecture as evidenced by interruptions in the otherwise continuous epithelial wall (asterisks). (5): Adhesion of the GBM to Bowman's capsule (centre of the micrograph), glomerular atrophy and tubular degeneration evidenced by loss of cuboidal epithelial cells. Scale bars - 40µm, light microscopy, captured field: renal cortex, H and E – Hematoxylin and Eosin.

Slika 5. Reprezentativna polja delov ledvic, zastrupljenih s TCA, zdravljenih z dieto, bogato z deviškimi kokosovim oljem.

(1) : nedotaknjen arhitekturni videz ledvične skorje z normalnim obrisom glomerularnega snopa (G), urinskega prostora (US) in enostavnega kuboidnega epitelijskega tubularnih celic (PCT, DCT). (2, 3): določena adhezija glomerularne bazalne membrane na Bowmanovo kapsulo (zgornji levi in srednji glomeruli pri 2, srednji in spodnji desni glomeruli pri 3), zadebelitev interlobularne arterije in obliteracija vaskularnega lumna pri 2 (IA), levkocitna infiltracija (črni krog), izguba tubularne arhitekture, kar je razvidno iz prekinitev v sicer neprekinjeni epitelijski steni v 2 (desni tubuli - zvezdica). Tubularna kongestija v 3. (4): Nagubane glomerularne kapilarne zanke (zgornji in spodnji desni glomeruli), izguba tubularne arhitekture, kar je razvidno iz prekinitev v sicer neprekinjeni epitelijski steni (zvezdice). (5): Adhezija GBM na Bowmanovo kapsulo (sredina mikrofotografije), glomerularna atrofija in tubularna degeneracija, ki jo dokazuje izguba kuboidnih epitelijskih celic. Merilna lestvica - 40 µm, svetlobna mikroskopija, zajeto polje: ledvična skorja, H in E – hematoksilin in eozin.

Discussion

The results of the present study are in harmony with previous findings on the deleterious effects that trichloroacetic acid has on the kidneys and liver of experimental animals (El Arem et al., 2013; Mokhamer et al., 2022).

The Aminotransferases— Alanine aminotransferase and Aspartate aminotransferase are indicators of hepatocellular injury; the level of ALT in normal liver is higher than AST (Lala et al., 2023). An increase in the circulating aminotransferases signifies that the membrane permeability of the liver cells is altered (Saad et al., 2014), as observed in the study. Moreover, the high level of circulating bilirubin in the TCA-assaulted rats also suggests that the liver functions were distorted, as established by Abdel-Hamid et al. (2011) and Mokhamer et al. (2022). TCA is a known chemical used to induce experimental hepatocellular injury by increasing the oxidative stress level and inflammation in the cells (Fouad et al., 2013), though a significant reduction in values associated with the above-named conditions was seen in groups treated with a VCO-rich diet.

Hepatocytes make an important layer that isolates sinusoidal blood from the bile in the canaliculi (Gissen and Arias, 2015). The histomorphological assessment of the liver queries the cellular arrangement and their integrity with or without exposure to TCA. The liver cells of the animals exposed to TCA had a deformed arrangement that can be seen in some cases of liver diseases. There was ballooning of the hepatocytes and pleomorphism, which indicates the presence of a tumour which could likely be cancerous. The noticeable cellular (leucocytes) infiltration and phagocytic kupffer cells are considered to be part of the cellular responses to injury (Alzergy et al., 2018) caused by the toxicity of TCA. Nonetheless, the intervention of a VCO-rich diet showed it has cytoprotective effects against the injury initiated by TCA, as demonstrated in the histomorphometric grading of the liver slides. It was observed that the suspected and/or possible tumour progression was altered in the animals treated with the VCO-rich diet.

Plasma urea and creatinine are useful markers in assessing renal functions (El Arem et al., 2013). An abnormal rise in plasma urea indicates a problem with the glomerular filtration rate (Kang et al., 2002; Michael and Sircar, 2010). TCA induction in the experimental animals caused reduced glomerular filtration rate and renal dysfunction, as evidenced by the elevated urea and creatinine levels in the blood. These findings are in alignment with various

works done using TCA (Pereira et al. 2001). Moreover, renal dysfunction in TCA-administered animals might be secondary because TCA distorted liver functions and might be unable to clear urea (which is the end product of protein catabolism) from the blood (El Arem et al., 2013). Leucocyte infiltration, adhesion of glomerular basement membrane to the Bowman's capsule, thickened interlobular artery, and loss of tubular architecture are clear indicators of the toxic effects that TCA had on renal integrity. Infiltration of leucocytes observed in the kidneys suggests that the glomeruli were inflamed. However, this renal dysfunction was evidently reversed in animals treated with VCO dose-dependently, which is also evidenced by the findings of the histomorphometric grading of the kidney slides.

The increased production and accumulation of reactive oxygen species and free radicals tend to disrupt the body's homeostasis (Granger and Kvietys, 2015). One of the mechanisms by which TCA induces its toxicity is via oxidative stress, which has been implicated in renal and hepatic injury (El Arem et al., 2013; Liu et al., 2016; Pinegin et al., 2018).

The antioxidant system of both enzyme and non-enzymatic defends the body against ROS effects (Paiva et al., 2018). Lipid peroxidation increases after the administration of TCA supports previous findings (Celik and Tuluze, 2007; Celik et al., 2009). Malondialdehyde (MDA) is a significant indicator of this activity, which is a result of released free radicals by TCA (Austin et al., 1996). However, a reduced level of MDA was observed in the groups treated with a VCO-rich diet, thus validating its anti-inflammatory potential.

Superoxide Dismutase (SOD) and Catalase (CAT) are primary antioxidant enzymes that defend cells against injury occasioned by the presence of increased free radicals (Srinivasan et al., 2007). One of the many functions of cells is the maintenance of an equilibrium between antioxidants and reactive oxygen species (ROS). Thus, oxidative stress in the cell must be at a minimal level to achieve and maintain redox homeostasis (Doulias et al., 2013; Aparicio-Trejo et al., 2019). To maintain this, various signalling pathways are employed by activating concerned transcription factors (Irazabal and Torres, 2020; Aranda-Rivera et al., 2022). Glutathione peroxidase (GPx) is one of the detoxifying enzymes that are modulated by upregulated Nuclear factor erythroid 2-related factor (Nrf2) (Kaspar et al., 2009; Cuadrado et al., 2014). There was an increase in the GPx quantified in the animals treated with a VCO-rich diet alongside up-regulated Nrf2, which indicates that VCO also employed this pathway (Nrf2/GPx) to establish its effects.

Anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) is one of the proteins that regulate cell death (Cory and Adams, 2002; Danial and Korsmeyer, 2004). The low expression of Nrf2 observed in the TCA-induced liver and kidney results in altered Bcl-2 induction and apoptosis. The rich expression of Bcl-2 can be stimulated by the binding of Nrf2 to the Antioxidant Response Element (ARE) (Kaspar et al., 2009; Niture and Jaiswal, 2012). This pathway helps in preventing chemical-induced cell death (and other types of cell death) (Niture and Jaiswal, 2011; Dodson et al., 2019). Consequently, there would be a reduced expression of proapoptotic proteins like Caspase 3 and 7. As seen in the results, a VCO-rich diet enhanced the expression of Bcl-2 while it reduced the activity of Caspase 3.

Mohan et al. (2020) and Liebman and Le (2021) reported that activation of Nrf2 reduces accumulated ROS levels and motivates hepatoprotection and nephroprotection. Both Nrf2 and NF- κ B have a close relationship in regulating inflammation in the liver and kidney (Alshehri et al., 2022). Li et al. (2021) and Deng et al. (2020) highlighted the NF- κ B/ Nrf2 signalling pathway in the liver and kidney of experimental animals; Nrf2 quashes inflammation through cytokine release regulation (Keleku-Lukwete et al., 2018).

Tumour Necrotic Factor- α (TNF- α) and Interleukin-1 β (IL-1 β) are examples of pro-inflammatory mediators (Colombo et al., 2018; Courtois and Fauvarque, 2018) which are activated by NF- κ B signalling pathway. These factors were up-regulated by TCA-induced toxicity in the liver and kidney. Interestingly, the administration of varying percentages of a VCO-rich diet reduced TNF- α , and IL-1 β levels, thereby explaining its capacity to reverse the liver and kidney damages induced by TCA.

NF- κ B is a main regulator of inflammation (Shih et al., 2015; Colomer et al., 2017) and one of the mediators of apop-

tosis by influencing the expression of apoptosis-related genes (Canbay et al., 2003). Reactive Oxygen Species can exacerbate inflammatory response, causing tissue damage and activation of the NF- κ B signalling pathway (Zhao and Wen, 2018). The production of apoptotic cells, which are later consumed by Kupffer cells, stimulates the expression of TNF- α , resulting in hepatic inflammation and hepatocyte apoptosis (Serhan et al., 2012; Wang and Lin, 2013). In this study, VCO probably de-activated the apoptosis cascade and inflammation by down-regulating the expression of NF- κ B, TNF- α , Caspase-3 and IL-1 β while stimulating Nrf2, Bcl-2 and GPx.

In conclusion, Virgin coconut oil enacted its anti-apoptotic, anti-inflammatory, and antioxidative effects, as demonstrated by the findings in this study. Although the 15% VCO/gram of feed appears to be most effective in the reversal of TCA-induced hepatic and renal damages, the suggestion of dose-dependent effects may still suffice.

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Data Availability

Data generated from this study can easily be made available on request.

Conflicts of Interest

The authors declare no conflict of interest.

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Original Research

Mikrobna obremenitev morske vode in peska na plaži med kopalno sezono

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Povzetek

Slovenija je ena od najbolj vodnatih evropskih držav, kjer je voda pomembna ne le za pitje, ampak tudi za različne rekreacijske dejavnosti, še posebej plavanje. Naravne rekreacijske morske vode so pomembno okolje, v katerem so kopalci izpostavljeni bakterijam, ki so odporne proti protimikrobnim učinkovinam ter številnim vrstam gliv. Poleg morske vode ljudje veliko časa preživijo tudi na obmorskih površinah (plaža s peskom) za katere se ne izvaja mikrobiološki monitoring. Da bi določili mikrobiološko obremenitev morske vode in peska na plaži, smo tekom kopalne sezone analizirali prisotnost bakterije *Escherichia coli* in oportuno patogenih gliv, ki se prenašajo preko človeka. *E. coli* je bila prisotna le v morski vodi znotraj dovoljenih meja čez celo kopalno sezono, z največjo frekvenco v avgustu. Poleg okoljskih sevov smo zaznali tudi seve iz skupine B2, seve s povečano zmožnostjo adhezije in sistemov za privzem železa ter seve z geni za odpornost proti antibiotikom iz skupin fluorokinolonov in beta-laktamov. Gliv v vodi tekom kopalne sezone z uporabljen metodo nismo osamili. V nasprotju pa smo v pesku na plaži zasledili glive *Candida parapsilosis*, *Geotrichum candidum* in *Trichosporon asahii*, ki so del kožne mikrobiote ljudi. Število *E. coli* v morski vodi in gliv v pesku je naraščalo s številom kopalcev in je doseglo vrh v avgustovskem vzorcu. Glede na dobljene rezultate je *E. coli* primeren parameter za spremljanje kakovosti morske vode medtem ko bi za spremljanje kakovosti peska na plaži lahko kot dodatni parameter uporabljali določene vrste gliv antropogenega izvora.

Ključne besede

pesek, *Escherichia coli*, glive, zdravje, indikatorji, morska voda, mestna plaža

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Microbial burden of seawater and beach sand during bathing season

Abstract

Slovenia is one of the most water-rich countries in Europe, where water is important not only as drinking water but also for various recreational activities, especially swimming. Natural marine waters are an important environment where swimmers are exposed to resistant bacteria and also fungi. In addition to seawater, people spend a lot of time on sandy beaches, that are currently not microbiologically monitored. To determine the microbiological burden of seawater and beach sand during the bathing season, we analyzed the presence of bacteria *Escherichia coli* and opportunistic pathogenic fungi transmitted by humans. *E. coli* was present in seawater only within the permissible limits throughout the bathing season, with the highest incidence in August. Besides environmental strains, strains of the B2 phylogenetic group, strains with increased adhesion and iron uptake systems, and strains with genes for resistance to antibiotics from the fluoroquinolone and beta-lactam groups were also detected. With the method used, we were unable to isolate any fungi in the water during the bathing season. On the contrary, we found human-transmitted fungal species *Candida parapsilosis*, *Geotrichum candidum*, and *Trichosporon asahii* in the beach sand. The number of *E. coli* in the seawater and fungi in the sand increased with the number of bathers, both reaching a peak in the August sample. According to the results, *E. coli* is a suitable parameter for monitoring seawater quality, while certain types of human-related fungi could be used as an additional parameter for monitoring beach sand quality.

Keywords

beach sand, *Escherichia coli*, fungi, health, indicators, seawater, urban beach

Uvod

V poletnem času številni obiskovalci uporabljajo morske kopalne vode in njihova obrežja za oddih in rekreacijo. Kopanje v površinskih vodah pa lahko predstavlja nevarnost za zdravje ljudi zaradi morebitne fizikalno-kemijske, predvsem pa mikrobiološke onesnaženosti vode (npr. komunalni izpusti, izplake iz živinoreje, spiranje površin) (NIJZ, 2020). Leta 2003 je Svetovna zdravstvena organizacija (WHO) objavila smernice za varno uporabo kopalne vode, tudi s priporočilom sledenja kakovosti obalnega peska, na katerem se zadržujejo ljudje (WHO, 2003). Tudi Evropska Direktiva o kopalnih vodah (2006/7/ES) v glavnem temelji na sledenju tradicionalnih fekalnih parametrov, kot so število enterokokov in *Escherichia coli*, ki so najpogostejše povezani s pojavom bolezni pri človeku po kopanju v naravnih kopalnih vodah (EEU, 2006). Vzroki fekalnega onesnaženja kopalnih voda so lahko točkovni ali razpršeni viri (Buer in sod., 2018; Kataržytė in sod., 2018). Točkovne vire je lažje izslediti in so običajno posledica enkratnega dogodka izpusta fekalij, medtem ko ima razpršeno onesnaženje običajno več bioloških in geografskih izvorov in je zato težje obvladljivo. K razpršenemu onesnaženju

prispevajo padavinsko spiranje okoliške prsti, lokalni manjši vodotoki ter prisotnost divjih in domačih živali (Brandão in sod., 2021). Poleg sledenja teh pa direktiva v 6. členu poziva k izvajanju „profila kopalnih voda“, s čemer bi identificirali in ocenili različne (ne le fekalne) vzroke onesnaženja, ki vplivajo na kakovost kopalne vode in zdravje kopalcev (EEU, 2006). Zaradi globalnega segrevanja, podnebnih sprememb, hitro rastoče človeške populacije in razvoja odpornosti proti antimikrobnim učinkovinam se v okolju pojavljajo tudi novi viri onesnaženja in potreba po sledenju drugim mikroorganizmom, ne le fekalnim indikatorjem (Weiskerger in sod., 2020), še posebej *E. coli*.

Kot mogoč parameter prihodnosti sledenja kakovosti kopalne vode in obalnega peska se v zadnjih nekaj letih omenjajo tudi glive. Pomen sledenja prisotnosti gliv in spreminjanja njihovega števila v morju in obalnem pesku je vezan na občutno povečanje glivnih bolezni kot so alergije, astma, dermatomikoze, sinusitis, otitis, micetomi in sistemske infekcije v svetu (de Hoog in sod., 2020) ter naraščajočo odpornostjo na protiglivične učinkovine v antimikotikih (WHO, 2022). Po dosedanjih podatkih se v morski vodi najpogostejše pojavljajo *Alternaria*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Cystofilobasidium*,

Didymella, *Exophiala*, *Hortaea*, *Meyerozyma*, *Penicillium*, *Rhodotorula* in *Wallemia* (Solo-Gabriele in sod., 2016; Brandão in sod., 2021; Novak Babič in sod., 2022; Cogliati in sod., 2023). Iz peska pa so bile najpogostejše osamljene filamentozne glive *Aspergillus*, *Cladosporium*, *Chrysosporium*, *Curvularia*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus*, *Scedosporium*, *Scopulariopsis*, *Scytalidium*, *Stachybotrys* in *Trichophyton*, ki so običajno prevladovala nad kvasovkami in kvasovkam podobnimi glivami iz rodov *Aureobasidium*, *Candida*, *Geotrichum*, *Exophiala*, *Metschnikowia*, *Rhodotorula* in *Yarrowia* (Solo-Gabriele in sod., 2016; Brandão in sod., 2021; Novak Babič in sod., 2022; Cogliati in sod., 2023). Prisotnost gliv je v veliki meri odvisna od vremenskih razmer, vodnih tokov, prisotnosti ljudi in živali in nenadnih, enkratnih dogodkov kot so poplave, potresi ali onesnaženje z nafto in njenimi derivati (Solo-Gabriele in sod., 2016; Romão in sod., 2015; Brandão in sod., 2021; Novak Babič in sod., 2022; Cogliati in sod., 2023).

Čeprav je prisotnost gliv v pesku plaž in v morju pogosto dokumentirana pa trenutno ni dovolj podatkov o glivnih skupinah, ki bi jih lahko uporabili kot kazalnike za sledenje različnim virom onesnaženja in tako tudi ni vzpostavljenega enotnega monitoringa za te mikroorganizme (Novak Babič in sod., 2022). Kot del iniciative »Mycosands«, smo zato v študiji želeli opredeliti pojav gliv in njihovo pogostost v pesku in morju v času kopalne sezone, hkrati pa smo spremljali tudi pojav in število *Escherichia coli* kot redno spremljan parameter fekalnega onesnaženja v kopalnih vodah. Poleg tega nas je zanimala tudi genotipska raznolikost izoliranih bakterij *E. coli* in njihov pripadajoči virulentni potencial.

Material in metode

Vzorčenje peska in morske vode

Vzorčenje peska in morske vode smo izvedli v letu 2022 v času kopalne sezone na Centralni plaži v Portorožu (Slovenija) v okviru širše evropske pobude "Mycosands II". Centralna plaža Portorož je nastala umetno in je zaradi svoje lege v središču urbanega okolja ena najbolj priljubljenih plaž v Sloveniji. Pesek je med kopalno sezono (od junija do septembra) vsakodnevno čiščen in mehansko prezračevan. Plaža redno prejema Modro Zastavo - certifikat kakovosti za čisto, varno in uporabniku prijazno plažo. Vzorčenje peska in morske vode je potekalo v kopalni sezoni, enkrat mesečno, od junija do septembra 2022 med 9. in 10. uro

dopoldan. Pesek smo vsakič vzorčili na petih točkah plaže, na robovih in na sredini, na globini 5 - 10 cm. Vzorce smo aseptično zbrali v sterilne 50 ml centrifugirke.

Morsko vodo smo vzorčili na pomolu nasproti plaže na globini 20 cm v vodnem stolpcu ~1 m. Vzorce morske vode smo aseptično zbirali v sterilne 500 ml posode (Golias, Slovenija). Vsi vzorci so bili označeni in v 2 urah po vzorčenju prepeljeni v laboratorij v hladnih pogojih, po vzoru Sabino in sodelavcev (2011).

Izolacija čistih glivnih kultur in trajno shranjevanje

V sterilne erlenmajerice smo zatehtali 40 g peska in nato dodali 40 ml sterilne destilirane vode (dH₂O). Glive smo is peska sprali s stresanjem pri 100 obratih na minuto, 30 minut. Po 200 in 100 µl suspenzije smo v treh ponovitvah konfluentno nacepili na Sabouraudov dekstrozni agar (SDA) (Biolife, Italija) in Mycosel agar (Becton Dickinson, Nemčija) z dodatkom cikloheksimida in kloramfenikola. Plošče smo nato inkubirali pri 30 in 37 °C, 5 - 7 dni (SDA) in do 21 dni (Mycosel). Enak postopek izolacije je bil uporabljen tudi za vzorce morske vode (Sabino in sod., 2011). Po inkubaciji smo prešteli morfološko enake kolonije in izračunali enote, ki tvorijo kolonije (CFU) v gramu peska in litru morske vode in jih podali kot povprečno vrednost triplikatov. Vsako od morfološko različnih kolonij gliv smo nato prenesli na svežo SDA ali Mycosel ploščo in inkubirali 5 - 20 dni do vidne rasti. Vse čiste kulture so bile trajno shranjene v zbirki ekstremofilnih mikroorganizmov Ex Infrastrukturnega centra Mycosmo, MRIC UL, Slovenija (<http://www.ex-gene-bank.com/>), na Oddelku za biologijo Biotehniške fakultete Univerze v Ljubljani.

Izolacija DNA in molekularno-genetska identifikacija izoliranih gliv

Čiste kulture gliv smo gojili na ploščah SDA ali Mycosel pri 25 °C, do vidne rasti. DNA nitastih gliv smo ekstrahirali po protokolu Van den Ende in de Hoog-a (1999) z mehansko lizo in ekstrakcijo s kloroformom. Kvasovke smo gojili na SDA pri 25 °C, 3 dni, nato pa DNA ekstrahirali z reagentom PrepMan Ultra (Applied Biosystems, Foster City, ZDA) po priloženih navodilih proizvajalca. Dobljene vzorce DNA smo shranili pri -20 °C.

Glive smo grobo identificirali z opazovanjem njihovih makro- in mikromorfoloških značilnosti. V skladu s tem smo

v nadaljevanju izvedli identifikacijo nitastih gliv na podlagi nukleotidnih sekvenc rDNA bodisi dela gena za aktin (*act*) (rod *Cladosporium*), dela gena za beta-tubulin (*benA*) (rodova *Aspergillus* in *Penicillium*), dela gena za translacijski elongacijski faktor 1-alfa (*tef*) (rod *Fusarium*) in celotno regijo notranjega distančnika (ITS = ITS1, 5.8S rDNA, ITS2) (vse ostale nitaste glive). Identifikacija kvasovk je potekala na podlagi DNA nukleotidnih zaporedij velike podenote ribosoma (LSU = delna 28S rDNA, domene D1/D2). Pri analizah smo uporabili sledeče oligonukleotidne začetnike: ACT-512F in ACT-783R (*act*) (Carbone in Kohn, 1999), Bt2a in Bt2b (*benA*) (Glass in Donaldson, 1995), EF1 in EF2 (*tef*) (O'Donnell in sod., 1998), ITS5 in ITS4 (ITS) (White in sod., 1990) ter NL1 in NL4 (LSU) (Boekhout in Kurtzman, 1996).

Določevanje nukleotidnega zaporedja je bilo izvedeno pri Microsynth AG, Avstrija. Zaporedja so bila nato pregledana s programsko opremo FinchTV 1.4 (Geospiza, Perkin-Elmer Inc., Seattle, ZDA) in Molecular Evolutionary Genetics Analysis (MEGA) različica 7.0 (Kumar in sod., 2016).

Identifikacija nukleotidnih zaporedij je bila izvedena z algoritmom BLAST na spletni strani NCBI (Altschul in sod., 1990) in primerjana s tipskimi sevi iz taksonomskih baz podatkov, kot sta Westerdijk Fungal Biodiversity Institute (Utrecht, Nizozemska) in Index Fungorum (www.indexfungorum.org). Sekvence sevov so deponirane v bazi podatkov zbirke Ex Infrastrukturnega centra Mycosmo (<http://www.ex-genebank.com/>).

Izolacija, identifikacija in karakterizacija sevov bakterijske vrste *E. coli*

Za osamitev bakterijske vrste *E. coli* iz peska smo se poslužili enakega postopka, opisanega pri izolaciji gliv iz peska. Na kratko, v sterilne erlenmajerice smo zatehtali 40 g peska in nato dodali 40 ml sterilne destilirane vode (dH₂O). Celokupne bakterije smo iz peska sprali s stresanjem pri 100 obratih na minuto, 30 minut. Po 50 µl suspenzije smo v treh ponovitvah konfluentno nacepili na gojišče UriSelect (UriSelect™ 4 Medium, BioRaD, FRA). Plošče smo inkubirali pri 37 °C 1-2 dni. Po inkubaciji smo prešteli morfološko enake kolonije in izračunali enote, ki tvorijo kolonije (CFU) v gramu peska.

Za osamitev čistih kultur bakterijske vrste *E. coli* iz morske vode smo uporabili dve različni selektivni kromogeni gojišči. Kot primarno selektivno gojišče smo za detekcijo *E. coli* v vodnih vzorcih uporabili gojišče Compact Dry EC (Nissui Pharmaceutical), kamor smo nanесли po 1 ml

vzorčene morske vode. Na tem kromogenem gojišču so kolonije *E. coli* modre barve. Sočasno smo morsko vodo tudi filtrirali preko nitroceluloznih filtrov Millipore s premerom por 0,45 µm. Filtrirali smo 50 ml in 100 ml vzorčene morske vode ter filtre aseptično položili na gojišče UriSelect (UriSelect™ 4 Medium, BioRaD, FRA), vse filtracije smo opravili v treh ponovitvah. Plošče smo inkubirali pri 37 °C 1-2 dni. Na tem kromogenem gojišču so kolonije *E. coli* rožnate barve. Porasle kolonije iz obeh tipov kromogenih gojišč smo prešteli in izračunali povprečno število kolonijski enot (CFU) na 50 oz. 100 ml morske vode.

Rožnate kolonije z gojišča UriSelect smo precepili do čistih kultur in jih shranili na trajen, metabolno neaktiven način v mikrobiološko zbirko ekstremofilnih mikoorganizmov Ex Infrastrukturnega centra Mycosmo, MRIC UL, Slovenija (<http://www.ex-genebank.com/>), na Oddelku za biologijo Biotehniške fakultete Univerze v Ljubljani.

Izolirane seve *E. coli* smo dodatno identificirali tudi z MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) masno spektrometrijo in potrdili, da gre za bakterije vrste *E. coli*. Vsem sevom smo pripravili bakterijske lizate, ki smo jih nato uporabili kot matrično / vzorčno DNA za verižno reakcijo s polimerazo. Z uporabo metode PCR smo seve tudi genotipsko opredelili. Na podlagi pridobljenih PCR-pomnožkov smo jih uvrstili v filogenetske skupine po prvotni (Clermont in sod., 2000) in prenovljeni metodi (Clermont in sod., 2013).

Vsem osamljenim sevom smo z metodo določanja prstnega odtisa DNA bakterij ERIC-PCR (angl. enterobacterial repetitive intergenic consensus polymerase chain reaction) ter uporabo specifičnih oligonukleotidnih začetnikov ERIC1R in ERIC2, ki pomnožujejo enterobakterijska ponavljajoča se medgenska ohranjena zaporedja, ugotavljali njihovo klonalnost na podlagi profilov, pridobljenih po agarozni gelski elektroforezi PCR pomnožkov. Hkrati smo izoliranim sevom z uporabo specifičnih začetnih oligonukleotidov definirali njihovo možno pripadnost 4 različnim sekvenčnim skupinam (ST angl. sequence type): ST69, ST73, ST95 in ST131 (Doumith in sod., 2015).

Neklonalnim sevom *E. coli* smo tako določili prisotnost genov za dejavnike virulence kot so adhezini (gen *fimH*; gene skupine *afa*, ki se vežejo na receptorje DAF (angl. decay-accelerating factor); gen *dra*, gen *papGII*, gen *papC* ter gen *iha*), avtotransporterji (gen *fluA*, gen *sat*, gen *vat*), protektini (gen *iss*, gen *kpsMTII*, gen *ompTAPEC* in gen *traT*), sistemi za privzem železa (gen *fyuA*, gen *iroN*, gen *irp2*, gen *iucD* in gen *iutA*) in toksini (gen *ups deg*). Pravtako

smo neklonalnim sevom *E. coli* določili prisotnost genov z zapisi za odpornostjo proti protimikrobnim učinkovinam, predvsem odpornost proti betalaktamskim antibiotikom (gen *bla*_{CTX-M-1}, gen *bla*_{CTX-M-2}, gen *bla*_{CTX-M-8}, gen *bla*_{CTX-M-9}, gen *bla*_{CTX-M-25}, gen *bla*_{SVH}, gen *bla*_{TEM}, in *bla*_{OXA}) in plazmidno posredovane odpornosti proti (fluoro)kinolonom (PMQR, angl. Plasmid-Mediated Quinolone Resistance) (gen *qnrA*, gen *qnrB* in *qnrS*).

Rezultati

Mikrobna raznolikost in številčnost v pesku mestne plaže med kopalno sezono 2022

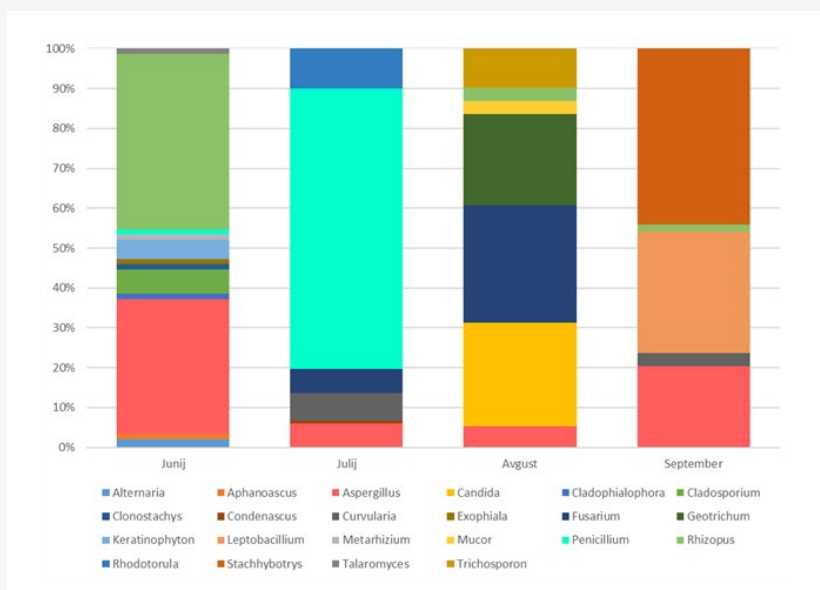
V času kopalne sezone, od junija do septembra 2022, smo mesečno vzorčili pesek na mestni portoroški plaži. V vseh štirih mesecih skupaj smo iz peska osamili 32 glivnih vrst, ki pripadajo 22 rodovom (Tabela 1, Slika 1). Najmanjše celokupno število gliv smo zabeležili junija (CFU/g = 145), hkrati pa smo v tem vzorcu opazili največjo raznolikost (15 vrst iz 12 rodov). Julijski in septembrski vzorec sta imela primerljivo številčnost ($\text{CFU/g}_{\text{september}} : \text{CFU/g}_{\text{julij}} = 985 : 997$), vendar je bila septembra zabeležena najmanjša raznolikost s 7 vrstami iz 5 rodov. Avgusta smo iz peska osamili 8 vrst gliv iz 10 rodov, pri čemer je bila številčnost v tem vzorcu najvišja (CFU/g = 1018) (Slika 1). Izmed vrst, osamljenih iz peska, *Aphanoascus fulvescens*, *Aspergillus flavus*, *Fusarium*

oxysporum, *F. solani*, *Rhizopus arrhizus* in *Trichosporon asahii* spadajo med oportuno patogene glive 2. varnostne stopnje (BSL-2). Največ teh gliv smo osamili avgusta (37,5% vseh vrst), najmanj pa junija in julija (oboje 20%).

Iz vzorcev peska smo v kopalni sezoni osamili številne bakterije. Na podlagi svetlo modre in temno modre barve kolonij na kromogenem gojišču UriSelect smo v vseh vzorcih peska fenotipsko potrdili prisotnost različnih vrst *Enterococcus* spp., *Klebsiella* spp. ter *Pseudomonas* spp. Najmanj prisotnih bakterij je bilo v junijskem vzorcu (26 CFU/g vzorca), medtem ko sta si vzorca julij in september po celokupnem številu bakterij primerljiva (58 CFU /g julijski vzorec peska in 64 CFU/g septemberski vzorec peska). Največje celokupno število bakterij smo zabeležili v vzorcu peska meseca avgusta (162 CFU/g). Iz vzorcev peska tekom kopalne sezone 2022 nismo osamili nobene bakterijske kolonije vrste *E. coli*.

Glive kot kazalniki človekovega vpliva na mikrobiološko kakovost peska plaže

V času največje obiskanosti mestne plaže (mesec avgust) smo poleg redno prisotnih rodov *Aspergillus*, *Fusarium* in *Rhizopus*, zabeležili tudi pojav humanih oportuno patogenih vrst *Candida parapsilosis*, *Geotrichum candidum* in *Trichosporon asahii*. Te vrste so bile osamljene le iz avgustovskega vzorca, njihovo celokupno število pa predstavlja 58,7 % vseh gliv iz tega vzorca (26,0 %, 22,9 % in 9,8 %) (Tabela 1, Slika 1).



Slika 1. Zastopanost glivnih rodov (%) v vzorcih peska vzorčenega mesečno med kopalno sezono leta 2022.

Figure 1. Representation of cultured fungal genera (%) in beach sand samples taken monthly during the bathing season in 2022.

Tabela 1. Glive, osamljene iz peska plaže v Portorožu (Slovenija) med kopalno sezono v letu 2022.

Table 1. Fungi, isolated from beach sand in Portorož (Slovenia) during the bathing season in 2022.

Molekularno-genetska identifikacija	Gojišče	Mesec vzorčenja	CFU/g	Genetski označevalec	EXF številka ¹	Varnostna stopnja (BSL) ²
<i>Alternaria alternata</i>	Mycosel	Junij	3	ITS	EXF-16980	BSL-1
<i>Aphanoascus fulvescens</i>	Mycosel	Junij	2	ITS	EXF-16983	BSL-2
<i>Aspergillus calidoustus</i>	Mycosel	Junij	5	<i>benA</i>	EXF-16978	BSL-1
<i>Aspergillus flavus</i>	SDA	Junij Avgust September	20 33 67	<i>benA</i>	EXF-16974 EXF-17030 EXF-17035 EXF-17040	BSL-2
<i>Aspergillus nidulans</i>	SDA	Junij Julij September	25 43 33	<i>benA</i>	EXF-16973 EXF-16990 EXF-17042	BSL-1
<i>Aspergillus niger</i>	SDA	Avgust	20	<i>benA</i>	EXF-17036	BSL-1
<i>Aspergillus tamarii</i>	SDA	Julij	13	<i>benA</i>	EXF-16991	BSL-1
<i>Aspergillus welwitschiae</i>	SDA	Julij September	3 100	<i>benA</i>	EXF-16992 EXF-17043	Ni podatka
<i>Candida parapsilosis</i>	SDA	Avgust	265	ITS, LSU	EXF-17031	BSL-1
<i>Cladophialophora immunda</i>	Mycosel	Junij	2	ITS	EXF-16981	BSL-1
<i>Cladosporium cladosporioides</i>	Mycosel	Junij	2	<i>act</i>	EXF-16979	BSL-1
<i>Cladosporium pseudocladosporioides</i>	Mycosel	Junij	7	<i>act</i>	EXF-16977	BSL-1
<i>Clonostachys rosea</i>	Mycosel	Junij	2	ITS	EXF-16984	BSL-1
<i>Condenascus tortuosus</i>	SDA	Julij	7	ITS	EXF-17004	Ni podatka
<i>Curvularia buchloes</i>	SDA	September	33	ITS	EXF-17041	Ni podatka
<i>Curvularia inaequalis</i>	SDA	Julij	70	ITS	EXF-16997	BSL-1
<i>Exophiala equina</i>	Mycosel	Junij	2	ITS	EXF-16985	BSL-1
<i>Fusarium delphinoides</i>	SDA	Julij Avgust	18 300	<i>tef</i>	EXF-16993 EXF-17033	BSL-1
<i>Fusarium oxysporum</i>	SDA	Julij	20	<i>tef</i>	EXF-17000 EXF-17001 EXF-17002	BSL-2
<i>Fusarium solani</i>	SDA	Julij	22	<i>tef</i>	EXF-16994 EXF-16996	BSL-2
<i>Geotrichum candidum</i>	Mycosel	Avgust	233	ITS	EXF-17038	BSL-1
<i>Keratinophyton indicum</i>	Mycosel	Junij	7	ITS	EXF-16986 EXF-16988	BSL-1
<i>Leptobacillium leprobactrum</i>	Mycosel	Junij	2	ITS	EXF-17045	Ni podatka
<i>Metarhizium robertsii</i>	Mycosel	Junij	2	ITS	EXF-16982	BSL-1
<i>Mucor circinelloides</i>	SDA	Avgust	33	ITS	EXF-17034	BSL-1
<i>Penicillium citrinum</i>	Mycosel	Junij	2	<i>benA</i>	EXF-16987	BSL-1
<i>Penicillium lanosocoeruleum</i>	SDA	Julij	700	<i>benA</i>	EXF-16999	BSL-1
<i>Rhizopus arrhizus</i>	SDA	Junij Avgust September	65 33 17	ITS	EXF-16975 EXF-17037 EXF-17039	BSL-2
<i>Rhodotorula mucilaginosa</i>	SDA	Julij	100	ITS, LSU	EXF-16998	BSL-1
<i>Stachybotrys sp.</i>	Mycosel	September	435	ITS	EXF-17044	BSL-1
<i>Talaromyces pinophilus</i>	Mycosel	September	300	ITS	EXF-16976	BSL-1
<i>Trichosporon asahii</i>	SDA	Avgust	100	ITS	EXF-17029	BSL-2

Legenda:

¹ EXF številka; zaporedna številka seva v zbirki ekstremofilnih mikroorganizmov Ex, IC Mycosmo Biotehniška fakulteta, Univerza v Ljubljani² Varnostna stopnja (ang. Biosafety Level; BSL); podatek povzet po de Hoog in sod. (2020) in ATCC (2023).

V morski vodi se tekom kopalne sezone pojavljajo genetsko različni sevi bakterije *E. coli*

Nasprotno z vzorci peska mestne plaže, smo bakterijsko vrsto *E. coli* zasledili v vseh vodnih vzorcih morske vode tekom kopalne sezone 2022. Skupno smo osamili 49 izolatov, v nadaljne analize pa smo vključili 37 (76%) neklonalnih izolatov, ki so imeli glede na rezultate ERIC-PCR enake profile in so se uvrstili v 9 različnih ERIC skupin in so bili neenakomerno porazdeljeni tekom kopalne sezone; 5 izolatov junija, 3 izolati julija in 29 izolatov avgusta. Pri razvrstitvi v filogenetske skupine po prvotni metodi (Clermont in sod., 2000) so se sevi iz morske vode najpogosteje uvrstili v skupino A0 (41,67%), nato so po pogostosti sledile skupine A1 (31,25%), B1 (16,67%), in B23 (10,42%). Pri razvrstitvi sevov *E. coli* v filogenetske skupine po prenovljeni metodi (Clermont in sod., 2013) je pričakovano prišlo do manjše prerazporeditve sevov v druge skupine; sevi iz morske vode so se še vedno najpogosteje uvrstili v skupino A (67,6%), nato so si sledile skupine B2 (13,5%), C (10,8%) in B1 (8,1%). Sevov, ki so se pri razvrščanju v filogenetske skupine po prenovljeni metodi prerazporedili v drugačno skupino od prvotne, je bilo 10,8%.

Pri vseh neklonalnih izolatih *E. coli* (N = 37) smo s pomočjo metode PCR preverili prisotnost 18 različnih genov z zapisi za dejavnike virulence (DV), kot so adhezini, avtotransporterji, toksini, dejavniki povezani z odpornostjo proti serumu in izogibanjem imunskemu sistemu gostitelja ter sistemi za privzem železa. Med njimi je kar v 60 % najpogosteje prisoten gen za DV *fimH*, ki sodeluje pri tvorbi adhezina na koncu fimbrij tipa I in je faktor kolonizacije z bakterijami pri izvenčrevesnih infekcijah, omogoča pa tudi tvorbo bakterijskih biofilmov. V 27% oziroma 24 % smo zasledili tudi pojavnost genov *fyuA* in *irp2*, oba sodelujeta v sistemih za privzem železa. Med geni za dejavnike virulence, povezane z izogibanjem imunskemu sistemu, je prevladovala prisotnost genov *traT* in *kpsMTII*; oba smo potrdili pri 6 (16,2%) sevih.

Bakterije lahko sintetizirajo tudi posebne toksine, kot so genotoksini, ki povzročajo poškodbe DNA. Pri uropatogenih sevih *E. coli* se lahko pojavlja gen *usp* (angl. uropathogenic-specific protein) za genotoksin z DNazno aktivnostjo, ki deluje na celice sesalcev. Njegovo prisotnost povezujejo z obolenji, kot so pielonefritis (okužba ledvic), prostatitis (vnetje prostate) in bakteriemija (prisotnost bakterij v krvi). Pri sevih, osamljenih iz morske vode, smo

prisotnost gena *usp* potrdili pri 5 (13,5%) sevih.

Razmeroma veliko (7) je bilo genov, ki so bili prisotni izključno pri sevih iz filogenetske podskupine B2₃. To so bili geni *iha*, *papC*, *papGII*, *sfa*, *sat*, *vat* ter *ibeA*. Prav tako je bilo veliko genov, ki so v filogenetski podskupini B2₃ najbolj prevladovali, v manjšem deležu pa smo jih potrdili tudi pri sevih iz ostalih skupin. Gre za gene *fluA*, *hlyA*, *usp*, *fyuA*, *iroN*, *irp2* in *sitA*.

Pri vseh izolatih smo z metodo PCR testirali njihovo uvrstitev v sekvenčne skupine ST69, ST73, ST95 in ST131. Izmed skupno 37 neklonalnih izolatov so se 4 (10,8%) uvrstili v skupino ST131, po 1 (2,7%) izolat v skupino ST69, in en (2,7%) izolat v skupino ST95. V skupino ST73 nismo uvrstili nobenega izolata.

Izolate smo testirali tudi na prisotnost genov za betalaktamaze iz skupin CTX, SHV, TEM in OXA ter genov PMQR. Izmed preučevanih skupin genov za betalaktamaze je najpogosteje prisotna skupina genov *bla*_{TEM} (10,8%). Ostalih testiranih genov *bla* (petih preučevanih skupin genov *bla*_{CTX-M}), *bla*_{TEM} in *bla*_{OXA} nismo zaznali v nobenem izmed testiranih izolatov.

V preiskovanih izolatih smo ugotavljali prisotnost treh genov *qnr*, ki omogočajo odpornost proti nižjim koncentracijam kinolonov. Genov *qnrA* in *qnrS* nismo zaznali v nobenem izmed testiranih izolatov, smo pa zaznali en izolat z zapisom za gen *qnrB*. Vsi sevi, pri katerih smo potrdili prisotnost genov za odpornost proti protimikrobnim učinkovinam, so se uvrščali v filogenetsko skupino A po prenovljeni metodi (Clermont in sod., 2013).

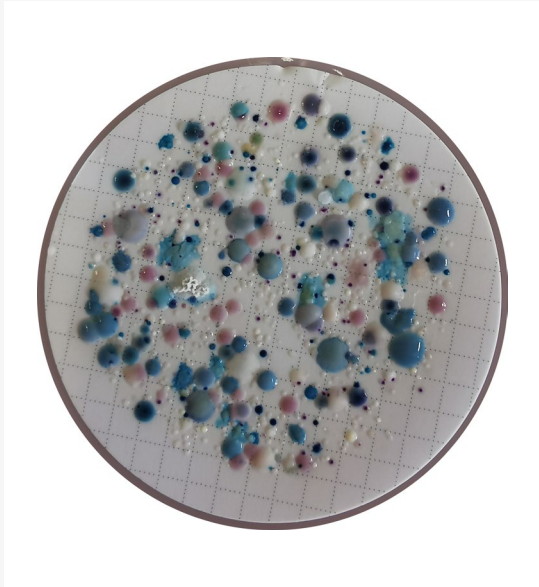
Razprava

Priljubljenost razvedrilnih dejavnosti, povezanih z vodo, narašča povsod po svetu. Hkrati pa tovrstna rekreacija omogoča stik s patogenimi mikroorganizmi. Možni načini za prenos le-teh na ljudi v kopalnih okoljih so preko kože, oči in sluhovoda ter zaužitjem kopalne vode ali vdihavanjem aerosolov (Solo-Gabriele et al. 2016). Kopalne vode (naravna rekreativna vodna okolja), kot jih definira direktiva kopalnih voda (European Environment Agency (EEA), so obalne ali celinske vode (reke, naravna jezera, zajetja in ribniki), v katerih je kopanje bodisi avtorizirano ali ni prepovedano in jih redno uporablja večje število kopalcev. V Sloveniji področje kakovosti kopalnih voda urejajo Zakon o vodah (ZV-1) (2002), Pravilnik o podrobnejših kriterijih za ugotavljanje kopalnih voda (2008) in Uredba

Tabela 2. Molekularna karakterizacija sevov *E. coli* iz morske vode. Klasifikacija preisovanih sevov v filogenetske skupine po prenovljeni metodi (Clermont in sod., 2013), prisotnost genov za dejavnike virulence ter prisotnost genov za odpornost proti protimikrobnim učinkovinom pri preiskovanih sevih.

Table 2. Molecular characterization of *E. coli* strains from seawater. Classification of the investigated strains into phylogenetic groups according to the revised method (Clermont et al., 2013), the presence of genes for virulence factors and the presence of genes for antimicrobial resistance in the investigated strains.

	MORJE N = 37 (100%) n (%)	
Filogenetske skupine	Clermont, 2013	
	A	25 (67,6%)
	B1	3 (8,1%)
	B2	5 (13,5%)
	C	4 (10,8%)
	D	0 (0%)
	E	0 (0%)
	F	0 (0%)
	Clade I/II	0 (0%)
Geni z zapisi za odpornost proti antibiotikom	Clade III/IV/V	0 (0%)
	Unknown	0 (0%)
	PMQR	
	<i>qnrA</i>	0 (0%)
	<i>qnrB</i>	1 (2,7%)
	<i>qnrS</i>	0 (0%)
	β-laktamaze	
	<i>bla</i> _{CTX-M-1}	0 (0%)
	<i>bla</i> _{CTX-M-2}	0 (0%)
	<i>bla</i> _{CTX-M-8}	0 (0%)
	<i>bla</i> _{CTX-M-9}	0 (0%)
	<i>bla</i> _{CTX-M-25}	0 (0%)
	<i>bla</i> _{TEM}	4 (10,8%)
	<i>bla</i> _{SHV}	0 (0%)
Geni z zapisi za dejavnike virulence	Adhezini	
	<i>afa/dra</i>	0 (0%)
	<i>fimH</i>	22 (59,5%)
	<i>iha</i>	0 (0%)
	<i>papC</i>	1 (2,7%)
	<i>papGII</i>	0 (0%)
	Avtotransporterji	
	<i>flaA</i>	1 (2,7%)
	<i>sat</i>	0 (0%)
	<i>vat</i>	5 (13,5%)
	Protektini	
	<i>iss</i>	0 (0%)
	<i>kpsMTII</i>	6 (16,2%)
	<i>ompTAPEC</i>	0 (0%)
	<i>traT</i>	6 (16,2%)
	Sistemi za privzem železa	
	<i>fyuA</i>	10 (27%)
	<i>iroN</i>	1 (2,7%)
	<i>irp2</i>	9 (24,3%)
	<i>iucD</i>	0 (0%)
	<i>iutA</i>	0 (0%)
	Toksini	
	<i>usp deg</i>	5 (13,5%)



Slika 2. Rast bakterijskih kolonij sevov *E. coli* iz morske vode. Filtracija 100 ml morske vode, vzorčene avgusta, preko nitroceluloznih filtrov Millipore s premerom por 0,45 µm in inokulacija in inkubacija na gojišču URISelect. Vrsta *E. coli* se na tem kromogenem gojišču obarva rožnato.

Figure 2. Bacterial colony growth of *E. coli* strains from seawater. Filtration of 100 mL of seawater sampled in August through millipore nitrocellulose filters with a pore diameter of 0.45 µm, inoculation, and incubation on URISelect medium. The *E. coli* species turns pink on this chromogenic medium.

o upravljanju kakovosti kopalnih voda (2008). Država zagotavlja spremljanje kakovosti kopalne vode na naravnih kopališčih in kopalnih območjih. Izdelan je tudi program monitoringa, ki določa parametre kakovosti, merilna mesta in pogostost spremljanja (Poje, 2021). Glede na 16. člen Uredbe lahko kopalno vodo razdelimo v štiri kakovostne razrede, in sicer na odlično, dobro, zadostno in na slabo. Ocena kakovosti se določi glede na mikrobiološko kakovost vode. Mikrobiološki parametri, ki se določajo v celinskih vodah, so enterokoki in bakterija *E. coli* (Uredba o upravljanju kakovosti kopalnih voda, 2008). V kopalnih vodah je pozornost namenjena predvsem prisotnosti fekalnih bakterij – *E. coli* in enterokokov, ki so v vodi kot posledica izpustov komunalnih čistilnih naprav, neurejene kanalizacije, spiranja obrežnih površin ob močnem dežju, iztrebkov živali in nenazadnje zaradi kopalcev. Ker te bakterije v slani vodi preživijo le kratek čas, nanje vpliva tudi sončna svetloba, jih v morju navadno ne zaznamo ali pa so njihove koncentracije zelo nizke (NLZOH, Kakovost morske kopalne vode, 2021). Smerne vrednosti za *E. coli* v morski vodi so do 500 CFU na 100 ml vzorčene vode (NLZOH, Kakovost morske kopalne vode, 2021). Glede na naše rezultate (5–29 CFU *E. coli* /100 ml morske vode) lahko sklepamo, da je bila morska voda v času našega vzorčenja skladna s parametri za dobro kakovost kopalne vode, kot jih določa metodologija za mikrobiološka vrednotenja po Uredbi o upravljanju kakovosti kopalnih voda (Uradni list RS, št. 25/08).

Bakterija *E. coli* je običajno komenzal v prebavnem traktu človeka. Plastičnost genoma zaradi horizontalnih prenosov genov in rekombinacij omogoča razvoj številnih, tudi za človeka patogenih sevov. Ti lahko povzročijo raznolika črevesna in zunaj črevesna obolenja. Nabor dodatnih genov za dejavnike virulence (DV) omogoča bakteriji kolonizacijo sicer sterilnih telesnih področij, izogibanje imunskemu sistemu, privzem železa iz okolja, izločanje toksinov ter posledično tudi boljše preživetje v različnih razmerah (Croxen in Finlay, 2010; Sarowska in sod., 2019). Da bi dobili vpogled v patogeni potencial *E. coli*, izolirane iz morske vode tekom kopalne sezone, smo pri vsakemu od 37 neklonalnih izolatov preverili prisotnost 18 genov, povezanih z DV. Neklonalni izolati so imeli različno število genov za DV (med 1 in 10). Kar 11 % vseh izolatov pa je imelo po 7 genov za DV in vsi so pripadali filogenetski skupini B2₃ po prvotni, oziroma B2 po prenovljeni metodi. Za to filogenetsko skupino je namreč značilno, da se vanje uvrščajo predvsem bolj virulentni sevi humanega izvora in

se posledično tipično pojavlja pri kliničnih izolatih *E. coli* (Clermont in sod., 2000). Hkrati je potrebno poudariti, da so bili vsi ti problematični izolati osamljeni iz vzorca vode v avgustu, ko je frekvenca kopalcev v morski vodi najvišja. Navkljub hipotezam pa natančnega vira kontaminacije oziroma organizma, iz katerega sev izhaja, ne moremo zagotovo potrditi. Poleg najverjetnejšega človeškega izvora, so pogost izvor patogenih bakterij lahko tudi galebi. V morebitnem nadaljevanju študije, bi bilo zato smiselno vzorčiti tudi iztrebke ptic (Ewbank in sod., 2022).

Poleg povezave med filogenetsko skupino sevov *E. coli* in njihovim izvorom lahko v literaturi zasledimo tudi navedbe o povezanosti filogenetskih skupin s tipom seva oziroma njegovim virulentnim potencialom (Ishii in sod., 2007). Sevi iz skupin A in B1 so običajno komenzali, z manjšim številom genov za dejavnike virulence, medtem ko so sevi iz filogenetske skupine B2 (in v manjši meri skupine D) pogosto bolj virulentni, zaradi večjega nabora genov za dejavnike virulence (Clermont in sod., 2000; Branger in sod., 2005). V naših vzorcih so prevladovali sevi iz filogenetske skupine A (68%), kateri so sledili sevi iz skupin B2 (14%), C (10%) in B1 (8%). Prisotnosti ostalih filogenetskih skupin nismo potrdili.

Horizontalni prenos genov (HGT) povezanih z geni, ki posredujejo odpornost proti protimikrobnim učinkovinam, predvsem antibiotikom, je eden glavnih razlogov za širjenje odpornosti proti protimikrobnim učinkovinam in vitro in in vivo okoljih, med katerimi imajo mobilni genetski elementi (MGE) pglavitno vlogo pri širjenju bakterijske odpornosti (Stadler in sod., 2017). Horizontalni prenosi genov omogočajo bakteriji *E. coli* hitro pridobivanje odpornosti tudi proti več protimikrobnim učinkovinam hkrati. Kinoloni in β-laktami so protimikrobne učinkovine, ki se po vsem svetu široko uporabljajo pri zdravljenju številnih nalezljivih bolezni, ki jih povzročajo okužbe s po Gramu negativnimi bakterijami, kot so *Enterobacteriaceae*, še zlasti z *E. coli*. Hkrati se v populaciji že pojavljajo sevi, ki so odporni proti navedenim antibiotikom, zlasti zaradi posedovanja genov za determinante odpornosti na mobilnih genetskih elementih. Pri izoliranih sevih smo preverili prisotnost plazmidno zapisanih genov, ki so povezani z nizko odpornostjo proti kinolonu (PMQR). Za testiranje smo izbrali gene *qnrA*, *qnrB* in *qnrS*. Med vsemi izolati smo samo pri enem sevu, izoliranem iz avgustovskega vzorca vode, potrdili zapis za *qnrB*, ki se med *qnr* geni najpogosteje pojavlja in je velikokrat tudi so-lokaliziran na istem plazmidu skupaj z geni *bla* (pretežno *qnrB1* in *bla*_{CTX-M-9}, *bla*_{CTX-M-3} ali *bla*_{SHV-12}) v Evropi, Združenih državah, Aziji in Afriki (Juraschek in sod., 2022). Pri tem

istem sevu drugih genov za odpornosti proti protimikrobnim učinkovinam nismo potrdili. V povezavi s pojavljanjem in širjenjem *E. coli*, odpornih proti protimikrobnim učinkovinam sodijo med najbolj problematične sevi, ki izločajo encime β -laktamaze z razširjenim spektrom delovanja (ESBL; angl. Extended spectrum β -lactamases). Sevi ESBL imajo lahko genetski zapis *bla* za encime skupine TEM, SHV ali CTX (Rasheed in sod., 1997). Med našimi izolati so bili prisotni sevi *E. coli*, ki so bili odporni proti protimikrobnim učinkovinam. Z genotipizacijo smo potrdili gen *bla*_{TEM} pri 11% izolatov, vsi so bili osamljeni v avgustovskem vzorcu vode in spadajo v filogenetsko skupino A1 po prenovljeni metodi. Prisotnost zgolj genskega zapisa *bla*_{TEM} nas ne preseneča, saj tudi v literaturi zasledimo podatke, da se omenjeni gen uvršča med pri *E. coli* najpogostejše prisotne zapise za β -laktamaze (Branger in sod., 2005; Bou in sod., 2002). Pri ostalih sevih nismo zasledili testiranih genov za odpornost.

Čeprav se sevi *E. coli* uvrščajo v številne sekvenčne tipe (ST), se približno polovica vseh izvenčrevesnih sevov *E. coli* uvršča v štiri prevladujoče tipe (ST69, ST73, ST95 in ST131) (Horner in sod., 2014). Med temi prevladujejo sevi ST131 z geni za številne virulentne dejavnike in odpornostjo proti različnim protimikrobnim učinkovinam (Hojabri in sod., 2019). Šestim izolatom smo lahko določili sekvenčno skupino, en je pripadel sekvenčni skupini ST69, drugi ST95, štirje neklonalni izolati (10,8 %) pa sekvenčni skupini ST131. Sekvenčna skupina ST131 se je pojavila šele po letu 2000 in predstavlja pomemben človeški patogen, ki se je močno razširil po vsem svetu in je odgovoren za hitro povečanje protimikrobne odpornosti med *E. coli* (Peirano in sod., 2010). Znano je, da ST131 povzroča zunaj črevesne okužbe, saj je odporen proti fluorokinolonom in je povezan s proizvodnjo beta laktamaz z razširjenim spektrom delovanja, najpogostejše zaradi prisotnosti gena *bla*_{CTX-M-15} (Nicolas-Chanoine et al., 2014). Vsi štirje neklonalni izolati ST131 so se uvrstili v filogenetsko skupino B2₃ po prvotni razporeditvi, imeli hkrati prisotnih med 7 in 10 genov za dejavnike virulence, medtem ko genov za odpornost proti protimikrobnim učinkovinam (CTX, TSO in PMQR) pri njih nismo potrdili.

Za razliko od bakterij in virusov so glive kot mikrobiološki pokazatelj ustreznosti vode popolnoma prezrte. Glive kot heterotrofi imajo ključno vlogo pri kroženju organskih snovi v okolju. Pomembne so predvsem z vidika razgradnje dolgoveriznih in kompleksnih ogljikovodikov kot so hitin, celuloza, lignin pa tudi nafta in njeni derivati, guma, silikoni in plastike (Lumibao in sod., 2018; Neto in sod., 2019).

Umetni materiali so zaradi naraščajoče človeške populacije, urbanizacije in industrializacije vedno pogostejše zastopani v naravnem okolju, eno od teh so tudi peščene plaže in morje (Pérez-Alvelo in sod., 2021; Bridson in sod., 2020). V zadnjih desetletjih so glive tudi vedno pogostejše povezane z različnimi človeškimi boleznimi, od blagih do kroničnih (de Hoog in sod., 2020; Pathakumari in sod., 2020). V nasprotju z virusnimi ali bakterijskimi okužbami se simptomi glivnih okužb pojavijo pozneje in jih je zato težje povezati s primarnim okoljskim virom (Bongomin in sod., 2017). Raziskave o njihovem številu in raznolikosti v naravnih okoljih so zato pomembne za zapolnitev vrzeli o izvoru in pogostosti vpliva na zdravje ljudi, k čemur poziva tudi dokument, izdan s strani WHO (WHO, 2022).

V vseevropski iniciativi »Mycosands« pri preučevanju gliv v pesku plaž in priobalnem morju sodeluje 13 držav, med njimi tudi Slovenija. Cilj je raziskati raznolikost in številčnost gliv v različnih okoljskih razmerah po evropski celini ter oceniti tveganje za zdravje, ki bi ga glive lahko predstavljale za ljudi (Brandão in sod., 2021). Vzorčenje v Sloveniji je potekalo na urbani plaži v centru Portoroža kot mesečni monitoring v času kopalne sezone med junijem in septembrom 2022. Plažo od morja ločuje betonska pešpot, ki preprečuje odplavljanje peska zaradi plimovanja. Pesek zaposleni redno čistijo, odstranjujejo odpadke in ga mehansko pregrabljajo. Plaža redno prejema nagrado Modra zastava kot sinonim za kakovost in varnost (FEE, 2023). V sklopu monitoringa smo vzorčili oboje, morsko vodo na pomolu nasproti plaže in pesek. Z uporabljeno metodo iz morske vode nismo izolirali gliv. Da bi uspeli izolirati tudi glive iz morja, bi morali uporabiti gojišča z dodatki soli, vodo pa filtrirati, s čemer bi vzorec skoncetrirali (Novak Babič in sod., 2022). Gojišča, predlagana v iniciativi »Mycosands« so specializirana za osamitev gliv iz peska. Tekom monitoringa smo tako osamili 32 glivnih vrst iz 22 rodov. Rezultati so primerljivi z monitoringom prejšnje študije (Novak Babič in sod., 2022) in drugih svetovnih študij, ki poročajo o velikem številu in pestrosti gliv, tudi ko je pesek suh, izpostavljen močnemu soncu in dobro prezračen (Weiskerger in sod., 2020 Londoño in sod., 2018; Frenkel in sod., 2020). V pesku so konstantno prisotne glive »jedrne mikrobiote« *Aspergillus*, *Aphanoascus*, *Fusarium* in *Rhizopus*, njihovo število pa variira glede na mesec vzorčenja (Slika 1). Vrsta raznolikost je bila najvišja junija, prisotne pa so bile glive, ki so povezane predvsem z rastlinami in žuželkami (Novak Babič in sod., 2022). Junija so vremenski pogoji najugodnejši za razrast in cvetenje okoliške

vegetacije, obisk ljudi pa je še razmeroma nizek. Nasprotno pa smo avgusta iz peska osamili oportuno patogene vrste *Candida parapsilosis*, *Geotrichum candidum* in *Trichosporon asahii*, ki poseljujejo človeško kožo, nohte in lase (de Hoog in sod., 2020). Prav tako so pogoste v urbanih področjih, v gospodinjstvih, odpadnih vodah in na domačih živalih, ki bi lahko poleg ljudi predstavljale vir vnosa teh gliv na plažo. Njihovo število je predstavljalo skoraj dve tretjini vseh gliv. Podatek je pomemben, saj smo drugič zapovrstjo v času kopalne sezone v pesku te plaže zasledili glive, ki so najpogostejše vezane na človeški vir ali domače živali (Novak Babič in sod., 2022). S tem smo potrdili rezultate prejšnje študije, ki opisuje pesek kot prehodni rezervoar za oportune patogene ljudi (Brandão in sod., 2021; Novak Babič in sod., 2022). Rodove *Candida*, *Geotrichum*, *Trichosporon* in *Meyerozyma* bi v prihodnosti lahko uvrstili na seznam indikatorskih mikroorganizmov za spremljanje kakovosti peska.

Sklepi

Svetovna zdravstvena organizacija in Evropska Unija spodbujata raziskave mikroorganizmov v okolju, da bi sledili virom porajajočih se patogenov. Prisotnost mikrobov (tudi gliv) v pesku urbanih in naravnih plaž je v zadnjih letih dobro raziskana. Medtem ko bakterije in viruse ponavadi povezujejo z viri fekalnega onesnaženja pa so glive pogosto povezane s prisotnostjo rastlin, divjimi in domačimi živalmi in sestavo peska na plažah. Na vrhuncu turistične sezone smo iz peska osamili oportuno patogene glive, medtem ko jih v morski vodi nismo zaznali. Obraten trend pa smo

zasledili pri bakteriji *E. coli*, katere prisotnost nismo potrdili v vzorcih peska, ampak le v morski vodi tekom celotne kopalne sezone v dovoljenih vrednostih. Pri osamljenih sevih *E. coli* smo potrdili različen nabor genov za dejavnike virulence, ki predstavljajo vir možnega širjenja v druge, zaenkrat še neproblematične bakterijske vrste. Medtem ko je *E. coli* ustrezen indikator za spremljanje kakovosti morske vode, pa rezultati študije nakazujejo potrebo po uvedbi dodatnih indikatorskih mikroorganizmov za nadzor kakovosti peska.

Author Contributions

Koncept, metodologija, validacija, analize, urejanje podatkov in pisanje prispevka J.Č.Z. in M.N.B. Vsi avtorji so prebrali objavljeno različico rokopisa in se z njo strinjali.

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Conflicts of Interest

Avtorji ne navajajo navzkrižja interesov.

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Original Research

A detailed survey on fungal distribution and characterization under Bankura District, West Bengal, India

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Abstract

Fungi are eukaryotic organisms that exhibit heterotrophic nutrition. The abundance of wild edible mushrooms during the rainy season offers a valuable opportunity for indigenous communities to capitalize on these natural resources to generate income and support their local economy. This study investigates fungal diversity in three unique forest ecosystems, specifically Bishnupur, Ramsagar, and Onda, through a comprehensive survey and documentation of their distinct fungal populations. A total of 24 fungal species were collected and identified, comprising eight edible, four poisonous, and 12 inedible species. The anatomical study showed that the gills are parallelly arranged, basidia mostly club-shaped, and spores were bilayered and brown. The Simpson's Diversity Index values were 0.22 for Bishnupur, 0.32 for Ramsagar, and 0.36 for Onda Forest. The collected fungi predominantly belonged to the division Basidiomycota, except for one species, *Daldinia concentrica*, which was classified under the family Xylariaceae (Ascomycota). The study revealed that the Agaricaceae family comprises the highest number of genera within the total fungal population. Basidiomycota members exhibited a dominant presence in the region, accounting for 95.8% of the total occurrence. In contrast, Ascomycota members had a relatively minor representation, accounting for only 4.2%.

Keywords

Fungal distribution, fungal morphology, fungal anatomy, Basidiomycota, Ascomycota

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Podroben pregled razširjenosti in značilnosti gliv v okrožju Bankura, Zahodna Bengalija, Indija

Izvleček

Glive so heterotrofni evkariontski organizmi. Obilje divjih užitnih gob v deževnem obdobju ponuja avtohtonim skupnostim dragoceno priložnost, da izkoristijo te naravne vire za ustvarjanje dohodka in podporo svojemu lokalnemu gospodarstvu. V študiji smo preverili raznolikost gliv v treh edinstvenih gozdnih ekosistemih, zlasti v Bishnupurju, Ramsagarju in Ondi. Skupaj je bilo zbranih in določenih 24 vrst gliv, od tega 8 užitnih, 4 strupene in 12 neužitnih vrst. Anatomska študija je pokazala, da so resice vzporedno razporejene, bazidiji večinoma paličaste oblike, spore so dvoslojne in rjave barve. Vrednosti Simpsonovega indeksa raznolikosti so bile 0,22 za Bishnupur, 0,32 za Ramsagar in 0,36 za gozd Onda. Zbrane glive so večinoma pripadale drevesu Basidiomycota, z izjemo vrste *Daldinia concentrica*, ki je bila uvrščena v družino Xylariaceae (Ascomycota). Študija je pokazala, da družina Agaricaceae obsega največ rodov v celotni populaciji gliv, pripadniki Basidiomycota pa so v regiji prevladovali, saj so predstavljali 95,7 % celotnega pojavljanja, medtem ko so bili pripadniki Ascomycota zastopani razmeroma malo, le 4,3 %.

Ključne besede

Razporeditev gliv, morfologija gliv, anatomija gliv, Basidiomycota, Ascomycota

Introduction

Fungi are the eukaryotic organism lacking chlorophyll. They may be saprophytic, parasitic or symbiotic or coprophilous. They are cosmopolitan in distribution. Fungi are seen abundantly in forest areas mainly due to the humus in soil. Out of 14,000 fungal flora, 7000 species have food value, among which 3000 edible fungi have been considered prime food.

On the other hand, the number of cultured fungi is approximately 200, the number of cultivated fungi is 100, even 60 species are used for commercial purposes, and 10 specimens have industrial properties (Chang & Miles, 2004). It has been reported that 10% of total fungi show poisonous activity, and 30 species are thought to be lethal (Deshmukh et al., 2006). According to Hawksworth & Lucking (2017), approximately 2.2-3.8 million fungal flora have been reported worldwide. In India, 850 species have been collected (Deshmukh et al., 2004). The macrofungi are collected in epigeous or hypogeous conditions; only fructifications are visible to the naked eye. The fructification easily attracts anyone because of its colourful pilus, which may vary from fungus to fungus. It is reported that most macrofungi belong to Basidiomycota or Ascomycota, but some are Zygomycota members (Yusran et al., 2021). It is

an important factor for biodegradation and facilitates other plants' growth. It acts as a bioindicator for the health of the forest ecosystem (Stametes, 2000). Fungi have been considered the second greatest community after insects (Yusran et al., 2021). Recently, it has been revealed that the tropical regions show greater diversity than the temperate regions (Suryanarayanan et al., 2003; Singha et al., 2017). Different fungi act as seasonal food, making a profitable income source for the local tribes throughout the rainy season. Some fungi also have medicinal uses (Nad et al., 2021). Panda et al. (2019) have reported 1,50,000 fungi worldwide, among them 14,000 considered as macro type. Few are used as ingredients in bakeries to make cakes and bread. In India, fungus is most underrated, thus getting less attention; as a result, people are unaware of its impact value. In West Bengal of India, the fungal population is widely distributed. At present, many mycologists are curious to know about their habitat, distribution, and social value. The macrofungal biodiversities and their morphological and anatomical studies, as collected from the Bankura District of West Bengal, India, have been focused in the present work.

Materials and Methods

The study area

Three places in Bankura District, respectively, Bishnupur, Ramsagar, and Onda were surveyed from September 2021 to October 2022 and the climatic condition of the entire study area (Table 1), which is favourable for the fungal population.

Bishnupur forest is naturally deciduous, where Sal (*Shorea robusta*), Segun (*Tectona grandis*), Mahogany (*Swietenia mahagoni*), Akashmoni (*Acacia auriculiformis*) are the dominating flora. Tropical climatic conditions and red laterite soil are important factors for floral growth (Ganguly et al., 2018a). Onda forest is also deciduous with a tropical savanna climate and laterite rocky soil. The vegetation mainly includes Sal (*Shorea robusta*), Sengun (*Tectona grandis*), Kendu (*Diospyros melanoxylon*), and Bamboo (*Bambusa sp.*). Ramsagar also represents a deciduous forest where laterite and sandy soil combine with the tropical environment to grow Sal, Segun, Mahogany, Bamboo, and Arjun (*Terminalia Arjuna*). The animal diversity of these study areas include different types of birds, snakes, wild lizards, ants, butterflies, beetles, jackals, foxes, honey bees, and rabbits.

Process of collection

The fungal species have been collected from the Bishnupur forest (extended almost 133 square kilometres), Ramsagar forest (covers an area of 60 square kilometres) and Onda forest (extended approximately 100 square kilometres) (Fig. 1).

The collection was made using the quadrant method, which used essential appliances like sharp knives, digging tools, gloves, and plastic bags. The fruit bodies have been collected from the underground mycelia with the help of a knife or sometimes out of the reproductive structure by digging the soil. Only healthy specimens have been collected for each species. During the survey, morphological characteristics were documented, and photographs of the macro-fungal species in their natural habitat were taken, capturing their original conditions and providing a visual record for further study and identification (Fig. 2).

Process of preservation

The collected wet fungi were preserved with 4% formalin solution in separate plastic jars, and dry fungi were preserved in plastic zipper pouches separately.

Preparation of Slide

The sectioned specimens were placed on the slide, and 2-3 drops of the lactophenol-cotton blue solution were added for staining. The stained specimen was then covered with a cover slip, and the extra solution was removed by blotting paper. Heat was applied to the slide for 1-2 minutes and the specimen was subsequently mounted by wax for microscopic study.

Method of identification

Specimens have been identified following the standard protocol (Ganguly et al., 2021; Holec & Kolarik, 2011; Gogoi and Vipin, 2015; Pradhan et al., 2009). The length and width of both spores and gills have been measured with the help of an Ocular and stage micrometre for fungal identification.

Statistical methods

The frequency has been calculated using the following formula (Aung et al. 2008).

$$\text{Frequency(\%)} = \frac{\text{presence of total number of a group}}{\text{total number of all groups}} \times 100$$

Simpson's Index was calculated for the individual location to estimate the diversity of the sporocarps.

Results

Observation of fungal distribution

The fungal population of Bankura District, including forest areas of Bishnupur, Ramsagar, and Onda, shows dominance throughout the rainy season, and it rules over other flowering plants as well as thallophyta at that time. Out of 24 fungal species that were collected, 23 genera belongs to 15 families, among which 18 fungi were identified up to species level (Table 2).

Table 1. The locations of the study area and the meteorological data

Tabela 1. Meteorološki podatki za vzorčne lokacije

Study Area	Latitude	Longitude	Altitude	Temp.	Humidity	Rainfall	Source of information
Bishnupur	23° 04' 48" N	87° 19' 12" E	59 m	23°C to 29°C (26°C average)	73-96% (85% average)	2289-6170 mm of rain approximately (average 4230mm precipitation)	Different journal papers and power access climate data
Ramsagar	23° 06' 22" N	86° 16' 37" E	64 m	26°C to 29°C (27.5°C average)	83-85% (84% average)	2288-6169 mm of rain approximately (average 4229 mm precipitation)	(https://power.larc.nasa.gov/data-access-viewer)
Onda	23° 08' 31" N	87° 12' 07" E	74 m	25°C to 28°C temperature (26.5°C average)	82-84% of humidity (83% average)	2288-6169 mm of rain approximately (average 4229 mm precipitation)	

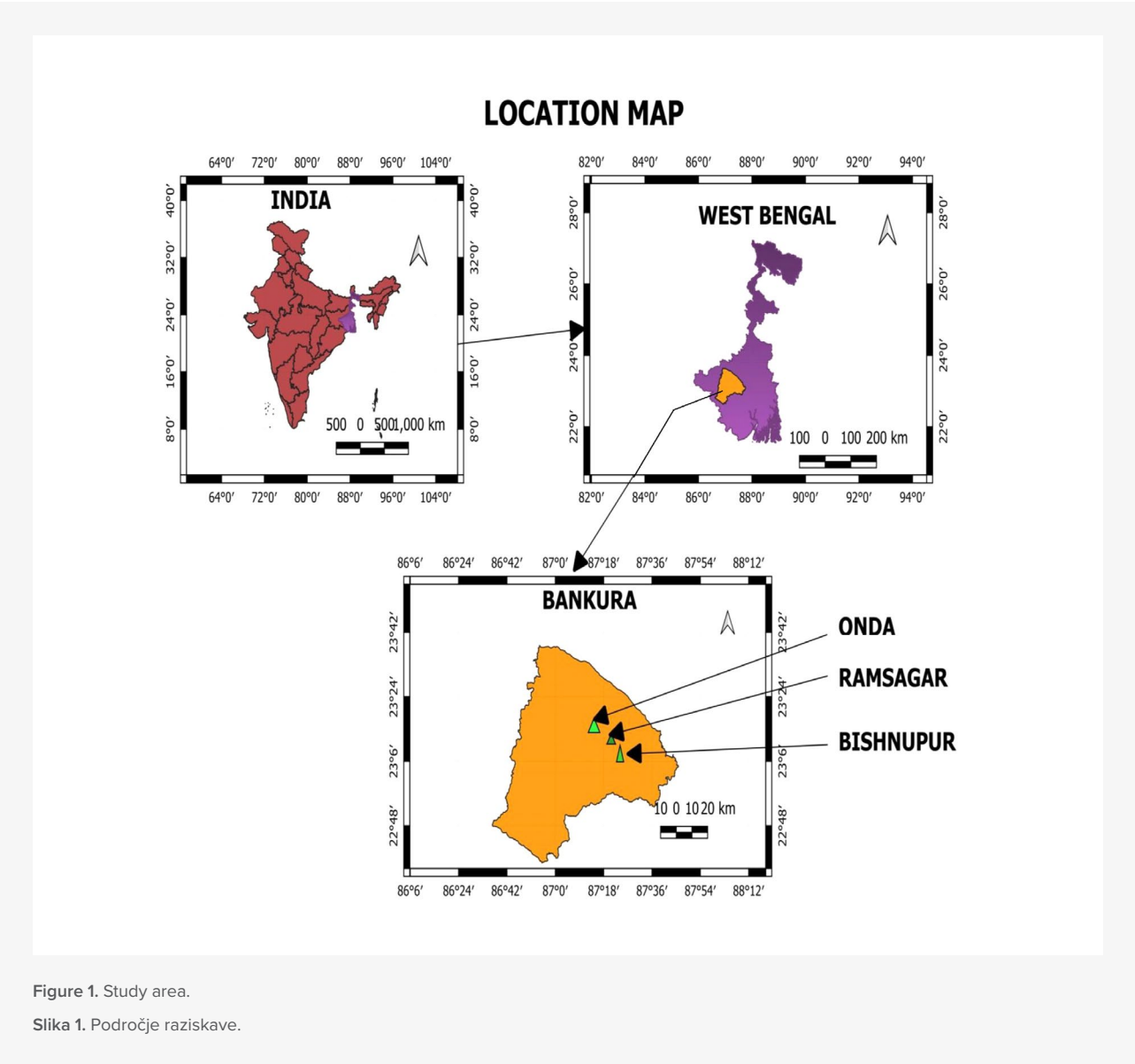


Figure 1. Study area.
Slika 1. Področje raziskave.

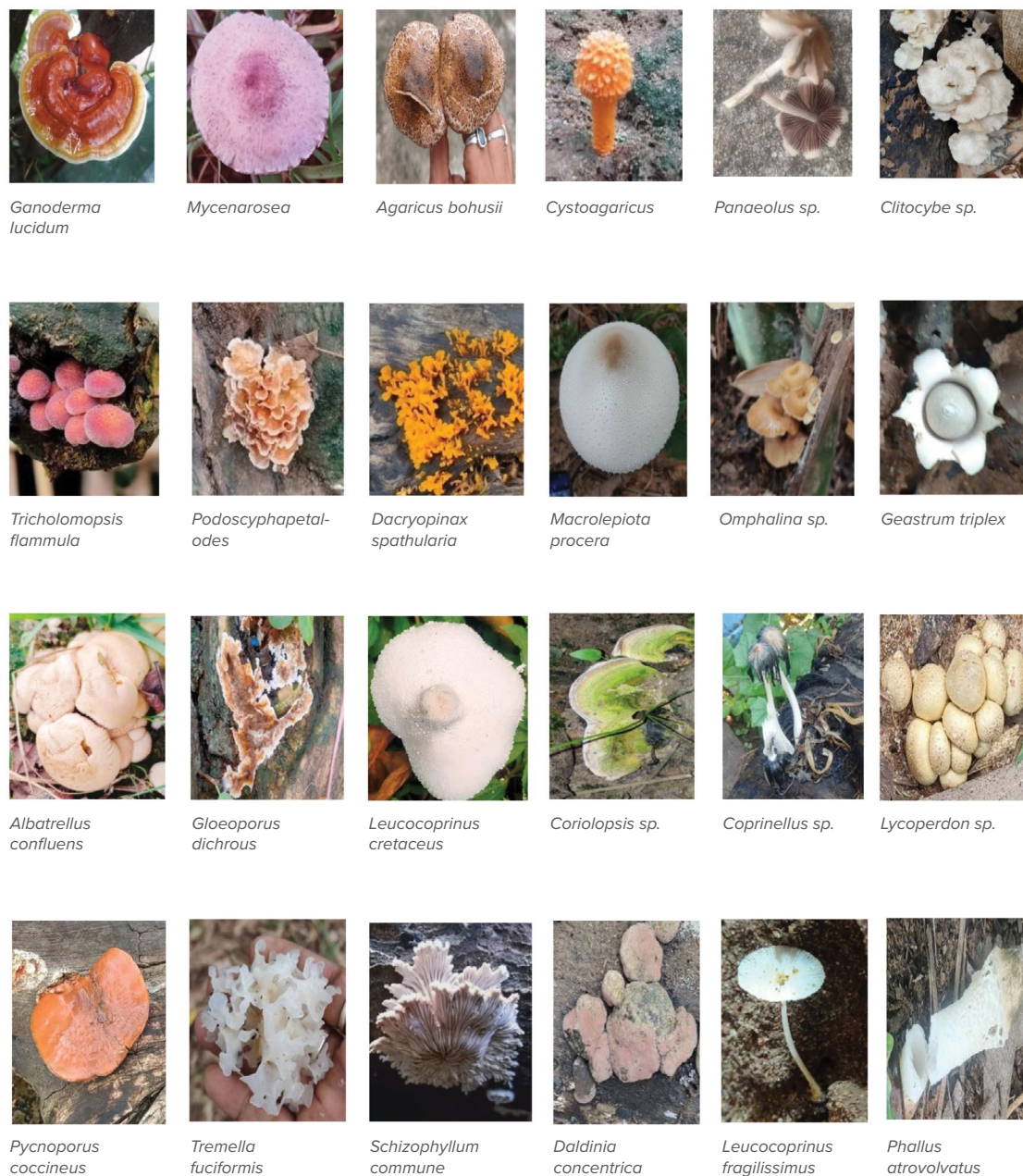


Figure 2. A collection of different fungi from the Bankura District.

Slika 2. Zbirka nabranih gliv iz okrožja Bankura.

Table 2. Collected fungi with their respective families and places of collection.

Tabela 2. Nabrane gljive, taksonomska družina in kraj najdbe.

Name	Family	Number of Individuals	Site of Collection	Status
<i>Ganoderma lucidum</i>	Ganodermataceae	7	Bishnupur, Ramsagar, Onda	Edible and medicinal
<i>Mycena rosea</i>	Mycenaceae	1	Onda	Poisonous
<i>Agaricus bohusii</i>	Agaricaceae	2	Bishnupur	Edible
<i>Cystoagaricus trisulphuratus</i>	Psathyrellaceae	1	Onda	Poisonous
<i>Tricholomopsis flammula</i>	Tricholomataceae	12	Ramsagar	Inedible
<i>Podocypha petalodes</i>	Meruliaceae	17	Ramsagar	Inedible
<i>Panaeolus sp</i>	Bolbitiaceae	4	Bishnupur	Edible
<i>Clitocybe sp</i>	Tricholomataceae	10	Ramsagar	Edible
<i>Coprinellus sp</i>	Agaricaceae	8	Bishnupur	Inedible
<i>Lycoperdon sp</i>	Agaricaceae	16	Onda	Inedible and Medicinal
<i>Pycnoporus coccineus</i>	Polyporaceae	3	Ramsagar	Inedible
<i>Tremella fuciformis</i>	Tremellaceae	4	Bishnupur	Edible
<i>Schizophyllum commune</i>	Schizophyllaceae	7	Bishnupur, Ramsagar	Edible and Medicinal
<i>Daldinia concentrica</i>	Xylariaceae	10	Ramsagar	Inedible and medicinal
<i>Leucocoprinus fragilissimus</i>	Agaricaceae	1	Onda	Inedible
<i>Phallus atrovolvatus</i>	Phallaceae	5	Bishnupur	Edible
<i>Dacryopinax spathularia</i>	Dacrymycetaceae	100	Ramsagar, Bishnupur	Inedible
<i>Macrolepiota procera</i>	Agaricaceae	1	Bishnupur	Poisonous
<i>Omphalina sp</i>	Tricholomataceae	6	Ramsagar	Inedible
<i>Geastrum triplex</i>	Geastraceae	2	Bishnupur	Inedible and medicinal
<i>Albatrellus confluens</i>	Albatrellaceae	6	Onda	Edible
<i>Gloeoporus dichrous</i>	Meruliaceae	2	Bishnupur	Inedible
<i>Leucocoprinus cretaceous</i>	Agaricaceae	3	Bishnupur	Poisonous
<i>Coriopsis sp</i>	Polyporaceae	3	Onda	Inedible

Among the 15 families, Agaricaceae is the most dominant family with about 21% of the total fungal population (Fig. 3) in the study area. These represent five different genera, respectively *Agaricus bohusii*, *Macrolepiota procera*, *Leucocoprinus sp*, *Coprinellus sp* and *Lycoperdon sp*. Of 23 genera, only *Leucocoprinus* has two species: *Leucocoprinus cretaceous* of Bishnupur forest and *Leucocoprinus fragilissimus* of Onda forest. A total of 70 individual species under 12 genera have been collected from the Bishnupur forest. A total of 132 individuals under nine genera were collected from the Ramsagar forest, and

a total of 29 individuals under 7 genera were collected from the Onda forest (Table 3).

Among the 24 species, *Dacryopinax spathularia* have been collected in the highest number, about 100 individuals. During the survey, it was noticed that Bishnupur had covered 43% of the total genera with Ramsagar covering 32% and Onda covering 25% of the total genera (Fig. 4) under the Bankura District. The Venn diagram shows a graphical representation of the fungal diversity across different study areas, highlighting the shared and distinct species found in each region (Fig. 5).

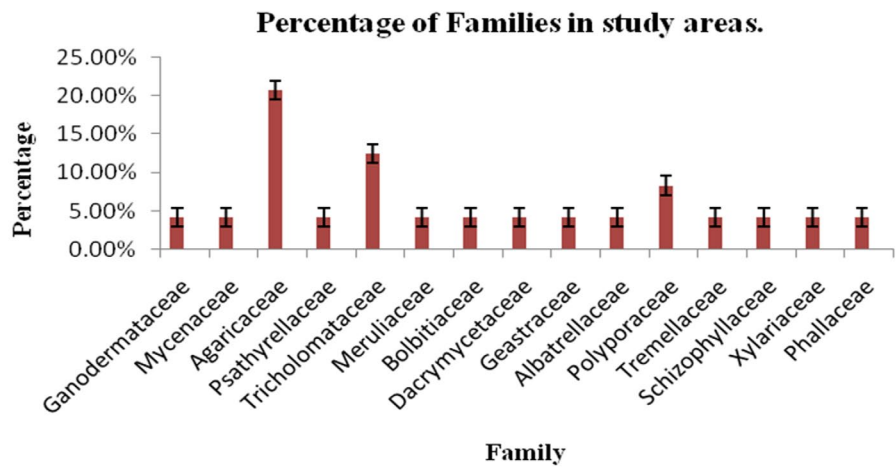


Figure 3. Percentage of families in study areas.
Slika 3. Procenti glivnih družin na področju vzročenja.

Table 3. The number of individual collected species in the study area.
Tabela 3. Število nabranih primerkov posamezne glivne vrste glede na vzorčno lokacijo.

Name of fungus	Area of collection		
	Bishnupur Forest	Ramsagar Forest	Onda Forest
<i>Ganoderma lucidum</i>	4	2	1
<i>Mycena rosea</i>	-	-	1
<i>Agaricus bohusii</i>	2	-	-
<i>Cystoagaricus trisulphuratus</i>	-	-	1
<i>Tricholomopsis flammula</i>	-	12	-
<i>Podocypha petalodes</i>	-	17	-
<i>Panaeolus sp</i>	4	-	-
<i>Clitocybe sp</i>	-	10	-
<i>Coprinellus sp</i>	8	-	-
<i>Lycoperdon sp</i>	-	-	16
<i>Pycnoporus coccineus</i>	-	3	-
<i>Tremella fuciformis</i>	4	-	-
<i>Schizophyllum commune</i>	5	2	-
<i>Daldinia concentrica</i>	-	10	-
<i>Leucocoprinus fragilissimus</i>	-	-	1
<i>Phallus atrovolvatus</i>	5	-	-
<i>Dacryopinax spathularia</i>	30	70	-
<i>Macrolepiota procera</i>	1	-	-
<i>Omphalina sp</i>	-	6	-
<i>Geastrum triplex</i>	2	-	-
<i>Albatrellus confluens</i>	-	-	6
<i>Gloeoporus dichrous</i>	2	-	-
<i>Leucocoprinus cretaceous</i>	3	-	-
<i>Corioloopsis sp</i>	-	-	3

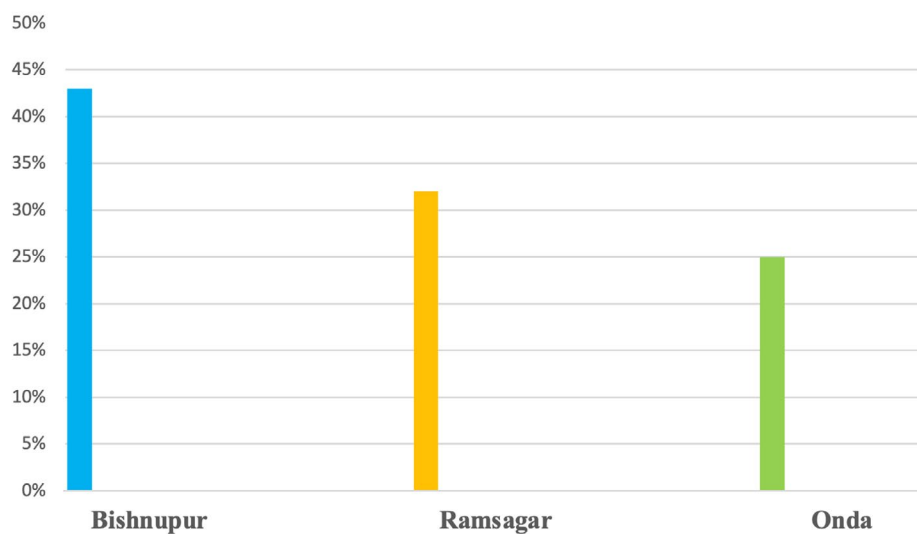


Figure 4. Percentage of total genera in a particular study area.

Slika 4. Skupni procenti glivnih rodov na posamezni vzorčni ploskvi.

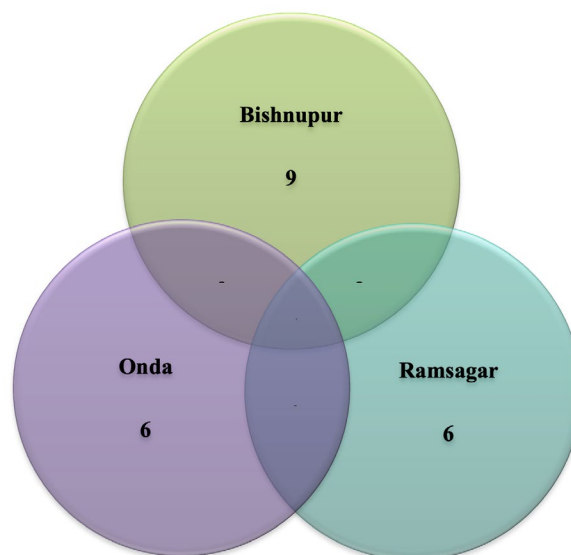


Figure 5. Venn diagram showing representation of fungal genera from different study areas.

Slika 5. Vennov diagram glivnih rodov po posamezni vzorčni ploskvi.

In respect to edibility, the fungi are 33%, the poisonous fungi are 17%, and inedible fungi are 50% all over the study area (Fig. 6). From the survey, it also observed that collected fungal species have not been distributed same in number in all the study areas because soil of Bishnupur forest is more fertile even moisture and rainfall quite high than other two study areas so Bishnupur have been shown higher fungal population. However, Onda forest is more remarkable for its unique fungal species than Bishnupur forest as *Mycena rosea* (about 3.4%) and *Cystoagaricus trisulphuratus* (about 3.4%) have been collected from Onda forest which are rarely seen where *Coprinellus sp* (about 11.4%) only unique fungus have collected from Bishnupur forest. It has also been found that *Ganoderma lucidum* is

the only species which was collected from all three study areas; similarly, only two fungal species, namely *Schizophyllum commune* and *Dacryopinax spathularia*, are common in both the forest areas of Bishnupur and Ramsagar. The Simpson's diversity index has been shown in Bishnupur forest as 0.22, in Ramsagar as 0.32 and in Onda as 0.36. Species richness has been reported in Bishnupur forest, and species evenness has been reported in Onda at about 0.88. This study has also revealed that 95.8% of specimens belong to the phylum Basidiomycota, and 4.2% of specimens belong to the phylum Ascomycota (*Daldinia concentrica*, Xylariaceae) as diversified in Bankura District (Fig. 7). The study has identified eight medicinal fungi, validating their traditional use by tribal communities (Table 4).

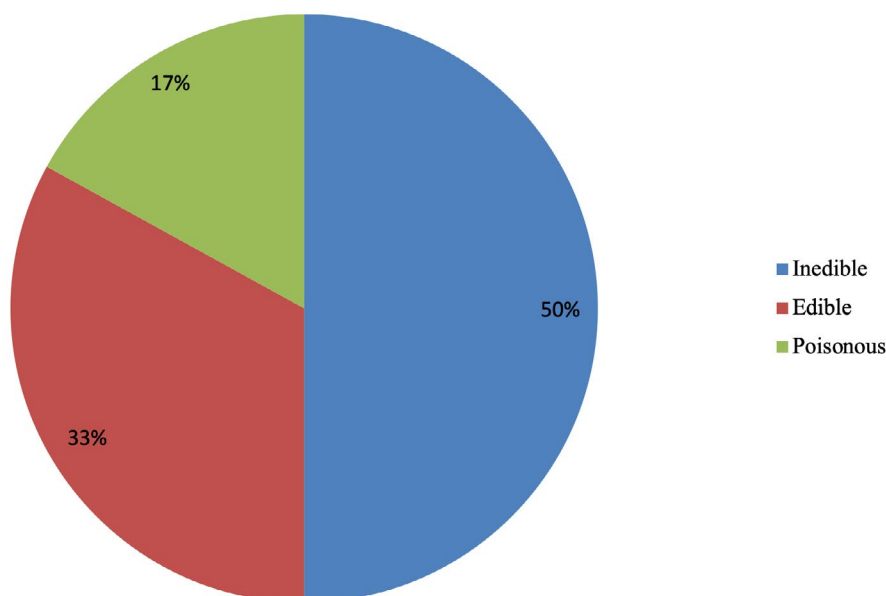


Figure 6. Percentage of collected fungi according to edibility.

Slika 6. Procenti nabranih gliv glede na užitnost.

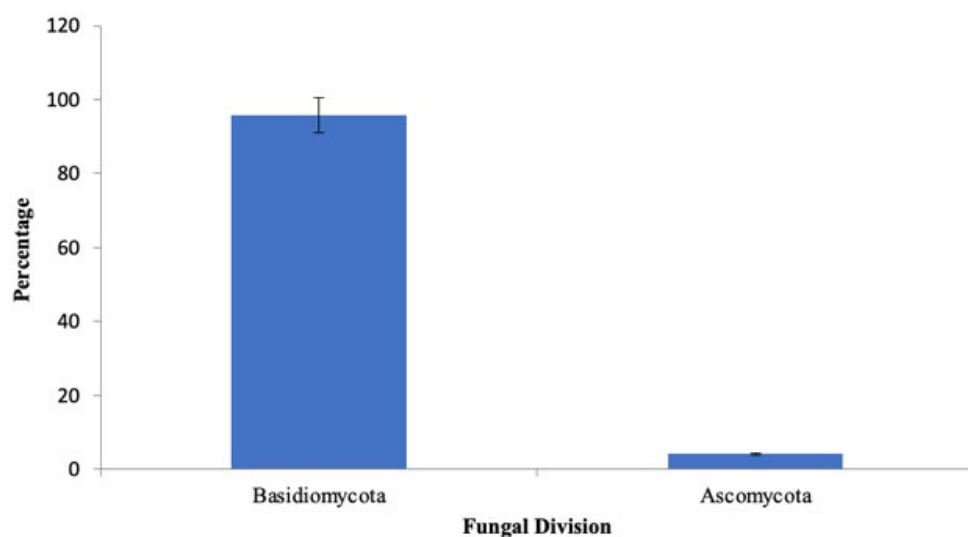


Figure 7. Percentage of fungal division.

Slika 7. Procent posameznih glivni taksomskih debel.

Table 3. The number of individual collected species in the study area.

Tabela 3. Število nabranih primerkov posamezne glivne vrste glede na vzorčno lokacijo.

Name	Used as	Use for	References
<i>Podoscypha petalodes</i>	Extract form.	Its extract contains fatty acid, known as Podoscyphic acid, which shows antiviral activity and plays an important role in suppressing leukaemia disease. It also helps to control the activity of Plasmodium, Leishmania, Trypanosoma,	Lorenzen & Anke (1998), Abugri et al. (2019)
<i>Clitocybe sp</i>	Extract form.	Few species of Clitocybe have anticancer properties. The extract of this mushroom is used for the treatment of lung, breast, and colon cancer.	Patel & Goyal (2012)
<i>Albatrellus confluens</i>	Extract form and soup form.	A secondary metabolite, Grifolin, is obtained from this mushroom, which helps to decrease the activity of some cancer cells.	Patel & Goyal (2012)
<i>Dacryopinax spathularia</i>	Extract form.	These mushrooms have antibacterial activity.	Kumar et al. (2019)
<i>Coriolopsis sp</i>	Extract form.	It shows anti-cancer activity, anti-tumour activity, antiviral activity, and even anti-inflammatory effects against various diseases.	Nguyen et al. (2020)
<i>Tremella fuciformis</i>	Extract form and soup form.	It is used as traditional medicine in China for the purpose of anti-ageing, anti-inflammatory, lower cholesterol, and anti-cancerous activity.	Shahrajabian (2020)
<i>Coprinellus sp</i>	Extract form.	It has antitumor, antibacterial, antifungal, and antiprotozoal activities.	Badalyan (2020)
<i>Phallus atrovolutus</i>	Extract form.	It contains different polysaccharides that have immune system-modulating activity and high anti-inflammatory activity.	Chun et al. (2021)

Discussion

Though wild fungi are visible in limited numbers throughout the year, they grow abundantly in the rainy season. Bankura District has favourable climatic conditions for fungal growth. Different fungal specimens have been collected from the study area during the survey. The climatic factors of Bishnupur forest are very helpful for fungal growth as approximately 23°C-27°C temperature, 2289-6170 mm of rain and 73-96% humidity available in the months of July to September (Table 1). It was also found that the soil of the Bishnupur forest is rich in humus, which is why it is congenial for a wider range of fungal growth than Ramsagar and Onda. It has been noticed that these three places of Bankura show different types of genera and species, which are different in colour and shape. Even all the genera of the same family are not grown at the same time in a place.

In some cases, it has been found that some fungal species do not grow in the same place for a second time. Not all the families were distributed equally among the three study areas. The fungal specimens have been found on soil, wood, and rock as their habitat. During the survey, morphological studies of the collected fungi played a crucial role in characterizing their features, including the colour and shape of the cap and stipe, gill arrangement, presence or absence of volva and scales, and the odour and colour changes of the fruiting bodies after collection. Additionally, anatomical studies revealed variations in basidia size, with some being immature, providing valuable insights into the diversity of fungal structures. Basidium looks long, club-shaped or spine-like. Spores are round to oval in structure, bilayered and deep brown coloured. Basidiospores are released after maturity from the broken basidium wall. Fungi have significant medicinal value, but in India, fungal medicine is not as popular as plant medicine now because people have very little knowledge of it and have low acceptance of myco-medicine. Even many people have the misconception that most fungi are poisonous, so only local tribal people commonly engage in medicinal practices involving fungi.

Among the collected fungi, five species have been known as medicinal sources, confirmed by the local tribal people in the study areas (Table 2). Those fungi are traditionally used for the treatment of tribal communities. *Ganoderma lucidum* is collected from the forests of Bishnupur, Ramsagar and Onda, which helps to increase

body healing ability (Jha et al., 2011). It also shows activity in decreasing diabetes and dizziness and even acts as a terminator of tumours, HIV, and cancer (Ganguly et al., 2021). It sometimes helps cure asthma (Dutta and Acharya, 2014; Debnath et al., 2019). *Daldinia concentrica* is collected from the Ramsagar forest, and it is very important in curing pneumonia wounds and relieving constipation. It is mixed in powdery form with oil or water to form a paste during treatment (Jha et al., 2011). It is taken in powdery form with coconut oil by many patients for the treatment of burning, itching and skin disease (Dutta and Acharya, 2014; Debnath et al., 2019) and also used for the treatment of hypertension (Vishwakarma and Tripathi, 2019). *Schizophyllum commune* is a common fungus collected from the forest of Bishnupur, Ramsagar. It is used to cure skin diseases and enhance the immune system. It also produces a polysaccharide named schizophyllan, known for its anti-cancer activity (Ganguly et al., 2021). *Schizophyllum commune* also helps to make tonics for patients (Dutta and Acharya, 2014; Debnath et al., 2019) and cure eye infections (Vishwakarma and Tripathi, 2019). *Lycoperdon sp* is collected from the Onda forest, which is important in recovering stomach pain, hallucinogenic and wounds (Jha et al., 2011). It is also used to cure the burning skin and wounds (Debnath et al., 2019). *Pycnoporus sp* is collected from the Ramsagar forest and used in dried form with mustard oil, a traditional treatment for ear infections in the childhood of local tribal people (Jha et al., 2011).

The present study has compiled a list of eight medicinal fungi, highlighting their therapeutic properties and confirming their traditional use by tribal populations in different areas. This underscores the significance of ethno-remediation and the potential for new discoveries. Ganguly et al. (2021) recorded 15 macrofungi that have medicinal uses. Debnath et al. (2019) have shown the data with 136 medicinal fungi. Yusran et al. (2021) found nine macrofungi used as medicine by the local tribes. Dutta and Acharya (2014) have reported five medicinal macrofungi. Jha et al. (2011) recorded 11 macrofungi that have medicinal value. Vishwakarma and Tripathi (2019) have shown 36 macrofungi species with medicinal properties. Besides medicine, fungi have great food value in India. Mushrooms are considered a balanced food due to their low cholesterol and fat content and high levels of proteins, vitamins, and minerals (Vishwakarma and Tripathi, 2019). Menstruating girls may be advised to consume edible mushrooms along with probiotic-enriched fermented foods to combat protein

deficiency (Ganguly et al., 2024; Ganguly & Midya, 2023; Ganguly et al., 2018b). Approximately 2189 fungal species have been reported as edible worldwide, and 283 edible fungi have been collected from India (Sarma et al., 2022). *Termitomyces heimii* (Karan chatu), *Agaricus bisporus* (Botam chatu), *Pleurotus ostreatus* (Oyster mushroom), *Volvariella volvacea* (Khar Chatu or Straw mushroom), *Russula sp* (Rangin chatu or Colour mushroom), *Astraeus hygrometricus* (Kurkuri chatu), *Agaricus campestris* (Field mushroom) are very common edible mushroom of India. Still, *Termitomyces heimii* and *Astraeus hygrometricus* are most popular than other edible fungi. A total of 8 edible fungi have been reported in the present study (Table 2) like *Ganoderma lucidum*, *Agaricus bohusii*, *Panaeolus sp*, *Clitocybe sp*, *Albatrellus confluens*, *Tremella fuciformis*, *Schizophyllum commune* and *Phallus atrovolvatus* but only three species namely *Agaricus bohusii*, *Panaeolus sp* and *Clitocybe sp* are consumed by tribal people which have mild taste. The previous study shows that Pramanik and Chaudhuri (2017) have reported 17 edible mushrooms from the Nadia District of West Bengal. Singha et al. (2017) have recorded 32 edible macrofungi from Paschim Medinipur of West Bengal. Chakraborty (2019) has reported 17 edible mushrooms from Dakshin Dinajpur of West Bengal. Dutta and Acharya (2014) have recorded 31 edible mushrooms from West Bengal. Sarma et al. (2022) have recorded 14 edible mushrooms from Jammu, India. Singha et al. (2020) have reported 12 macrofungi as edible from Gurguripal of West Bengal. Das et al. (2015) have reported 16 edible mushrooms from the Eastern Chata Nagpur Plateau of West Bengal. Semwal et al. (2014) have recorded 23 species of edible mushrooms from Northwestern Himalayas. During the survey, four poisonous fungi also have been collected: *Mycena rosea*, *Cystoagaricus trisulphuratus*, *Macrolepiota procera*, and *Leucocoprinus cretaceus*, which are limited in number but responsible for food poisoning and allergy in the human body even sometimes edible fungi become harmful to consume due to toxicity of its nearby poisonous fungi which is confirmed by local people. However, the focus of the recent study is to explore fungal diversity, increase the medicinal and food value among the people and break the misconception regarding fungi. Recent studies have revealed that Bankura is enriched in macrofungi with high nutritional value and medicinal properties, and it can even increase the social value of myco-medicine in the future. However, further surveys must be conducted to learn about the unknown fungi and their economic values.

Conclusion

The present survey on fungal distribution in the three forests of Bankura District, Bishnupur forest, Onda forest and Ramsagar forest, indicates the presence of rich fungal diversity with 95.7% members of Basidiomycota and 4.3% members of Ascomycota. The number of edible and poisonous members was 8 and 4, respectively. The morphological and anatomical studies of those fungi help their differentiation from one another.

Based on food value, some members of Agaricaceae and the entire puffball (*Lycoperdon*) attract the attention of local people. Due to their high source of protein, they are sold by people at high market prices. Some gill fungi are very popular among children; they love to eat it, and those fungi are very helpful for their growth. So now, many fungi have been cultivated artificially to fulfil public demand. Even today, it is the most demanding ingredient in many industries. Though medicinally fungi stay behind, their progress must be seen in future. More investigation is needed on a broad scale to know the social value of fungi for future economic development. Fungi are an important factor for reforestation programs worldwide (Wongchalee and Pukahute, 2012), but they get less attention, so nowadays, many fungi have become endangered, which causes infertility of soil. There are many causes behind the subsidence of fungal populations, like pollution, overuse of a particular fungus, deforestation, and the absence of organic matter in the soil. In India, research on fungal diversity, as well as ethno-medicinal study, is very limited due to the lack of interest of researchers. The general public's interest in studying wild fungi is often deterred by the fear of snake bites, which is a legitimate concern given the number of fatalities reported yearly. Additionally, some vegetarians may be hesitant to consider fungi as non-veg, further limiting the exploration and utilization of the potential benefits of fungi.

This research aims to enlighten people about fungi's distribution, morphological, and anatomical characteristics in the Bankura District, including their edibility, toxicity, and ethno-medicinal properties. It seeks to raise awareness among the local population about wild fungi's importance and potential uses, promoting a deeper understanding and appreciation of wild fungi. The investigation revealed that the Bankura district holds great promise for future research, poised to become a hub of interest for scientists and researchers seeking to explore and discover new fungal species, thereby enriching our understanding of the fungal kingdom.

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Author's contributions

Conceptualization- P.M. and S.K.M; methodology- S.K.M and S.K.M; software- A.M.; validation- S.K.M, A.M. and S.K.M; formal analysis- P.M. and A.G.; investigation- P.M. and A.M.; data curation- P.M. and R.G.; writing— P.M. and A.G, writing—review and editing- A.G and S.K.M.; visualization- R.G. supervision- S.K.M.

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Research Article

Significant records of plants, algae, fungi, and animals in SE Europe and adjacent regions 2

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Simona Strgulc Krajšek^{1*}

Abstract

In this paper, we present two significant records of mosses in Slovenia: *Bryum canariense* and *Fissidens fontanus*, and the first record of an animal species, *Porcellio obsoletus*, from the Slovene Coast.

Keywords

Bryum canariense; *Fissidens fontanus*; *Porcellio obsoletus*; bryophytes; mosses, flora; fauna; Isopoda; isopods; Slovenia

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Bryum canariense Brid., fam. Bryaceae (moss)

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Leg.	Aljaž Jakob
Country	Slovenia
Statement of significance	The first recent record of a data deficient species in Slovenia.
Locality description	Primorska region, Classical Karst, Lipica, 390 m a. s. l.
Habitat	Quercus pubescens forest with admixed Pinus nigra, on the forest floor under Juniperus communis
Date of observation	2024-03-25
GPS	N 45.66687°, E 13.86696°
Voucher	Authors personal herbarium, 203888293

Bryum canariense is a data deficient species of Slovenian flora, previously only collected and reported in 1882 from Orleška draga (Martinčič, 2024). The New site lies 4 km southeast of the historical site at Orleška Draga in similar vegetation type (thermophilous *Quercus pubescens* forests with admixture of *Pinus nigra* in rather early stages of succession).

B. canariense is a Mediterranean-Atlantic species (Hill et al., 2007), occurring in Europe from Great Britain in the north to the Mediterranean basin and Madeira in the South and from the Azores to Turkey. It is absent from some Mediterranean islands. In the immediate vicinity of the presented locality, it grows in Friuli-Venezia Giulia (Italy) and Croatia but is absent from Austria and Hungary. Its conservation status is least concern in Europe (Hogetts & Lockhart, 2020).

The abandonment of land use from the late 19th century to the present is unfavourable for this moss. Previously, the area was predominantly a stony, open grassland and current reforestation (Kaligarič & Ivajnšič, 2014) of the area is likely reducing the habitats suitable for this heliophilic species of open spaces (Hill et al., 2007).

B. canariense differs from all other species of Bryaceae by its arrangement of leaves into 2–3 comal tufts, where each year's rosette is placed above the last year's rosette. Some other rosulate species are similar, but all lack this arrangement, for example, the species of *Rhodobryum* have stolons, so tufts are not arranged above each other. *Ptychostomum capillare* and *P. torquescens* are smaller and lack a serrate border of the leaf tip, and the dry leaves of *P. capillare* are often strongly spirally twisted (erect at *B. canariense*). The leaves of both *Ptychostomum* species have a stronger border up to the leaf tip, sometimes with small teeth, whereas *B. canariense* has a weak border that usually disappears in the upper quarter of the leaf, where it is replaced by strong teeth (Casas et al., 2006; Holyoak, 2013).

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Fissidens fontanus (P. Karst.) Bas, fam. Fissidentaceae (moss)

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Leg.	Urška Kuhar
Country	Slovenia
Statement of significance	The second and third known localities in Slovenia, the first two in Central Slovenia.
Locality description	(1) Central Slovenia, Ljubljana, Moste, Ljubljana river, between the bridge of Grablovičeva street and the pedestrian bridge connecting Zaloška street and street Ob Ljubljani, 300 m a.s.l. (2) Central Slovenia, Ljubljana, water channel Gruberjev prekop, between the bridge of Kajuhova street and the pedestrian bridge Mekinčeva brv, 300 m a.s.l.
Habitat	Submersed in the water on a rock substrate
Date of observation	2022-07-25
GPS	(1) N 46.055172, E 14.533501 (approximate coordinates) (2) N 46.051042, E 14.540049 (approximate coordinates)
Voucher	Herbarium LJU (s.n.)

Fissidens fontanus is an aquatic moss species growing on submerged rocks or wood in mesotrophic lowland lakes and rivers (Frey et al., 2006). It has remote and narrow leaves, which are much longer than the leaves of other representatives of the genus. The upper one-layered part of the leaf is up to 3 times as long as the sheathing part, the leaf is unbordered, and the costa ends before the apex (Frey et al., 2006; Godfrey, 2010).

F. fontanus is an European temperate species (Hill & Preston, 1998), distributed from the Mediterranean to S. Scandinavia (Frey et al., 2006). It is also present in North America and in the Southern Hemisphere (Hill & Preston, 1998). The species is considered least concern (LC) in the European Red List (Hodgetts et al., 2019). It is present in all neighbouring countries of Slovenia except Hungary (Hodgetts & Lochhart, 2020; Aleffi et al., 2020; Alegro et al., 2019). In Slovenia, it has been only reported for the sub-Pannonial phytogeographical region, where it was found in 2018 in river Dravinja near Makole by Martinčič (Martinčič, 2024), so our records are the first in the pre-Alpine phytogeographical region of Slovenia.

The specimens in the river Ljubljana and the channel Gruberjev prekop were found during the monitoring of macrophytes for the assessment of the ecological status of surface waters. Macrophytes were sampled in 100 m long river stretch by wading across the channel or from the shore or from the boat when wading is not possible.

Funding

Slovenian Environment Agency financially supported the monitoring of macrophytes.

Porcellio obsoletus Budde-Lund, 1885, fam. Porcellionidae (animal)

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Leg.	Miloš Vittori
Country	Slovenia
Statement of significance	This is the first record of <i>Porcellio obsoletus</i> in Slovenia.
Locality description	Primorska region, Piran, Trubarjeva ulica, 10 m a. s. l.
Habitat	street with stone pavement
Date of observation	2019-06-14
GPS	N 45.529361°, E 13.567500°
Voucher	The specimen is kept in the isopod collection of Miloš Vittori, University of Ljubljana, Biotechnical Faculty, Department of Biology, Večna pot 111, 1000 Ljubljana, Slovenia.

This is the first report of the occurrence of *Porcellio obsoletus* Budde-Lund, 1885 in Slovenia. The species was observed in a synanthropic habitat on the Slovenian coast.

A single adult *P. obsoletus* female was spotted at night in a narrow street in the town of Piran in June 2019. The individual was collected and preserved in 96% ethanol. The species can be recognized by its weakly granulated tergites (Figure 1a), the position of the glandular pore fields on the anterior edges of pereon epimera (Figure 1b), a furrow running along articles four and five of the second antenna (Figure 1c), and broad, flat uropod exopodites (Strouhal, 1968).

P. obsoletus is distributed in the central and eastern Mediterranean region, Iran, and the Crimea (Schmalfuss, 2003). In the territory of former Yugoslavia, it has previously been reported from Bosnia and Herzegovina as well as from the Croatian coast and islands (Dollfus, 1895; Rogenhofer, 1908; Potočnik, 1989). It has not yet been reported this far north in the Adriatic region. Given the proximity of its known distribution area, there is no reason to assume that its presence in Slovenia is due to human introduction. Previously, six species of *Porcellio* were known to occur in Slovenia (Vittori et al., 2023), making *P. obsoletus* the seventh recorded species of the genus and raising the total number of known Oniscidea species in the country to 75.

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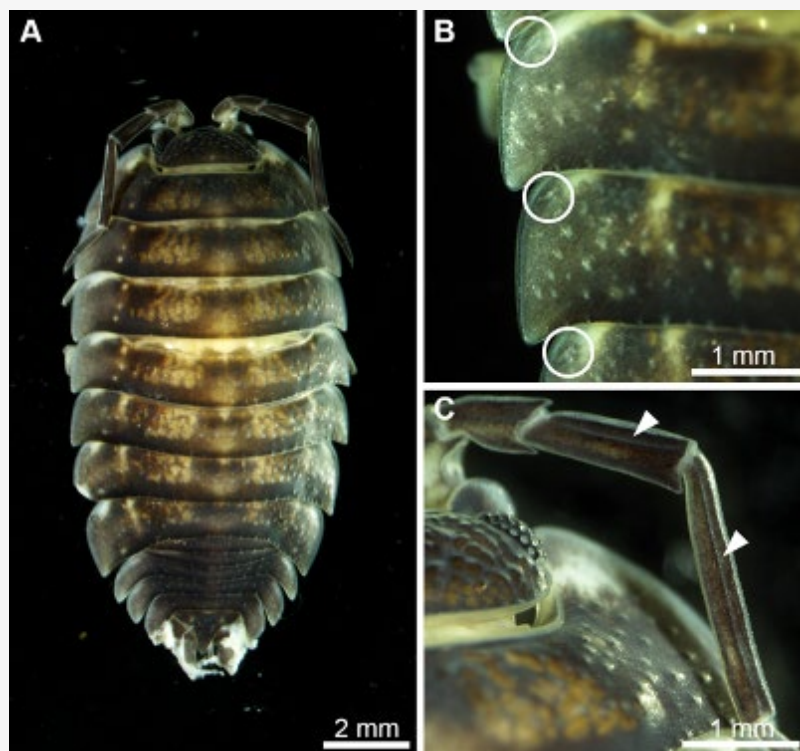


Figure 1. *Porcellio obsoletus*, female, collected in Piran. A: Habitus. B: Position of glandular pore fields (circled). C: Second antenna with a furrow (arrowheads).

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Review

Menstruation in Adolescent Girls: Myths & Taboos

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Abstract

Menstruation is a natural process experienced by women. It signifies the natural commencement of puberty. Yet, it is shrouded with taboos and myths across various cultures. Menstruation related taboos not only ostracize women from socio-cultural activities but also affect their emotional well-being, mental condition, lifestyle and overall health. Tackling these deeply entrenched beliefs presents considerable challenges, particularly due to the lack of knowledge among girls about puberty, menstruation, and reproductive health. Therefore, there's a crucial need for a strategic approach to tackle the issues. This paper aims at delving into prevalent menstruation-related myths and taboos in India, their detrimental impact on women's lives, the importance of addressing them in primary care, and an overview of various strategies to combat these taboos. Furthermore, the article proposes strategies to improve menstrual health and hygiene among adolescent girls. The study finds that cultural and social attitudes toward menstruation are influenced by factors such as girls' education, attitudes, family environment, cultural background and beliefs.

Keywords

Adolescent, menarche, menstruation, myth, social & cultural practices, taboos.

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Menstruacija pri mladostnicah: miti in tabuji

Izvleček

Menstruacija je naraven proces, ki ga doživljajo ženske, vendar je v različnih kulturah zavita v tabuje, povezane z menstruacijo, ne le da odvrta ženske od družbeno-kulturnih dejavnosti, temveč vpliva tudi na njihovo duševno stanje, življenjski slog in splošno zdravje. Reševanje teh globoko zakoreninjenih prepričanj predstavlja velik izziv, zlasti zaradi pomanjkanja znanja med dekleti o puberteti, menstruaciji in reproduktivnem zdravju. Namen tega prispevka je poglobiti se v prevladujoče mite in tabuje v zvezi z menstruacijo v Indiji, njihov negativen vpliv na življenje žensk, pomen obravnave le-teh v primarni oskrbi in pregled različnih strategij za boj proti tem tabujem. Poleg tega članek predlaga strategije za izboljšanje menstrualnega zdravja in higiene med mladostnicami. Študija ugotavlja, da na kulturni in družbeni odnos do menstruacije vplivajo dejavniki, kot so izobrazba deklet, družinsko okolje, kulturno ozadje in prepričanja.

Ključne besede

Adolescenca, menstruacija, miti, socialni in kulturni običaji, tabuji

Introduction

Menstruation is a natural physiological phenomenon of normal healthy females who experience shedding of blood from the uterine endometrium in every month from the age of menarche until menopause. It typically commences in girls aged 11-14 years and serves as a key indicator that puberty is underway. During the puberty phase, there occur notable changes in one's physical, psychological and cognitive abilities. For women, the onset of menstruation, known as menarche, is one of the most significant and memorable events of adolescence (Alharbi et al., 2018). Menarche marks the beginning of a normal biological process during which a girl gradually matures. Despite being a natural process exclusive to adolescent girls and women, menstruation has long been obscured by myths and taboos across various cultures in the third world countries, especially in India (Kaur et al., 2018). These societal attitudes have marginalized women from participating fully in various aspects of socio-cultural life. The pervasive taboos surrounding menstruation have far-reaching consequences, impacting the emotional well-being, mindset, lifestyle and, most importantly, the health of girls and women. Ganguly and Midya (2023) reported various cultural and religious restrictions that are imposed upon the school-going early adolescent girls in Bankura, West Bengal. Limited awareness and understanding of puberty, menstruation and reproductive health among girls further

perpetuate these socio-cultural taboos and myths. Consequently, addressing these deeply ingrained issues necessitates a deliberate and comprehensive strategy. This paper aims at shedding light on the prevalent beliefs surrounding menstruation in India, their detrimental effect on women's lives, and the imperative need to address these concerns within primary care settings.

Myths and taboos related to menstruation

In Nepal, during the menstruation, women are considered filthy, impure and untouchable (Amatya et al., 2018). These social misconceptions prevent girls from participating in their daily activities, such as touching kitchen items, livestock, men, and water resources; consuming certain foods like milk and other dairy products; and visiting specific public places, including temples, prayer rooms, and cultural ceremonies (House et al., 2013). Additionally, girls are often forced to sleep alone in cattle sheds, known as Chhaupadi, where personal hygiene is severely lacking (Thakuri et al., 2021). Nonetheless, Chhaupadi custom is not only common during menstruation but also during postpartum period. When living in the Chhaupadi huts during menstrual cycle, women/girls experience physical as well as psychological anguishes. In the western hills of Nepal, several incidents have been documented during

the practice of living in Chhaupadi huts, including rape, sexual assault, illness, and bites from snakes or scorpions (Thakuri et al., 2021). These incidents were connected to inadequate security and unhygienic surroundings (Thapa & Aro, 2021). Poor hygiene and sanitation standards in Chhaupadi are also a result of a lack of sanitary napkins, an unclean environment, and limited access to water and sanitation services (Sharma et al., 2022). Poor menstrual hygienic practices adversely affect one's health, increasing the risk of the genitourinary and reproductive tract infections, depression, anxiety and cervical cancer (Thakuri et al., 2021). According to a study conducted on Tanzanian adolescent girls (aged 16–19 years), the most prevalent menstrual "taboo" is the possibility of infertility as a form of punishment and improper disposal of menstrual waste (Sommer, 2013). Taboos include beliefs such as "If one flushes her pad down the toilet, she can never become pregnant," and "If her brother finds out that she has menstrual blood, she can never become a parent in her entire life" (Sommer, 2013). Conversely, a significant portion of Cambodian girls were advised by their mothers to retain their initial used menstrual sanitary material, as it was believed to provide protection against malicious intentions, to act as an antidote for snakebites, and to enhance skin smoothness (Sommer et al., 2015). Menstruating women and girls in Western Uganda are often restricted from drinking milk due to a belief that it could impact milk production from cows (Mohammed and Larsen-Reindorf, 2020). Similar prohibitions exist in Eastern Uganda, where it is thought that seeding groundnuts during menstruation would reduce crop productivity (Mohammed and Larsen-Reindorf, 2020).

In India, discussing menstruation has long been considered taboo, and even today, cultural and social influences continue to pose hurdles to the advancement of knowledge on the subject. A significant majority of the 355 million menstruating women and girls in India encounter uncomfortable and undignified experiences with menstrual hygiene management (Sharma et al. 2020). In many parts of India, menstruation is still culturally viewed as unclean and impure. It is often accompanied with ancient myths. The origin of these myths can be traced back to the Vedic era, often associated with the stories like Indra's slaying of Vritra. The Veda declares that the guilt of killing a Brāhmana, akin to murder, manifests monthly as the menstrual flow in women (Janet, 1994). According to Samvarta, it is said that "Father, mother, and brother, all

these three go to hell if they see a maiden in puberty." In the Hindu faith, women are traditionally forbidden from engaging themselves in regular activities during menstruation. They have to undergo a process of purification before rejoining their families and resuming their daily tasks. A class of neuropeptides known as kisspeptin is essential for controlling ovulation and bringing on puberty in sexually mature females (Xie et al., 2022). There is no rational basis for considering menstruating women as 'impure'. Many women and girls in India face several restrictions in their daily lives due to menstruation (Table 1). For urban females, the main limitation during menstruation is to stay out of the prayer room and ritual places. Rural girls face restrictions in entering kitchen and store rooms or applying Kohl (Joshi and Fawcett, 2001). There is a misconception that menstruating women are unhygienic and unclean, leading to concerns about food contamination when they prepare or handle it (Bhalgat, 2023). There is a myth that during menstruation, the body emits a specific odour or radiation that spoils preserved food (Kumar and Srivastava, 2011). Consequently, they have to avoid handling sour foods like pickles during this time with the belief that such food may be spoiled.

Dysmenorrhea, a frequently encountered gynecological condition, is characterized by excruciating menstrual cramps that originate in the uterus (Itani et al., 2022). Swain and Nayak (2018) identified dysmenorrhea as the most common issue among menstruating girls in Balda village in Koraput district, Odisha. Girls are often prohibited from touching holy books and men during menstruation. A pubertal ceremony is a ritual that is celebrated when a girl attains Puberty. In the Paraja and Rana communities, menstruating girl is referred to as Bartaman Jhia, and her pubertal ceremony is called Uthani. Her family members then consult Dishari; a traditional healer, who follows religious calendar (Pāñji) and guide the devotees about the rituals and customs to follow. Dishari advises them to sacrifice animals, typically a black hen or a male goat in order to ward off the evil spirit associated with it. Girls are instructed to spend 7-9 days at indoor during the period of menstruation which is considered as pollutant period. After that, the family members take her to bathe at the holy river in order to purify her. Simultaneously, the Dishari performs some defined rites and sacrifices animal at the river bank. Once the ceremonies are completed, the family invites relatives and villagers for a feast. The girl uses pieces of clothing instead of sanitary napkins as going out to buy sanitary

napkins is considered as a sin (Swain & Nayak, 2018). In the Paraja community, when a girl attains menarche, her family either arranges a party or offers a coconut to God at home. Menstruating girls in the Rana community are bound by a number of socio-cultural restrictions. During their menstruation, they are not allowed to go into the kitchen or do any cooking. They are also prohibited from going inside the temples and other places of worship. They must also avoid touching the men in the society and taking food from kitchen during menstruation. They are not allowed to consume sour, spicy, or Prasāda (Vegetarian foods especially for devotees). They are only allowed to consume rice, jaggery, and green vegetables at this time because they believe that eating sweets will cause profuse bleeding (Saga). Additionally, they are not allowed to cross Dhinki (the traditional wooden rice meal) as they consider it to be extremely bleeding (Swain and Nayak, 2018). The Rana community observes these limitations as a part of their cultural views and customs surrounding menstruation.

Cultural norms and religious taboos surrounding menstruation are often aggravated by traditional belief about evil spirits, as well as feelings of shame and embarrassment related to sexual reproduction (Garg and Anand, 2015). In some societies, women bury their cloths used during menstruation as a means of protecting them from perceived influences of evil spirits (Sivakami et al., 2019). In Suriname, there is a belief that menstrual blood holds a dangerous power, and individuals with malicious intent can harm a menstruating woman or girl by using black magic ("wisi"). Additionally, it is believed that a woman can exert control over a man by using her menstrual blood to impose her will (Garg and Anand, 2015). It's interesting to note that such beliefs are still observed in Asia, including India (Khatuja et al., 2019). However, there is no logical or scientific explanation for these beliefs.

In some parts of India, strict dietary restrictions are also observed during menstruation, with menstruating girls typically avoiding sour foods like curd, tamarind and pickles (Puri and Kapoor, 2006). It is believed that consuming such foods may disturb or even stop the menstrual flow (Sadiq and Salih, 2013). A common misconception among teenage girls is that physical activity during menstruation aggravates dysmenorrhea (John et al. 2021). However, in reality, exercise can actually help to alleviate premenstrual syndrome and dysmenorrhea (Agarwal & Agarwal, 2010). Additionally, exercise triggers the release of serotonin, which can enhance mood and overall well-being (Sadiq

and Salih, 2013; Garg and Anand, 2015). In Brazil, a study found that menstruating women were prohibited from touching, harvesting, or preparing medicinal plants due to beliefs that it could harm the plants or reduce their medicinal properties (Siqueira et al. 2021).

In almost all parts of India, perception of Hinduism revolves around the concept of purity and pollution. Bodily excretions are considered polluting as are the bodies when they produce them. All caste groups experience ritual impurity due to menstruation and childbirth. Water is frequently employed as the primary means of purification. Preserving water sources from such contamination, as they are considered the physical embodiment of Hindu deities, becomes a prime concern. This sheds light on prohibiting menstruating girls and women from performing many daily works. There is a belief that if a girl or woman in her menstrual period touches a cow, it may render the cow infertile (Garg and Anand, 2015). This association often leads girls to perceive their own bodies as carriers of curse and impurity. Muslim girls reportedly avoid touching religious books, performing prayer, and visiting mosques (Fischer, 1978). Similarly, girls from the Sarna tribe also avoid visiting their places of worship during menstruation. Christian girls, on the contrary, actively participate in worship and attend church services even during their menstrual period (Maharaj and Winkler, 2020).

There is a long-standing custom among the Lohra tribe that mothers do not discuss menstruation with their daughters (Kumar and Srivastava, 2011). Kayastha is a prominent and influential caste in India and Nepal, traditionally associated with administrative and religious duties, and considered the direct offspring of a Vedic deity (Kumar and Srivastava, 2011). It's a common superstition among Kayastha families that handling handmade ghee or vinegar while a woman is menstruating will ruin it.

A menstruating female is not allowed to greet guests, sit on the couch, enter kitchen or serve meals in Marwari households because she is considered impure (Chawla, 1992). The Oraon tribe holds a belief that when a girl attains menarche and if her mother shreds a piece of cloth into three equal parts in one breath and offers it to her daughter, it will alleviate abdominal pain (Kumari et al., 2021). Among the Bhargav Brahmins, it is a tradition for menstruating girls to refrain from touching iron objects like locks and keys (Eram & Tamanna, 2016).

Impact of taboos related to menstruation on women's life

Taboos surrounding menstruation in Indian societies (Fig. 1) profoundly affect the emotional wellbeing, mental health, lifestyle and, most crucially, the physical health of girls and women (Garg and Anand, 2015). The early onset of menstruation (before age 12) reduces school enrolment by 13% (Khanna, 2019). Despite being in the 21st century, millions of women across India still encounter substantial obstacles to achieve comfortable and dignified experiences with menstrual health (Yadav and Pandey, 2023). Moreover, the monthly menstruation period also presents obstacles for female students. Gender-unfriendly school culture, a lack of female toilet facilities, and insufficient menstrual

protection further exacerbate the situation. In India, more than 77% of women and girls who are menstruating use old cloths (Garg and Anand, 2015). A study on a Muslim community in India reported that more than 80 percent women and girls use old cloths (Adak and Midya, 2020). Insufficient protection and inadequate washing facilities can increase susceptibility to infection, while the odour of menstrual blood may subject girls to stigmatization, which affects their mental health (Parker et al., 2014). The task of confronting socio-cultural taboos and beliefs surrounding menstruation becomes even more complex due to limited knowledge and understanding of puberty, menstruation, and reproductive health (Puri and Kapoor, 2006, Ahuja et al., 2018). There are different perceptions among girls regarding the cause of menstruation (Table 2).

Table 1. Restriction of daily activities during menstruation.

Tabela 1. Omejitev vsakodnevnih opravil med menstruacijo.

Researchers	Setting	Number of participants	Percentage of responded facing restrictions from different activities												
			Religious occasion	Physical activity/playing	Schooling	Attending family functions	Household work	Food restriction	Talk to boys	Visit to others home	Sleep on routine bed	Touch stored food	Enter kitchen	Separated	No restrictions at all
Mudey et al., 2010		300	87	18.6	12.6	14.3	7	2.6		23.6					17
Thakre et al., 2011	Rural	146	73.2	28.7	7.5		28.7				30.8	41.5		43.1	23.2
	Urban	241	67.6	19.5	3.7		24.9				23.6	26.9		26.9	28.2
Seenivasan et al., 2016		500	92.4		9.2		55.7				51.8	44.0			
Maji, 2016		100	100									57			
Hakim et al., 2017	Government	250	97.2					15.6			3.2				0
	Non-Govt.	250	100					12			2.8				0
Mathiyalagen et al., 2017		242	44.6	21.9	34.7	7.9	33.1				58.7				
Sultan and Sahu, 2017		350	76	12	5		8	22			15				
Chauhan et al., 2019		165	88.4		1.8			6							11.5
Das and Tasa, 2019		107	82.2	47.6	45.7	62.6	52.3				53.2	59.8	42.9		
Parle and Khatoon, 2019		600	88.9				35.1								
Shoor, 2017		352	53.9	11	0.8	7.9		37.7	2.27	5.39			7.1	0.2	11
Patel et al., 2019		273	62.6	10.6	1.8	20.9			4.4		23.1		12.1	8.4	28.6

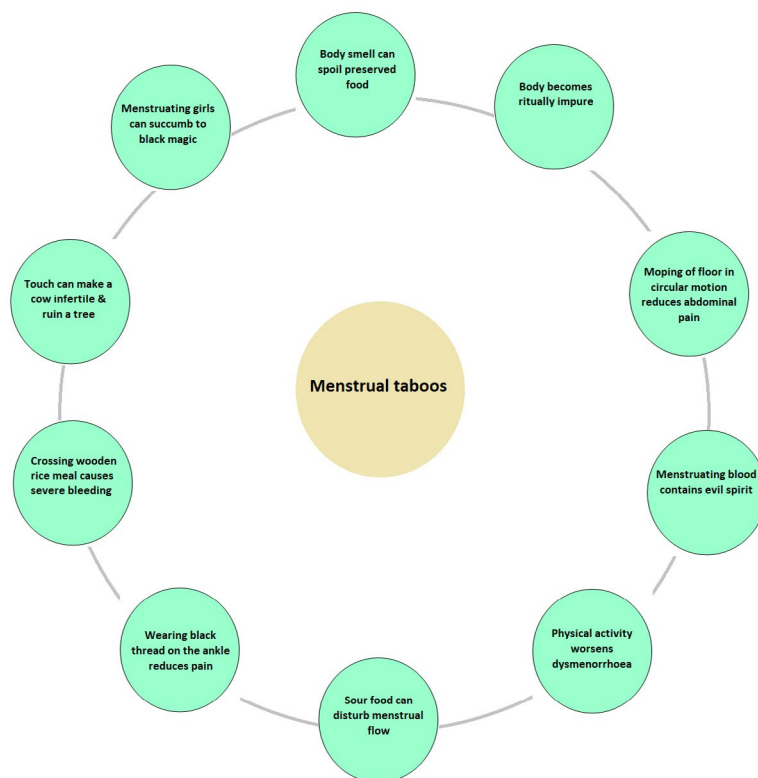


Figure 1. Menstrual taboos that persist in Indian society.

Slika 1. Tabuji v povezavi z menstruacijo, ki so še vedno prisotni v indijski kulturi.

Table 2. Overview of socio-cultural perceptions about the cause of menstruation in India.

Tabela 2. Pregled socialno-kulturnega dojemanja menstruacije v Indiji.

No of participants	Menstruation perceived as				References
	Physiological/ natural process (%)	Curse of God (%)	Disease (%)	Don't Know (%)	
387	18.4	1.0		80.6	Thakre et al., 2011
506	74.2	17			Shanbhag et al., 2012
798	41.1				Bhattacharjee et al., 2013
50	84	2	2	12	Dixit et al., 2016
100	38	16		46	Maji, 2016
242	71.5	6.6	2.9	18.6	Mathiyalagen et al., 2017
350	37	22			Sultan and Sahu, 2017
165	18.6		4	55.8	Chauhan et al., 2019
110	38.2	15.5		46.4	Das and Tasa, 2019
600	68.1	3.8	1.2	26.8	Parle and Khatoon, 2019
273	35.9	27.1	1.5	35.5	Patel et al., 2019
1963	74.6	15.2	1.3	7.5	Tamphasana et al., 2020

Strategies to combat menstruation related myths

The beginning of menstruation marks a significant milestone in female puberty, triggering substantial psychological and behavioural changes in adolescent girls. Indian society, that is most religiously and ethnically diverse, generates a lot of traditions, myths, misconceptions and superstition regarding menstruation. Adolescent girls often get succumb with reproductive tract infections due to poor hygiene and health. Based on the available evidences, it is crucial to adopt a strategy to combat the myths and social taboos associated with menstruation. It is essential to enhance the reproductive health of adolescent girls and women. The primary strategy in this regard should be to raise awareness among adolescent girls regarding menstrual health and hygiene (Garg and Anand, 2015). A comprehensive education involving schools, parents, and health personnel is essential to improve knowledge and practices related to menstrual hygiene (Rastogi et al. 2019). Additionally, promoting open discussions and creating supportive environments in schools and communities can further enhance their confidence and knowledge, leading to better health outcomes and overall well-being.

Often, young girls grow up with limited and/or mis-information about menstruation as their mothers and other women feel embarrassed to discuss these issues with them. The mothers and other women may also be unaware of biological facts and good hygienic practices, and they rather pass on cultural taboos and restriction to be observed. Empowering women through education and increasing their participation in decision-making processes is crucial for tackling a range of societal issues like taboos associated with menstruation. Women and girls often find themselves excluded from decision-making positions as a result of lower literacy rates. Raising women's educational attainment is, therefore, crucial for eradicating persistent cultural barriers as well as for promoting improved community health. Access to sanitary napkins and appropriate sanitation facilities, along with washing amenities, should be guaranteed with a gender-sensitive approach. Schools, governments, and non-governmental organizations should collaborate to provide these essential resources. Schools can ensure that these facilities are available on their premises, while governments can create policies and allocate funds to support menstrual health initiatives. NGOs can play a crucial role in advocacy, education, and

distribution of sanitary products, especially in underserved communities. On the other hand, initiatives should be taken to debunk misconceptions surrounding menstruation.

In rural and slum areas, where access to sanitary products is challenging, distribution of locally produced and distributed low-cost sanitary napkins can be an effective solution (Kumar and Srivastava, 2011). The initiative was initially launched in 2011 across 107 selected districts in 17 states in India, offering a pack of six sanitary napkins named "Free days" to rural adolescent girls just at \$ 0.072. Since 2014, funds have been allocated to States and Union Territories under the National Health Mission for decentralized procurement of sanitary napkin packs, which are then provided to rural adolescent girls at a subsidized rate. Accredited Social Health Activists (ASHAs) play a crucial role in India's rural healthcare system, serving as frontline workers linking communities to health services. They play a key role in distribution, with an incentive of receiving \$0.012 per sold pack, along with a free pack of napkins every month for their personal use (<https://nhm.gov.in/index1.php?lang=1&level=3&sublinkid=1021&lid=391>). Expanding the involvement of male partners and addressing their belief system is crucial in challenging entrenched social norms and cultural taboos. Despite typically having less knowledge about menstruation (Cultural taboos and stigma, lack of education, Socioeconomic factors, Gender Inequality, Misinformation, Personal discomfort etc) it's essential for men and boys to comprehend it to effectively support their wives, daughters, mothers, students, employees and peers (Kirk and Sommer, 2006).

Menstruating girls must be advised to consume small indigenous fish or probiotic-enriched local fermented foods on regular basis to fulfil their nutritional requirements, improvement of gut health, proper nutrient absorption, development of immune function, maintaining hormonal balance, reduced inflammation and improvement of mental health (Ganguly et al., 2018a; Ganguly et al., 2024). Probiotics are beneficial microorganisms which when consumed in adequate amount confer health benefit to the host (Ganguly et al., 2024; Ganguly et al., 2018b; Ganguly et al., 2019). They improve the quality of intestinal microflora which helps to combat various bacterial and viral infections (Chattaraj et al., 2022; Ganguly et al., 2018c). Countries where a large section is battling with protein deficiency and malnutrition (Stephenson et al. 2000), it may hold potential in uplifting the general health status of the populace (Ganguly et al., 2018d). Multisectoral approaches involve

collaboration among various stakeholders and sectors to achieve shared health outcomes, optimizing resource use and improving program effectiveness (Salunke & Lal, 2017). A study conducted in Nigeria demonstrated that integrating menstrual health education into school curricula enhanced students' understanding and attitudes toward menstruation (Uzoечи et al., 2023). In India, an educational program targeting both girls and boys effectively reduced menstrual stigma. A study in Uttar Pradesh engaged men and boys in menstrual hygiene management, sensitizing them and training male teachers to provide MHM services in schools (Sahin et al., 2015). Another intervention involving classroom discussions on gender equality for adolescents over two years resulted in more supportive attitudes towards gender equality, with effects persisting two years after the program ended (Dhar et al., 2018). A comprehensive review focusing on low- and middle-income countries revealed that initiatives such as healthcare provider training and community outreach significantly improved menstrual health outcomes (Hennegan et al., 2019). Similarly, in Tanzania, a program providing menstrual health services and education to adolescent girls led to notable improvements in their sexual and reproductive health (Njee et al., 2024). A Ghanaian study found that females' education raises their overall academic achievement and school attendance (Montgomery et al. 2012). Meanwhile, a community-based program in Kenya addressed menstrual stigma and provided menstrual products, resulting in improved school attendance and self-esteem among girls (Wood et al., 22). In Bangladesh, engaging religious leaders and community members in menstrual health education successfully reduced stigma and promoted better menstrual hygiene practices (Warrington et al., 2021). In the United States, exposure to menstrual-themed media was

found to reduce stigma and foster positive attitudes toward menstruation (Ramaiyer et al., 2023).

Connecting physical infrastructure such as water and sanitation projects with health education and reproductive health programme is essential. It's important to address the issue in a more integrated and holistic manner. Menstruation is nothing but a very normal biological phenomenon, and adolescent girls and women should understand that they have the power of procreation only because of this virtue.

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The authors declare that there is 'no conflict of interest' to publication and authorship of this article.

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Short Note

Naravna selekcija, naravni izbor, naravno odbiranje ali naravno izbiranje?

Peter Trontelj^{1*}

Izvleček

Desetletja je veljalo, da osrednji evolucijski proces v slovenščini poimenujemo s tujko *naravna selekcija* in z domačim imenom *naravni izbor*. Z novim prevodom Darwinove knjige »O nastanku vrst« leta 2009 je prevajalec vpeljal nov izraz, *naravno odbiranje*, ki naj bi kot ustrežnejši zamenjal prejšnja dva. Od takrat so v rabi različna imena, izpeljanke in kombinacije imen. Premalo prodorna priporočila stroke so večinoma ostala spregledana. Predvsem v šolstvu je zavladata negotovost glede pomena in ustreznosti različnih imen. V prispevku sta skupaj z argumenti podana predloga za enotno poimenovanje tega evolucijskega procesa z domačim imenom *naravno izbiranje* in s tujko *naravna selekcija*.

Ključne besede

naravna selekcija, naravno izbiranje, biologija v šoli, evolucija

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A plea for a proper use of the Slovenian term for 'natural selection'

Abstract

In Darwin's year, 2009, a new Slovenian translation of *The Origin* has introduced a hitherto unused combination of words to name the process of natural selection. This proposal was deemed by the translator as superior to the previously well-established terms but was rejected by the leading Slovenian authority on evolutionary biology. Not knowing about this rejection, and due to the central position of Darwin's book among the evolutionary literature, several authors, teachers and other users adopted the new term while some are using combinations of both. This has caused uncertainty about the proper use and meaning of different variants, especially among teachers. The present contribution is a plea for a stable Slovenian naming for natural selection based on semantic considerations.

Keywords

natural selection, Slovenian terminology, teaching biology, evolution

Besedne zveze iz naslova opisujejo enega najpomembnejših procesov za razumevanje živega sveta in človeštva po trenutno sprejeti znanstveni razlagi v sodobni družbi. Tega pomena se zahodna civilizacija zaveda od Darwina (1859) dalje. V slovenščini je najkasneje od prvega prevoda tega dela izpod prevajalskega peresa Ružene Škerlj iz leta 1954 veljalo popolno soglasje o tem, da se proces poimenuje z domačim izrazom naravni izbor. Tako je bilo vse do proslavljanja 150-te obletnice izida knjige, ki ga je Znanstvenoraziskovalni center Slovenske akademije znanosti in umetnosti obeležil z novim slovenskim prevodom prevajalca Bogdana Gradišnika (2009). Gradišnik je radikalno posegel v domače strokovno izrazoslovje. Ohranil je le koren pridevnika, celoten izraz pa prekoval v naravno odbiranje. Jezikovno novost je takoj podprl pisec spremne besede, biolog Matjaž Kuntner (Kuntner, 2009).

Med biologi, učitelji biologije in preštevilnimi drugimi strokovnjaki in nestrokovnjaki, ki sem ter tja zapišejo ali izrečejo slovensko ime osrednjega evolucijskega procesa, je zavladala negotovost. Ali lahko nestrokovnjak narekuje stroki, kako naj komunicira? Zakaj se nihče drug ne oglasi, zakaj ni širše strokovne razprave, ki je nujna, ko gre za tako korenit poseg v jezik in predvsem za pojem s tako usodnim pomenom? Po presenetljivo dolgem zatišju se je oglašil starosta slovenske evolucijske biologije, profesor Boris Sket (2015), ki je imenu *naravno odbiranje* odločno oporekal. Javna strokovna razprava je torej bila, čeprav ne ravno široka. Ker se ni odzval nihče drug, bi načeloma moralo veljati, da je stroka odklonila Gradišnikov predlog *naravno odbiranje*. V formalnem smislu bi morala biti

debata zaključena z jasnim izidom: predlog zavrnjen.

Toda v praksi se je zgodilo drugače. Razvoj dogodkov sta pravilno napovedala Kranjc in Razpet (2016), sicer fizika in pedagoga. Gradišnikovo novo poimenovanje sta opisala kot »zanimiv primer poseganja laika v znanstveno terminologijo« in napovedala, »da bosta vsaj nekaj časa v uporabi oba izraza in sejala dvom, kaj pomeni eden, kaj drugi«. Napovedala sta tudi zaplete in daljnosežne posledice, ker je z novostjo treba uskladiti vso drugo literaturo vključno z učbeniki.

Napovedane posledice so že tu, in sicer v obliki precejšnje zmede v aktualni slovenski strokovni literaturi, kjer na primer Gogala (2010) in Rutar (2020) uporabljata *naravno odbiranje*, Kuščer (2023) *naravni izbor*, avtorji gimnazijskega učbenika pa so se v obupu zatekli k himeri *odbiranje z naravnim izborom* (Stušek et al., 2010). V času nastajanja tega prispevka potekajo priprave na prenovu nacionalnih učnih načrtov, in učitelji biologije so izrazili negotovost, kateri slovenski izraz zapisati v učni načrt. Koncept je preveč bistven, da bi si kot družba lahko privoščili dvom o njegovem pravem pomenu. Najkasneje pri učnih načrtih in učbenikih je potrebna popolna nedvoumnost glede vsebine. To pa v strokovnem jeziku zagotavlja le stabilno enoznačno poimenovanje. Kranjc in Razpet (2016) sta povedala naravnost: »V vsakdanjem jeziku je mnogoterost sopomenk bogastvo, ki ga posebej leposlovje izkorišča za to, da je besedilo jezikovno zanimivejše in lepše, opisi raznolikeyši in za bralca privlačnejši. V znanstveni terminologiji dvojno ali večkratno poimenovanje istega pojma vnaša negotovost in dvom glede pravega pomena izrazov. Zato ni prednost, marveč

slabost.« Velika slabost je, kadar so v rabi kar štiri različna imena za isti naravni proces (Naravni izbor, Wikipedija).

Nastali položaj, predvsem negotovost glede učnih načrtov in s tem strokovnega izrazja v šolskem sistemu, terja jasno in argumentirano stališče stroke. Dobra novica je, da se enostavna rešitev ponuja sama od sebe. Imeni *naravni izbor* in *naravno odbiranje* sta namreč semantično neustrezni, s čimer ostaneta v izboru le še slovensko ime naravno izbiranje in pa seveda tujka naravna selekcija. Tujka je, kakršna je, nerodna ali nelepa, a vendar nevtralna in nepogrešljiva v žargonu. V učnih načrtih ter novih poljudnoznanstvenih in drugih domačih besedilih pa se bomo v idealnem primeru srečevali le še s pomensko ustreznim imenom *naravno izbiranje*. V nadaljevanju so podani bistveni argumenti v prid temu imenu, skupaj s pojasnili, zakaj sta alternativni imeni manj posrečeni. A začnimo pri tujki.

NARAVNA SELEKCIJA

Po Gradišniku (2009) je izraz problematičen zaradi nerodnosti pri uporabi glagola »selekcionirati« (narava selekcionira...). Z leposlovnega stališča je to nemara res lahko razlog za zavrnitev. Za strokovno rabo pa je inertna tujka najbolj priporočljiva, ker kot jasno definiran terminus technicus ni podvržena napačnim interpretacijam antropomorfiziranih prisposodob izbiranja ali odbiranja. V strogo tehničnem smislu niti ni priporočljivo zapisati, da narava selekcionira, ker to ne ustreza objektivnim dejstvom. Dejstva so razlike v preživetju in razmnoževanju, kdo koga selekcionira, pa je stvar subjektivne perspektive. Da s pojmom *naravna selekcija* ne moremo izvajati vse mogoče jezikovne akrobatike, v resnici ni njegova šibkost, temveč dobrodošla stranska posledica in razlog več, da tujko obdržimo v domačem besednjaku. Nenazadnje je beseda selekcija sama ali v raznih drugih kombinacijah tako dobro udomačena, da je pogosto sploh ne zaznavamo več kot tujko.

NARAVNI IZBOR

Je desetletja uveljavljeno in splošno sprejeto slovensko ime, s katerim so spoznavale evolucijo povojne in po-osamosvojitvene generacije dijakov in biologov. Gradišnik je opozoril na časovno omejenost, torej dovršno naravo glagola *izbrati*, ki je dejavnost, ki vodi do izbora. Argumentiral je, da je za ponazoritev stalnosti dogajanja treba uporabiti samostalnik oz. glagolnik, ki se naslanja na nedovršno obliko glagola. Prevajalcu novega slovenskega prevoda *On the origin of species* gredo vse zasluge, da smo biologi dojeli jezikovno

razliko med samostalnikom *izbor* in glagolnikom *izbiranje*. Vendar je Gradišnik iz ne najboljše pojasnjenega razloga ob uvedbi novega strokovnega izraza temu zamenjal osnovo in iz njega naredil *naravno odbiranje*.

NARAVNO ODBIRANJE

Vpeljava novega glagolnika *odbiranje* naj bi po Gradišniku nudila veliko prednost pred zvezama s samostalnikoma *selekcija* in *izbor*, ker omogoča tvorbo raznih zvez in povedi, kot so *narava odbira*; *naravno, umetno, spolno odbiranje*; *narava je odbrala*; *naravno odbran-a*. A ob tem je prevajalec namerno ali nenamerno spregledal, da vse to v najmanj enaki meri velja tudi za zvezo *naravno izbiranje*. Kot že omenjeno, so *naravno izbiranje* in vse sopomenke metafore. Z njimi poimenujemo proces, verno opisati pa ga ne moremo. Seveda pomaga, če je metafora čim bolj nazorna in ne zavaja. Predponi *iz-* in *od-* imata v slovenščini različen pomen. Izbira se iz kateregakoli dela skupine, odbira se od roba, *od* začetka ali *od* konca porazdelitve. Slednje drži za usmerjeno selekcijo, a je to le del resnice. Druge oblike selekcije dajejo prednost vmesnim fenotipom. Lahko bi rekli, da jih narava *izbere iz* sredine porazdelitve, medtem ko *odbrati od* sredine ne zveni slovensko. Nenazadnje je *izbiranje* tesno povezano z izbirčnostjo, ki, spet kot metafora, opisuje selekcijski proces, takrat ko ga vrši organizem s svojim nagnjenjem za na primer določen plen ali lastnost spolnega partnerja.

NARAVNO IZBIRANJE

Poleg tega, da bolj točno opiše naravo delovanja evolucijskega procesa kot *odbiranje*, z njim bistveno manj posegamo v ustaljeno terminologijo. Pojem *izbiranje* je v slovenščini veliko bolj domač kot srbohrvatizem *od(a) biranje*; razmerje zadetkov v spletnem iskalniku je 30 : 1. Če ostanemo pri *izbiranju*, obdržimo jedro izraza, ki je že tako dolgo v rabi in tako udomačen v stroki in širše.

Zaključek je, če smem govoriti v imenu kolegic in kolegov, da biologi hvaležno sprejemamo Gradišnikov poduk o nedovršnosti glagolnika, ki bolje opisuje trajen proces kot samostalnik, izhajajoč iz dovršnega glagola. Zato je prav, da ime *naravni izbor* postopoma izgine iz učbenikov, učnih načrtov in spletnih strani, ter ga zamenja bolj ustrezen izraz *naravno izbiranje*, kot alternativa pa se obdrži uveljavljena tujka *naravna selekcija*. Predlog *naravno odbiranje* ni le nepotreben, temveč je v več pogledih neprimeren in zavajajoč, zaradi česar ga je treba odločno odkloniti.

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