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APPLICATION OF DIFFERENT INSECTICIDES BY DRIP IRRIGATION METHOD AGAINST EUROPEAN CORN BORER ON CORN CROPS

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Key words: corn, European corn borer (ECB), mass flight, caterpillar, phytophagous, insecticides, drip irrigation, insectigation.

Abstract

The peculiarities of the development and dynamics of the population density of European corn borer (ECB) on corn crops under irrigation were studied in 2019–2021 in conditions of Steppe of Ukraine. Field investigations were conducted according to generally accepted methods. The size of the experimental plots in the field experiments was 50 m^2 (10.4 x 4.8 m), the replication was 4 times. Allocation of plots was randomized. The beginning of the first adults flight was observed in the 1st decade – in the middle of June at average. Mass laying of eggs and the flight of more than 50% of the butterfly population occurred in the 3rd decade of June, which coincided with the phase of pollen shedding of corn plants. Mass flight of the butterfly (flight of more than 75% of the population) was observed at the end of the 3rd decade of June – the beginning of the 1st decade of July. The revival of caterpillars began in the third decade of June and continued until the end of July. The average population density of European corn borer caterpillars in 2019–2021 were 1.6 and 1.8 specimens per stem on corn crops. The European corn borer is a dangerous pest of corn in the Steppe zone of Ukraine, where it's caterpillars damage 34.5–37.8% stems and ears of this crop. Application of Coragen 20 KS, Calypso 480 SC and Actara 25 WG at higher from recommended rates against the ECB contributed to the reduction of plants damage in 8–15 times, compared to untreated crops.

Introduction

The yield formation of agricultural crops in agrocenosis depends on the influence of many factors (pests, weeds and diseases). The anthropo-

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genic factor has a direct impact on the functioning of the agrocenosis. First of all, it is the choice of the *agricultural producer* in the application of certain elements of crop cultivation technology. Applying of the agricultural technology and the use of chemical measures of protection against harmful objects are related to all elements of the agrocenosis structure and significantly affected its productivity. From the point of view of plant protection to obtain a qualitative yield of a specific crop (including corn) is to reduce the number of harmful organisms to the level of their economic threshold (DÖRING at al. 2012).

The corn planting area in Ukraine exceeds 5 million hectares in 2021. Crop productivity is a complex factor that depends on the relationship between non-biological, biological factors and various components of plants structure. The yield of corn grain varies from 7.9 to 12.2 t ha^{-1} from year to year. As known, the corn can reach potential yield in 15–18 t ha^{-1} in different climatic zones of Ukraine. Unfavorable hydrothermal conditions of the vegetative seasons observed in recent years may be among the main reasons for crop losses (up to 30%). Low reserves of productive moisture in the one-meter layer of soil under crop determine the insufficient moisture supply of corn plants in the most critical phases of their growth and development, that leads to problems with grain filling (KLISHCHENKO at al. 2006, LAVRYNENKO et al. 2014).

Abnormally high temperatures during the development of the generative organs of this crop, in particular during the tasseling – pollen shedding at the absence of effective precipitation, starting from mid-June, determine the main reasons for the loses of corn grain yield: drying of pollen, insufficient pollination. Plants are the most sensitive to heat between the appearance of cob silks and milky kernel maturity. Four days of severe heat stress during this period can lead to a loss of 40–50% yield of the crop (ROMASHCHENKO at al. 2015, USHKARENKO et al. 2015, SHATKOVS'KYY 2018, LYKHOVYD at al. 2019).

Unsatisfactory phytosanitary condition of the corn agrocenosis is not less important factor for obtaining a low level of yield and its quality. Thus, yield losses can reach up to 40% at insufficiently effective and untimely control of corn pests. Therefore, in order to save grain from potential losses, it is extremely important to pay increased attention to the insecticide protection of this crop against harmful organisms. Spraying corn crops by insecticides ensures high biological and economic efficiency. But a small number of producers think about the consequences of such a wide use of chemicals. The selection of insecticides and the methods of their application should be rational and aimed to increase the delivering environmental safety of the agrocenosis in order to obtain environmentally safe prod-

ucts (MASON at al. 1996, TRYBEL′ at al. 2009, GARDNER at al. 2011, MELNICHUK at al. 2017).

Irrigation is one of the main factors for the intensification of the agriculture in areas with insufficient and unstable natural moistening. That is why artificial watering became widespread in the 20th century. Currently, in the world more than 270 million hectares are under irrigation. And irrigated lands provide more than 40% of the world crop production, including only 18% of the area of agricultural land (DUDKA 2013).

In arid regions the irrigation is an important factor that guarantees high corn yields. It affects not only on the conditions of plant growth, but also on the development of all living organisms in the soil, plants, and in the vegetation zone. During irrigation, the microclimate of the surface layer of the soil and atmosphere changes significantly. Irrigation creates more favorable conditions for the most pests of hydrophilic and mesophilic ecological groups (ELLERS at al. 2018). The last ones in all stages of their development are not associated with the soil and live in the zone of the plant layer. Pests whose numbers increase at irrigation also include the European corn borer (*Ostrinia nubilalis* Hübner, 1796).

Drip irrigation is a method of watering in which water is moving directly to the root feeding zone of each plant in accordance with its biological and age peculiarities. The main principle of providing plants with water is to moisten only a definite volume of soil in which the root system is located. The volume of drip irrigation usage began to grow rapidly only at the beginning of the 90s of the XX century. Because it became clear in practice that drip irrigation, in addition to saving water resources, is also significantly increasing the yield of agricultural crops. The particularly dynamic development of drip irrigation was observed in the early 2000s, when the areas of drip irrigation in Ukraine grew up to 30–70% every year (ELANGO and NISHA PRADEEPA 2017).

Despite the overall success of foliar spraying by insecticides for controlling harmful insects, there are several negative moments, including risks to human health and the environment. That is why insectigation (the use of insecticides together with water by the method of drip irrigation) is becoming more and more widespread worldwide (GHIDIU et al. 2009).

Systemic insecticides applied by drip irrigation enter directly into the root zone of plants, where they are absorbed by the roots and moved to various plant tissues. Since insecticide residues located in the vascular system of plants and not on their surface, only insects that feed directly on the plant are affected. Therefore, this method is considered as safer for non-target organisms in agricultural fields, such as entomophages or bees. Beside with the effectiveness of insecticides for target objects, their safety for

the main components of agrocenoses is actual. So, it is important to evaluate the action of insecticides of several chemical classes recommended for the protection crops against pests, to identify among them active ingredients that are safe to beneficial arthropods of agrocenoses (UMETSU and SHIRAI 2020).

Analysis of recent research and publications. The aim of improvement of the insecticides range is to increase their environmental safety in agrocenosis, in particular – to reduce the toxic load. Insecticides of a new class of chemical compounds – neonicotinoids appeared on the world market at the end of the 20th century. Their active ingredients were obtained based on natural toxins of plant (nicotine) and animal (anabasin) origin (PREETHA and STANLEY 2012).

The most suitable insecticides for use together with irrigation water are active ingredients from the class of neonicotinoids (such as thiamethoxam, thiacloprid, imidacloprid, acetamiprid) and anthranilic diamides (such as cyantraniliprole, chlorantraniliprole, lambda-cyhalothrin). Neonicotinoids have a systemic effect. They can move to the plant from the soil through the root system (root-systemic acropetal action), so they can be applied with drip irrigation. Neonicotinoids have a high level of mobility in the vascular system together with nutrients mainly in the leaves, but practically do not enter the grain and fruits. The duration of the protective effect of these insecticides is up to 6 weeks, and their effectiveness does not depend on changes in temperature and humidity (PEI-CHEN at al. 2013).

Investigating the dynamics of the decomposition of thiamethoxam and lambda-cyhalothrin in plants, degradation to undetermined amounts was established on 14th day after treatment. An active ingredient lambda-cyhalothrin degraded on the 28th day after treatment, and the concentration of its metabolites continued to decrease. Redistribution of the chlorantraniliprole in the plant occurs due to its translaminar movement through the cells of the stem epidermis and the conducting xylem vessels, which contributes to the entry of this active ingredient into the new growth. A high level of protective effect of chlorantraniliprole with drip application against the European corn borer was noted in foreign studies. As a result of research conducted with the use of an insecticide containing chlorantraniliprole to protect corn crops, it was established the presence of residual amounts of the insecticides active ingredient mainly in plants, not in the soil (LEPESHKYN at al. 2015, HLADIK et al. 2014, SANCHEZ-BAYO and GOKA 2014, SCHAAFSMA at al. 2015).

Relevance of research. The combination of using insecticides together with meliorated water using the drip irrigation method has

advantages compare with traditional spraying due to the rational use of irrigation water and pesticides to save the crop yield. The active ingredients of insecticides from the classes of neonicotinoids and anthranilic diamides are fit for use in drip irrigation systems. Because they have excellent solubility in water and, accordingly, quickly reallocate into the roots, further moving through the vascular system of the plant. Also, during insectigation, dependence on high air temperatures is eliminated.

The purpose of our research was to clarify the peculiarities of the ECB development and to study the effectiveness of application insecticides from the classes of neonicotinoids and anthranilic diamides together with irrigation water by drip irrigation to limit the development of this pest and control its number on corn crops in the Steppe zone of Ukraine.

Materials and Methods

Field investigations were conducted on corn crops according to generally accepted methods (Trybel′ at al. 2001) during 2019–2021 in the conditions of the Kherson Region, State Enterprise Experimental Farm 'Brylivske' of the Institute of Water Problems and Land Reclamation of the National Academy of Agrarian Sciences of Ukraine (coordinates 46.404028° N latitude, 33.112361° E longitude).

The emergence of the imago and, accordingly, the beginning and mass flight of the ECB on the fields were recorded using fermental traps with molasses and pheromone traps. According to the pest development cycle, the female eggs on plants were counted. To do this, 10 plants from two parallel rows were observed in 10 places of the field, calculating their average number per 1 square meter.

The size of the experimental plots in the field experiments was 50 m^2 (10.4 x 4.8 m), the replication was 4 times. Allocation of plots was randomized. Corn hybrid is P8816 (FAO 280). The technical efficiency of insecticides was determined by the reduction in the number of caterpillars in corn stems during the harvest period and damage of stems, selecting 50 plants from each plot (5 in 10 places). Corn stems were cut lengthwise, and the number of caterpillars was counted.

Investigated insecticides were used according to the experiment scheme (Table 1). Most of studied active ingredients are not registered for use by drip irrigation in Ukraine.

Table 1
Scheme of the trial on corn crops under different application rates of insecticides and irrigation systems (2019–2021)

Preparation name	Active ingredient	Type of irrigation	
		sprinkler	drip
		rate [l ha^{-1}]	
Control	(treatment by water)	–	–
Coragen 20 SC	chlorantraniliprole, 200 g l^{-1}	0.4	0.6
Actara 25 WG	thiamethoxam, 250 g kg^{-1}	0.3	0.6
Mospilan 20 SP	acetamiprid, 200 g kg^{-1}	0.05	0.1
Calypso 480 SC	thiacloprid, 480 g l^{-1}	0.375	0.5
Confidor 200 SL	imidacloprid, 200 g l^{-1}	0.25	0.6

Insecticides were applied by sprinkler irrigation and drip irrigation, depending on their physical and chemical properties (solubility in water and mobility in the soil). Some preparations were added into irrigative water in the first third, others in the second third of the volume of water provided for irrigation. The mother solution of the insecticide was prepared in a container connected to the irrigation system (volume is 200 l) and application was started. After using the insecticide, the system must be washed with the amount of clean water equal to the volume of the whole irrigative system. Execution of this condition will ensure the distribution of the full insecticide dose on the experimental plot and will prevent the accumulation of its unused residues in the irrigation system.

The technological process of applying plant protection products, fertilizers and chemical reagents with irrigation water in drip irrigation systems is regulated by the State standard of Ukraine 7937:2015 *Irrigation. Bringing of fertilizers with water in systems of microirrigation. General requirements*. This normative document is active from 2016 and was developed by the Institute of Water Problems and Land Reclamation of the National Academy of Agrarian Sciences together with the National University of Life and Environmental Sciences of Ukraine and the National Scientific Center 'Institute for Soil Science and Agrochemistry Research named after O.N. Sokolovsky'.

Hydrothermal coefficient of Selianinov (HTC) calculated by the formula:

$$K = R \cdot 10/\Sigma t,$$

where:
R – the sum of precipitation in millimeters for a period with temperatures above +10°C
Σt – the sum of temperatures in degrees Celsius [°C] for the same time.

The lower the HTC, the drier the area (USHKARENKO et al. 2014, MELADZE and MELADZE 2017, STOYANOVA and GEORGIEV 2017, LYKHOVYD 2019).

The degree-days were calculated using the formula for the average daily temperature, calculated from the daily maximum and minimum temperatures, minus the development threshold (baseline temperature) (HERMS 2004, MUSAYEVA and YAXYAYEV 2020). Temperature +10°C was taken as development threshold for ECB.

Results and Discussion

Constant monitoring of the pest number during the growing season was provided for the planning and timely implementation of crop protective measures from the caterpillars of the ECB. Therefore, during 2019–2021, we observed the flight dynamics of adults and recorded the dates of egg laying by the females and the appearance of caterpillars.

The development of the ECB in the conditions of the Kherson region took place in two generations. At the same time, the development of the second facultative generation was noted at the end of August – September. Monitoring of fermental traps with molasses, as well as pheromone traps in 2019 showed that the flight of the first adults of the ECB occurred in the middle of the first decade of June (369.2 GDD) – Table 2.

Table 2

Biological features of the European corn borer

Stage of ECB development	Date of stage beginning (GDD)		
	2019	2020	2021
Beginning of flight first adults of ECB. Start of egg laying	06.06 (369.2)	16.06 (374.8)	21.06 (379.5)
Mass egg laying	11.06 (435.1)	21.06 (441.5)	25.06 (442.0)
Mass flight (> 50% of population)	18.06 (536.5)	28.06 (543.0)	01.07 (532.1)
Mass flight (> 75% of population)	23.06 (621.4)	03.07 (624.9)	08.07 (623.5)

The increase in the density of the phytophagous population occurred at increasing of air temperature. An activity of the imago flight increased with significant warming, and its mass flight was observed at the end of the second decade of June (536.5–621.4 GDD).

The flight of the first adults of the ECB in 2020–2021 was observed in mid-June (374.8–379.5 GDD). The mass laying of eggs and flight of more than 50% of the butterfly population occurred in the third decade of the

month (441.5–543.0 GDD), which coincided with the phase of pollen shedding by corn tassels. The mass flight of the butterfly (flight of more than 75% of the population) was observed at the end of the 3rd decade of June – the beginning of the 1st decade of July (624.9–623.5 GDD). The revival of caterpillars began in the third decade of June and lasted until the end of July.

Analyzing the meteorological conditions of the research period, we noted that the sum of active temperatures in 2019–2020 exceeded the long-term indicator by 205–220°C (Table 3). The vegetation period of 2019 and 2021 was characterized by sufficient moisture supply (HTC – 1.26–1.41), while in 2020 it was dry (HTC – 0.70). The dynamics of the butterfly's population over the years depended on weather conditions. A large amount of precipitation without heavy rains was favorable for the massive reproduction of the pest. While dry weather limited its number, at the same time speeding up the phases of growth and development of corn plants.

Table 3

Meteorological conditions of the vegetative season

Weather indicator	2019	2020	2021	Long-term average
Sum of active temperatures (SAT) for IV–IX months [°C]	3470.3	3455.5	3208.9	3250.0
Sum of effective temperatures (SET) for IV–IX months [°C]	1780.3	1765.5	1618.9	1527.0
Sum of precipitation for IV–IX months [mm]	437.8	242.0	452.8	247.0
HTC	1.26	0.70	1.41	0.76

Protection against the ECB is quite complicated. So it is important to determine the correct time of spraying with a very extended period of the butterfly's flight. As can be seen from Table 2, time duration is almost 20 days from the appearance of the first adults to the flight of 75% of the pest population. Caterpillars of older and younger instars were found at the same time at observations and scores. In addition, for the larvae's is native a hidden lifestyle, and therefore they become not available for the action of insecticides.

The caterpillars of the ECB are very dangerous phytophagous of corn during the period of pollen shedding and grain formation. Afterwards its damage of stems and ears will cause a significant threat of yield reducing. Taking this into account, insecticides were applied by sprinkling and drip application to control the pest number and its harmfulness. The preparations were used in the phase of the beginning of pollen shedding by corn

tassels. At dissecting plant stems, the number of pests on the control variants was 1.8 specimens per stem at sprinkling and 1.6 specimens per stem at drip application.

The results showed the effectiveness of all insecticides reached 77.2–94.2% by decreasing damage of plants by caterpillars at the level of 2.0–8.6%. In particular, the insecticides Coragen 20, KS, Calypso 480 SC and Actara 25 WG at higher rates contributed to the reduction of phytophagous plant damage to 2.0–4.5%, which is 8–15 times less compare to the control (Table 4). Damage of corn stems on the control variant was at average 34.5–37.8%.

Table 4

Effectiveness of the insecticides application on corn against the European corn borer at different application rates and irrigation systems (average for 2019–2021)

Variant	Application rate [l ha^{-1}, kg ha^{-1}]	Damage of plants [%]		Efficiency [%]	
		sprinkler	drip	sprinkler	drip
Control	–	37.8	34,5	–	–
Coragen 20 SC	0.4	3.2	2.8	91.5	91.9
	0.6	2.4	2.3	93.7	93.3
Actara 25 WG	0.3	4.5	4.1	88.1	88.1
	0.6	2.8	2.0	92.6	94.2
Mospilan 20 SP	0.05	8.6	6.1	77.2	82.3
	0.1	4.7	4.6	87.6	86.7
Calypso 480 SC	0.375	4.4	4.2	88.4	87.8
	0.5	2.8	2.4	92.6	93.0
Confidor 200 SL	0.25	6.4	4.5	83.1	87.0
	0.6	3.3	2.6	91.3	92.5
LSD (at $p < 0.05$)	–	1.2	1.04	2.7	2.49

The maximum protection of the crop against the ECB was noted for the drip application of insecticides at two application rates. Thus, insecticides based on the active ingredients of thiamethoxam, thiacloprid and chlorantraniliprole controlled the pest, significantly reducing its number. Insecticide Actara 25 WG reliably decreased the damage of corn plants by caterpillars with an efficiency of 88.1–94.2%. The somewhat lower efficiency was obtained at applying by drip irrigation method of the insecticide Coragen 20 SC with the application rates of 0.4–0.6 l ha^{-1} – 91.9–93.3%. The lowest control of the number of caterpillars of the ECB was noted at application of the insecticide Mospilan 20 SP (active ingredient acetamiprid). Its level of protection was 82.3–86.7%.

The data obtained during the harvesting of the yield also indicate significant level of protection by the tested insecticides applied against this pest (Table 5). Thus, a significant reducing of density of stems was found (62.3–65.2 thousand ha^{-1}) on control. Corn plant stems were broken because of caterpillar's damage. This led to a lack of yield of corn grain due to the inability of the grain-harvesting header to pick up the ears that fell on the soil surface. Before harvest, plant density was in 1.2–1.35 times higher on the insecticide-treated plots compared to the control.

Table 5

The effect of the application of insecticides and their rates on productive indicators of corn under different types of irrigation (average for 2019–2021)

Variant	Application rate [l ha^{-1}, kg ha^{-1}]	Plant density before harvesting [thousands ha^{-1}]		Yield [t ha^{-1}]		± To control [t ha^{-1}]	
		sprinkler	drip	sprinkler	drip	sprinkler	drip
Control	–	62.3	65.2	13.0	13.9	–	–
Coragen 20 SC	0.4	78.3	83.0	16.7	17.4	3.7	3.5
	0.6	84.1	84.4	17.3	17.8	4.3	3.9
Actara 25 WG	0.3	78.5	83.0	16.4	17.6	3.4	3.7
	0.6	83.3	84.5	17.0	18.1	4.0	4.2
Mospilan 20 SP	0.05	80.5	81.5	15.9	16.8	2.9	2.9
	0.1	83.3	83.5	16.6	17.2	3.6	3.3
Calypso 480 SC	0.375	77.3	79.5	15.9	16.4	2.9	2.5
	0.5	80.3	83.0	16.7	17.3	3.7	3.4
Confidor 200 SL	0.25	81.8	83.5	16.2	17.3	3.2	3.4
	0.6	83.8	85.3	16.8	18.0	3.8	4.1

In the variant with the use of chemical protection, damage by the caterpillars of ECB to crop plants was reduced. This made it possible to save a significant share of the grain yield, compared to untreated plants. The highest level of yield (17.8–18.1 t ha^{-1}), compared to the control, was obtained on the plots with the use of higher rates of insecticides Coragen 20 SC, Confidor 200 SL and Actara 25 WG at applying them with irrigation water. Therefore, 3.9–4.3 t ha^{-1} of grain was additionally obtained.

The use of insecticides together with irrigation water. in comparison with traditional spraying, helps to improve the ecological condition of the agrocenosis. Also it improves the sanitary and hygienic condition of the working area of employees, and at the same time allows to grow ecologically clean products. The disadvantage of this method is the limited list of registered active ingredients that can be used. So, the research of modern

and new active ingredients of insecticides regarding the possibility of their application with water in irrigation systems is the promising direction of investigations.

Conclusions

1. The beginning of the ECB first imago flight in 2019–2021 was observed in the 1st decade – mid-June (369.2–379.5 GDD). The mass laying of eggs and flight of more than 50% of the butterfly population occurred in the II–III decades of the month (435.1–543.0 GDD), which coincided with the phase of pollen shedding by corn plants.

2. The mass flight of the imago (flight of more than 75% of the population) was observed at the end of the 3rd decade of June – the beginning of the 1st decade of July (621.4–624.9 GDD). The revival of caterpillars began in the third decade of June and continued until the end of July. The average number of larvae of the European corn borer in 2019–2021 was 1.6 and 1.8 specimens per stem in corn crops.

3. The European corn borer is a dangerous pest of corn in the Steppe zone of Ukraine, where it's caterpillars damage 34.5–37.8% stems and ears of this crop. Application of Coragen 20 KS, Calypso 480 SC and Actara 25 WG at higher from recommended rates against the ECB contributed to the reduction of plants damage by the pests in 8–15 times, compared to untreated crops.

4. Due to the reduction of corn plants damage by ECB caterpillars before harvesting, the density of plants was 1.2–1.35 times higher in the areas treated with insecticides, compared to the control. Treatment corn crops with insecticides makes possible to save the corn grain yield share at the level of 3.9–4.3 t ha^{-1}.

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SOYBEAN MEAL ALTERNATIVES IN RABBIT DIETS – A REVIEW OF CENTRAL EUROPEAN RESEARCH

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Abstract

Soybean meal (SBM) is a by-product of soybean processing, and it is widely used as a component of diets for various livestock species, including rabbits. However, at the beginning of the 21st century, researchers began to search for alternative high-protein feeds that could replace SBM in rabbit diets. The aim of the present study was to review, in chronological order, the literature on alternative feed ingredients as substitutes for SBM in rabbit diets in Central Europe in the 21st century. The authors reviewed studies investigating the replacement of SBM in rabbit diets with plant-based protein sources such as maize and wheat distillers' dried grains with solubles (DDGS), rapeseed meal, rapeseed cake, white lupine seeds, peas and silkworm pupae and mealworm larvae meals.

The analysis revealed that SBM in rabbit diets can be completely or partially replaced with other high-protein plant ingredients and insect meals without compromising the performance of animals.

The soybean (*Glycine max*) is an annual plant of the family Fabaceae. Its most productive part is the seed pod containing up to four seeds. The soybean was first domesticated in China in the 11th century BCE, and this crop was introduced to both Americas and Europe only in the 18th century CE. At present, the soybean is one of the leading crops in the global economy, and the interest in this plant species continues to increase (ANDERSON et al. 2019). The world's leading producers and exporters of soybeans are the United States, Brazil, Argentina, India, China,

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Canada, and Paraguay (LAGOS and STEIN 2017, IBÁÑEZ et al. 2020). Soybean production is still low in Europe, mainly due to unfavorable climatic conditions, but its cultivation has increased in recent years. Genetically modified (GM) soybeans were introduced to the global market in the 1990s. The distinguishing feature of GM soybeans was their resistance to the Roundup herbicide. New GM soybean varieties that are resistant to other plant protection products and pests were developed in the following years. At present, more than 80% of soybeans cultivated worldwide are GM plants, and GM soybeans occupied 50% of the global area under modified crops in 2018 (SIERADZKI et al. 2021). In the European Union, genetic modification of crops for food and feed production continues to stir considerable controversy. However, conventional (non-GM) soybeans are more expensive to produce, and they are cultivated mainly in the more affluent European countries and in Japan (GUILPART et al. 2022).

The global popularity of soybeans can be attributed to their high protein content. On average, soybeans contain 35–38% of protein with a highly favorable amino acid profile for feed production (KARR-LILIENTHAL et al. 2004). This crop is also cultivated for the production of food, and it can be used as a replacement for animal protein sources in vegetarian and vegan diets. Soybeans contain approximately 18% of fat rich in essential unsaturated fatty acids such as linoleic, oleic and linolenic acids. Soybean oil is used in the food processing industry and in the production of lubricants and biofuels. Soybean oil is also incorporated into animal diets to enhance their energy value and fatty acid content (GÜZELER and YILDIRIM 2016, ANDERSON et al. 2019).

The by-products of soybean oil extraction are used in animal nutrition, including in rabbit diets. These include soybean meal (SBM), soybean hulls, and soy protein concentrates. Soybean meal is the most popular by-product of soybean oil processing. It is a rich source of protein and exogenous amino acids for fast-growing farm animals during intensive fattening and for high-yielding livestock. The protein content of SBM ranges from 42% to 50%, and it is usually determined at 45.0–49.5%, depending on the soybean cultivation method, variety, and geographic origin. For example, SBM produced in the US sometimes contains more crude protein, fewer oligosaccharides, and is characterized by lower trypsin inhibitor activity than SBM produced in Argentina or Brazil, which results in higher apparent ileal digestibility of nitrogen and higher energy value. It should also be noted that thermal processing reduces the content of anti-nutritional factors in soybeans (KARR-LILIENTHAL et al. 2004, DE COCA-SINOVA et al. 2008, RAVINDRAN et al. 2014, LAGOS and STEIN 2017, IBÁÑEZ et al. 2020).

The chemical composition and energy value of SBM fed to rabbits in experiments conducted in Central Europe are shown in Table 1. In all tables, the results are presented on a dry-matter (DM) or a fresh-matter (FM) basis, and energy value is expressed as gross energy (GE) or digestible energy (DE), as in the cited studies. All abbreviations are spelled out under the tables. The protein content of SBM ranged from 36.82% (STRYCHALSKI et al. 2020) to 47.10% (VOLEK et al. 2018b) on a FM basis, and from 50.26% (KOWALSKA et al. 2020b, KOWALSKA et al. 2021, STRYCHALSKI et al. 2021) to 53.20% (ZWOLIŃSKI et al. 2017) on a DM basis.

Table 1

Chemical composition and energy value of soybean meal (according to the authors cited in the study

DM	CP	EE	CF	NFE	NDF	ADF	ADL	GE	Authors
[%]								[MJ/kg]	
86.80	43.80	2.30	–	–	10.80	6.70	2.50	–	VOLEK and MAROUNEK (2009)** VOLEK et al. (2014)**
88.24	45.93	2.46	–	–	16.97	7.41	–	18.11	STRYCHALSKI et al. (2014)**
88.20	53.20	2.30	4.20	–	19.20	8.40	–	17.10	GUGOŁEK et al. (2015)*
90.11	50.15	2.05	–	35.70	15.67	6.92	5.56	19.45	GUGOŁEK et al. (2017)*
89.66	50.40	20.64	–	–	15.41	7.56	4.23	14.53	ZWOLIŃSKI et al. (2017)*
87.30	47.10	1.90	–	–	9.90	5.80	1.40	–	VOLEK et al. (2018b)**
90.10	45.20	1.80	4.60	32.20	–	–	–	12.50[3)]	GUGOŁEK et al. (2018)**
88.68	46.56	1.98	4.44	34.12	10.63	6.25	1.15	17.36	GUGOŁEK et al. (2019)** GUGOŁEK et al. (2021)**
90.11	45.19	1.85	–	–	14.12	6.24	5.01	17.53	KOWALSKA et al. (2020a)**
89.92	36.82	2.17	–	–	16.57	7.36	5.08	17.13	STRYCHALSKI et al. (2020)**
89.10	46.50	2.40	–	–	11.70	7.00	–	–	VOLEK et al. (2021)**
89.35	50.26	2.15	–	–	15.02	7.84	3.96	16.38	KOWALSKA et al. (2020b)* KOWALSKA et al. (2021)* STRYCHALSKI et al. (2021)*
–	46.50	–	–	–	–	–	–	–	NOWAKOWICZ-DĘBEK et al. (2021)** WLAZŁO et al. (2021)**

* – in DM; ** – in FM; *** – digestible energy

DM – dry matter; CP – crude protein; EE – ether extract; CF – crude fiber; NFE – N-free extract; NDF – neutral detergent fiber; ADF – acid detergent fiber; ADL – acid detergent lignin; GE – gross energy; FM – fresh matter

Soybean meal is a popular protein source in complete pellets for rabbits. Most studies addressing this issue were conducted in the 1990s (NASR et al. 1996, PRASAD and KARIM 1998). Previously, peanut meal and animal meals had been included in livestock diets to increase protein content (GUGOŁEK and KOWALSKA 2022).

In addition to SBM from defatted flakes, soy protein concentrates are also fed to farm animals, including rabbits. Soy protein concentrates contain up to 90% of crude protein, and they are frequently added to diets for pregnant and lactating does (GARCÍA et al. 1999, GUTIÉRREZ et al. 2003, CHAMORRO et al. 2007).

Soybean hulls are yet another by-product of soybean oil extraction. Soybean hulls contain approximately 12–13% of crude protein and 2–3% of crude fat. They also contain around 36% of crude fiber, and can be used as a crude fiber source in rabbit diets (GARCÍA et al. 1999, NICODEMUS et al. 2007).

The by-products of the extraction of oil from soybean seeds, in particular SBM, are widely used as components of diets for broiler rabbits. However, at the beginning of the 21st century, researchers and breeders began to search for alternative high-protein feeds that could replace SBM in rabbit diets. This is because the production of SBM is expensive outside countries that are the world's leading soybean suppliers, including in Central Europe. Genetically modified (GM) soybeans also raise numerous concerns. Moreover, some countries have announced their plans to ban soybean cultivation for the production of feedstuffs (CHRISTIANSEN et al. 2019, JIANG 2020, SCHEITRUM et al. 2020).

Numerous studies have been conducted in Central Europe, i.e. in Poland and Czechia, to test other vegetable protein sources as potential SBM substitutes in rabbit diets, such as oilseed meals (STRYCHALSKI et al. 2014, GUGOŁEK et al. 2015, GUGOŁEK et al. 2017, ZWOLIŃSKI et al. 2017, VOLEK et al. 2018b, NOWAKOWICZ-DĘBEK et al. 2021, WLAZŁO et al. 2021), DDGS (CHEŁMIŃSKA and KOWALSKA 2013, STRYCHALSKI et al. 2014, GUGOŁEK et al. 2015), legume seeds (VOLEK and MAROUNEK 2009, VOLEK et al. 2014, GUGOŁEK et al. 2017, ZWOLIŃSKI et al. 2017, VOLEK et al. 2018a, VOLEK et al. 2018b, UHLÍŘOVÁ and VOLEK 2019, STRYCHALSKI et al. 2020), and insect meals (GUGOŁEK et al. 2019, KOWALSKA et al. 2020b, KOWALSKA et al. 2021, GUGOŁEK et al. 2021, STRYCHALSKI et al. 2021, VOLEK et al. 2021). The chemical composition and energy value of feed components used as SBM alternatives in rabbit diets, described in the above studies, are presented in Table 2.

Table 2

Chemical composition and energy value of feed components used as substitutes for soybean meal

Feed	DM	CP	EE	CF	NFE	NDF	ADF	ADL	GE	Authors
	[%]								[MJ/kg]	
Maize DDGS	88.88	23.59	12.63	5.45	–	–	–	–	–	Chełmińska and Kowalska (2013)**
Wheat DDGS	93.53	29.13	7.36	–	–	39.98	3.42	–	20.46	Strychalski et al. (2014)**
	93.50	39.30	7.80	8.50	–	42.80	3.60	–	18.50	Gugołek et al. (2015)*
DWLS	88.70	43.00	11.50	–	–	12.70	10.80	3.80	–	Volek et al. (2018a)**
FRM	87.87	36.15	1.63	14.16	–	–	–	–	–	Nowakowicz-Dębek et al. (2021)** Wlazło et al. (2021)**
MLM	94.30	51.34	27.95	–	–	11.42	7.59	1.26	22.50	Kowalska et al. (2020b)* Kowalska et al. (2021)* Strychalski et al. (2021*
	59.40	40.00	15.00	–	–	11.50	8.30	–	–	Volek et al. (2021)**
PS	88.14	25.25	0.96	–	63.51	12.77	7.9	5.34	18.83	Gugołek et al. (2017)*
	88.85	24.29	1.29	–	–	11.71	7.35	4.12	12.09	Zwoliński et al. (2017)*
	88.10	22.30	0.90	5.70	55.80	–	–		12.30[3)]	Gugołek et al. (2018)**
	88.14	22.26	0.85	–	–	6.96	11.26	4.71	16.60	Kowalska et al. (2020a)**
	88.85	29.61	1.26	–	–	14.63	7.72	4.60	16.48	Strychalski et al. (2020)**
RPM	91.02	37.98	3.99	–	33.35	26.29	14.62	6.42	17.48	Gugołek et al. (2017)*
	90.11	36.46	4.20	–	–	24.07	14.25	4.98	11.79	Zwoliński et al. (2017)*
	91.00	34.60	3.60	15.20	30.40	–	–	–	9.90[3)]	Gugołek et al. (2018)**
	87.80	34.50	–	–	–	28.30	20.10	8.50	–	Volek et al. (2018b)**
	91.02	34.57	3.63	–	–	23.93	13.31	5.84	15.91	Kowalska et al. (2020a)**
RSC	90.28	31.58	10.84	–	–	22.19	12.61	–	20.25	Strychalski et al. (2014)**
	90.20	35.50	12.00	13.40	–	24.60	14.00	–	18.90	Gugołek et al. (2015)*
SFM	88.70	27.50	2.80	–	–	38.60	28.00	9.50	–	Volek and Marounek (2009)** Volek et al. (2014) **
	88.50	27.30				39.90	28.70	9.80	–	Volek et al. (2018b)**
SPM	93.19	51.58	26.49	3.73	7.46	31.35	9.34	2.95	24.69	Gugołek et al. (2019)** Gugołek et al. (2021)**
	94.40	51.75	24.19	–	–	6.49	5.49	2.46	23.94	Kowalska et al. (2020b)* Kowalska et al. (2021)* Strychalski et al. (2021)*
WLS	88.30	29.70	11.40	–	–	33.00	23.00	5.90	–	Volek and Marounek (2009)** Volek et al. (2014)**
	88.79	46.28	2.27		31.64	31.41	22.12	5.99	18.77	Gugołek et al. (2017)**
	90.34	43.37	4.45	–	–	29.32	21.68	5.09	12.34	Zwoliński et al. (2017)**
	88.80	41.10	5.00	13.50	25.10	–	–	–	10.30[3)]	Gugołek et al. (2018)**
	89.00	34.90	–	–	–	22.70	17.00	6.50	–	Volek et al. (2018b)**
	88.79	41.09	2.02	–	–	27.89	19.64	5.32	16.67	Kowalska et al. (2020a)**
	90.13	29.61	2.64	–	–	28.85	16.93	5.52	16.65	Strychalski et al. (2020)**

* – in DM; ** – in FM; *** – digestible energy

DM – dry matter; CP – crude protein; EE – ether extract; CF – crude fiber; NFE – N–free extract; NDF – neutral detergent fiber; ADF – acid detergent fiber; ADL – acid detergent lignin; GE – gross energy; FM – fresh matter; DDGS – distillers' dried grains with solubles; DWLS – dehulled white lupine seeds; FRSM – fermented rapeseed meal; MLM – mealworm larvae meal; PS – pea seeds; RPM – rapeseed meal; RSC – rapeseed cake; SBM – soybean meal; SFM – sunflower meal; SPM – silkworm pupae meal; WLS – white lupine seeds

The aim of the present study was to review, in chronological order, the literature on alternative feed ingredients as substitutes for SBM in rabbit diets in Central Europe (Poland, Czech Republic) in the 21st century. Studies investigating SBM substitution in rabbit diets and substitution levels are listed in Table 3. The main findings and conclusions from these studies are discussed chronologically in subsequent sections.

Table 3

Studies investigating soybean meal substitution in rabbit diets and substitution levels

SBM in diet [%]	SBM substitution with other feed components [%]	Authors
10.0	1) WLS 15.0 2) SFM 17.0	Volek and Marounek (2009)
12.0	1) SBM 7.0+maize DDGS 5.0 2) SBM 2.0+maize DDGS 10.0	Chełmińska and Kowalska (2013)
1) SBM 13.0+SFM 5.0 2) 7.0	1) WLS 25.0 2) WLS 12.0	Volek et al. (2014)
5.0	1) wheat DDGS 5.0 2) RSC 5.0 3) wheat DDGS 2.5+RSC 2.5	Strychalski et al. (2014) Gugołek et al. (2015)
15.0	1) SBM 7.5+RSC 5.0+WLS 4.0+PS 3.0 2) RSC 10.0+WLS 8.0+PS 6.0	Gugołek et al. (2017) Zwoliński et al. (2017) Gugołek et al. (2018)
7.0	1) DWLS 7.0	Volek et al. (2018a)
1) SBM 13.0+SFM 5.0 2) 7.0	1) RPM 10.0+WLS 14.0 2) RPM 6.0+WLS 4.0	Volek et al. (2018b)
1) SBM13.0+SFM 5.0 2) 7.0	1) DWLS 18.0 2) DWLS 7.0	Uhlířová and Volek (2019)
10.0	1) SBM 5.0+SPM 5.0 2) SPM 10.0	Gugołek et al. (2019)
10.0	1) SBM 5.0+WLS 3.5+PS 1.5 2) WLS 7.0+PS 3.0	Strychalski et al. (2020)
15.0	1) SBM 7.0+RPM 5.0+WLS 4.0+PS 3.0 2) RPM 10.0+WLS 8.0+PS 6.0	Kowalska et al. (2020a)
10.0	1) SBM 5.0+SPM 4.0 2) SBM 5.0+MLM 4.0	Kowalska et al. (2020b) Kowalska et al. (2021) Strychalski et al. (2021)
6.0	1) MLM 3.0	Volek et al. (2021)
10.0	1) SBM 5.0+SPM 5.0 2) SPM 10.0	Gugołek et al. (2021)
10.85	1) SBM 7.85+FRSM 4.0 2) SBM 4.88+FRSM 8.0 3) SBM 1.90+FRSM 12.0	Wlazło et al. (2021) Nowakowicz-Dębek et al. (2021)

SBM – soybean meal; WLS – white lupine seeds; SFM – sunflower meal; DDGS – distillers' dried grains with solubles; RSC – rapeseed cake; PS – pea seeds; DWLS – dehulled white lupine seeds; RPM – rapeseed meal; SPM – silkworm pupae meal; MLM – mealworm larvae meal; FRSM – fermented rapeseed meal

VOLEK and MAROUNEK (2009) were the first Central European researchers to explore SBM replacement in rabbit diets. They analyzed whether white lupine seeds or sunflower meal could be effective dietary protein sources for broiler rabbits. Three diets were prepared: a control diet containing SBM, and two experimental diets where SBM was replaced with white lupine seeds (first experimental diet) and sunflower meal (second experimental diet). No significant differences in body weight gain or the feed conversion ratio (FCR) were found between the groups, but feed intake was higher in the sunflower meal group. Carcass yield was higher in rabbits receiving white lupine seeds, compared with the other two groups. The digestibility of DM and crude protein was not affected by the dietary treatments. However, the digestibility coefficient of neutral detergent fiber was significantly lower, and the digestibility coefficients of acid detergent fiber and energy were lower (non-significant differences) in rabbits fed sunflower meal, relative to the other two diets. Increased cecal concentration of lactic acid was observed in rabbits fed white lupine seeds. The study demonstrated that both white lupine seeds and sunflower meal can effectively replace SBM in diets for growing rabbits raised for meat. The next study by VOLEK and MAROUNEK (2011) did not focus on SBM substitution, but its results are worth mentioning. The authors compared the effects of white lupine seeds (15%) and sunflower meal (12%) added to rabbit diets on the fatty acid composition of hind leg meat and perirenal fat. The diet containing white lupine seeds had a beneficial influence on the fatty acid profile of both hind leg meat and perirenal fat. These tissues had higher concentrations of monounsaturated fatty acids than the tissues of rabbits fed sunflower meal.

CHEŁMIŃSKA and KOWALSKA (2013) determined the effect of two inclusion levels of maize DDGS in rabbit diets. The addition of DDGS at 5% had no negative influence on body weight gain, feed intake or meat quality. However, a diet containing 10% of DDGS was not safe for rabbits due to its high mycotoxin content. Mycotoxin concentrations remained high in this diet, despite the use of detoxifiers, which considerably deteriorated meat quality. The study has shown that maize DDGS fed to rabbits should be analyzed to establish safe mycotoxin levels.

In their experiment, VOLEK et al. (2014) examined the effect of white lupine seeds fed to lactating does on the yield and fatty acid composition of milk, and the growth rate and health status of their offspring. No significant differences in feed intake or litter weight gain were noted between the groups. However, does fed a diet with white lupine seeds were characterized by higher milk yields and a more favorable fatty acid profile of milk. Morbidity and mortality rates due to digestive diseases were lower

in the group receiving white lupine seeds, compared with the groups fed SBM and sunflower meal. It was found that white lupine seeds can be a viable alternative to conventional protein sources in diets for lactating does, and that they can improve the fatty acid profile of milk.

STRYCHALSKI et al. (2014) investigated the efficacy of rapeseed cake and wheat DDGS fed to growing Californian rabbits. Four diets were analyzed: a control diet containing 5% SBM, a diet containing 5% rapeseed cake, a diet containing 5% wheat DDGS, and a diet containing 2.5% rapeseed cake and 2.5% wheat DDGS. No significant differences in rabbit performance were found between the groups. Nutrient and energy digestibility was highest in the control group, and lowest in the group fed a diet with 5% wheat DDGS. The relative weight of the small intestine and digesta was highest in the latter group. The coefficients of cecal digesta hydration and bulking as well as pH values were highest, and the cecal concentrations of volatile fatty acids were lowest in rabbits fed a diet with 5% addition of rapeseed cake. Enhanced enzyme activity of colonic microbiota, which increased nutrient digestibility, was noted in rabbits receiving a diet with 2.5% rapeseed cake and 2.5% wheat DDGS, relative to the group fed 5% wheat DDGS. The study demonstrated that SBM can be effectively replaced with 5% rapeseed cake as well as 2.5% rapeseed cake combined with 2.5% wheat DDGS in diets for young rabbits.

In a study by MATUSEVIČIUS et al. (2014), sunflower meal was partially replaced with rapeseed meal in diets for growing rabbits to determine their gastrointestinal tract response and growth performance. The results of this study are interesting because both meals can also be used as SMB substitutes. Desirable changes were noted in the gastrointestinal tract of rabbits fed diets containing 5% and 10% rapeseed meal, including a decrease in pH and ammonia concentration, and an increase in DM digestibility, compared with the control group. However, rapeseed meal had no significant effect on feed intake and utilization or live weight gain. It was found that the addition of rapeseed meal to rabbit diets can minimize the negative effect of sunflower meal on fermentation processes in the gastrointestinal tract, but it does not improve the growth performance of animals.

GUGOŁEK et al. (2015) evaluated the productivity and gastrointestinal tract response of meat-type rabbits fed diets containing rapeseed cake and wheat DDGS. The administered diets were identical to those described by STRYCHALSKI et al. (2014). Productivity was highest in the control group, and lowest in the group receiving a diet with 5% wheat DDGS. Similar tendencies were noted for nutrient digestibility and nitrogen retention. No significant differences in carcass dressing percentage were observed

between the groups. Animals fed 5% wheat DDGS were characterized by undesirable gut responses, including excess digesta hydration in the small intestine, increased ammonia concentration in the cecum and colon and enhanced activity of potentially pathogenic bacteria. Such responses were not noted in rabbits fed other diets. A decrease in DM concentration in the small intestine was observed only in the group receiving a diet with the addition of 2.5% rapeseed cake and 2.5% wheat DDGS. The results of this experiment indicate that SBM can be replaced with rapeseed cake in rabbit diets. Since the animals receiving 2.5% rapeseed cake and 2.5% wheat DDGS were characterized by comparable productivity, such a combination is also possible. However, the dietary inclusion of 5% wheat DDGS negatively affected most of performance parameters in growing rabbits.

In their later study, GUGOŁEK et al. (2017) analyzed whether SBM could be replaced with rapeseed meal and legume seeds in rabbit diets. The influence of such a substitution on productivity and gastrointestinal function was evaluated. Three diets were formulated: a control diet containing 15% SBM, experimental diet 1 containing 7.5% SBM, 5% rapeseed meal, 4% white lupine seeds and 3% pea seeds, and experimental diet 2 where SBM was completely replaced with 10% rapeseed meal, 8% white lupine seeds and 6% pea seeds. Productivity was comparable in all groups. Changes were noted in the enzyme activity of large gut microbiota, in particular increased secretion of glycoside hydrolases by bacterial cells, in rabbits fed a diet without SBM, which probably contributed to their high growth performance. The study revealed that SBM can be replaced, partially or completely, with a mixture of rapeseed meal, white lupine seeds and pea seeds.

ZWOLIŃSKI et al. (2017) also evaluated the effect of a mixture of rapeseed meal, white lupine seeds and pea seeds as a substitute for SBM on performance parameters, nutrient digestibility and nitrogen retention in rabbits. They found that animals fed diets containing rapeseed meal, white lupine seeds and pea seeds were characterized by similar productivity as those fed SBM-based diets.

VOLEK et al. (2018a) investigated the effect of dehulled white lupine seeds added to rabbit diets on carcass traits and meat quality. The control group was fed a diet containing SBM. Many quality attributes of raw and cooked meat were higher in rabbits fed a diet with dehulled white lupine seeds.

In their subsequent experiment, VOLEK et al. (2018b) analyzed whether SBM and sunflower meal can be replaced with white lupine seeds and rapeseed meal in diets for female rabbits, and whether such a substitution would affect milk yield and composition as well as the performance of their

offspring. Two diets for lactating does and two diets for growing rabbits were formulated. The first lactation diet contained 13% SBM and 5% sunflower meal as the main protein sources; the second lactation contained 10% rapeseed meal and 14% white lupine seeds; the first grower diet contained 7% SBM; the second grower diet contained 6% rapeseed meal and 4% white lupine seeds. Does feeding their young received one of the two lactation diets, and kittens aged 17 to 42 days received one of the two grower diets. No significant differences in milk yield, the body weights of animals or feed intake were found between the dietary treatments. However, diets with white lupine seeds and rapeseed meal contributed to a more favorable fatty acid profile of milk, including higher concentrations of polyunsaturated fatty acids. No negative side effects of feeding the experimental diets were noted, which suggests that rabbits can be successfully fed diets with white lupine seeds and rapeseed meal.

GUGOŁEK et al. (2018) evaluated the physiological response of rabbits to diets containing rapeseed meal, white lupine seeds and pea seeds as SBM substitutes. The diets were identical to those formulated by GUGOŁEK et al. (2017). No significant differences in the growth rate of animals, selected morphological and biochemical blood parameters or carcass traits were noted between experimental and control groups. In addition, white lupine seeds exerted a beneficial influence on gastrointestinal function by improving fermentation processes in the gut. The results of this study clearly indicated that SBM can be successfully replaced with white lupine seeds, pea seeds and rapeseed meal in rabbit diets.

UHLÍŘOVÁ and VOLEK (2019) performed an experiment to determine the effect of dehulled white lupine seeds added to feed on milk production and composition in does, and on the growth performance of their litters before weaning. Two lactation diets were formulated: a control diet containing 13% SBM and 5% sunflower meal and an experimental diet containing 18% dehulled white lupine seeds. No significant differences in milk production were observed between the treatments. An increase in the concentrations of polyunsaturated fatty acids was observed in milk from does receiving dehulled white lupine seeds. Their kittens were also characterized by higher milk intake. It was found that dehulled white lupine seeds can be used as a protein source in diets for lactating does without compromising their milk performance or the growth performance of their offspring. It should also be noted that a study by VOLEK et al. (2020) revealed that narrow-leaved lupine seeds were a less effective dietary protein source for fattening rabbits than white lupine seeds.

GUGOŁEK et al. (2019) analyzed the growth performance of broiler rabbits and the chemical composition of their meat in response to different

dietary levels of silkworm pupae meal. The control diet contained 10% SBM, the first experimental diet contained 5% SBM and 5% silkworm pupae meal, and the second experimental diet contained 10% silkworm pupae meal. In both experimental groups, rabbits were characterized by slightly lower final body weights, lower average daily gains, and lower feed intake, as well as lower dressing percentage and carcass weight, relative to the control group. In turn, silkworm pupae meal contributed to improving the FCR. Experimental diets had no significant influence on the chemical composition of rabbit meat, but they affected its fatty acid profile. The cited authors concluded that insect meals can constitute novel protein and fat sources for rabbit raised for meat. Moreover, the use of silkworm pupae meal for feed production can partially solve the problem of environmentally-friendly sericultural waste management.

A study by STRYCHALSKI et al. (2020) aimed to determine the effect of replacing SBM with a mixture of white lupine seeds and pea seeds on growth performance, nutrient digestibility and nitrogen retention in rabbits. The control diet contained 10% SBM, the first experimental diet contained 5% SBM and a mixture of white lupine seeds and pea seeds, whereas in the second experimental diet, SMB was completely replaced with white lupine and pea seeds. Neither partial nor complete substitution of legume seeds for SBM negatively affected the growth performance of rabbits, nutrient digestibility or nitrogen retention.

Similarly to GUGOŁEK et al. (2017), KOWALSKA et al. (2020a) investigated whether a combination of rapeseed meal, white lupine seeds and pea seeds, used as substitutes for SBM in rabbit diets, affected the growth performance of animals and the fatty acid composition of their meat and fat. The tested diets had no significant effect on the final body weights of rabbits. Rabbits fed diets containing rapeseed meal, white lupine seeds and pea seeds were characterized by higher feed efficiency and higher protein content of thigh muscle. Dressing percentage was higher in the experimental group fed a diet without SBM than in the control group. The proportion of saturated fatty acids decreased, and the proportion of polyunsaturated fatty acids increased in perirenal fat with decreasing dietary inclusion levels of SBM. The study demonstrated that SBM can be effectively replaced with a mixture of rapeseed meal, white lupine seeds and pea seeds in rabbit diets.

In another experiment, KOWALSKA et al. (2020b) evaluated the efficacy of insect meals in rabbit nutrition, including their effect on slaughter parameters and meat quality. The following diets were formulated: an SBM-based control diet, an experimental diet containing silkworm pupae meal and an experimental diet containing mealworm larvae meal. Insect

meals increased the final body weights of rabbits and carcass dressing percentage. They also affected the amino acid composition of meat by modifying the levels of lysine, tryptophan and isoleucine, although the total concentrations of essential amino acids remained unchanged. The content of saturated fatty acids in meat was comparable in all groups. The meat of rabbits fed silkworm pupae meal had higher levels of polyunsaturated fatty acids and lower cholesterol levels. It was concluded that insect meal can be included in rabbit diets at 4% without compromising the growth performance of animals or meat quality.

Set out to determine whether SBM could be replaced with yellow mealworm larvae meal in rabbit diets, VOLEK et al. (2021) formulated two diets, of which one contained 6% SBM and the other contained 3% yellow mealworm larvae meal as protein sources, and their effects on average daily gains, nutrient digestibility and nitrogen retention were compared. Daily feed intake was significantly lower in rabbits fed the diet with insect meal. Average daily gains between days 32 and 53 of the experiment were lower in the experimental group than in the control group. However, at the end of the study, no significant differences in this parameter or in the final body weights of animals were found between the groups. Feed utilization, nutrient digestibility and N excretion were also comparable in both groups. Rabbits receiving yellow mealworm larvae meal were characterized by lower total nitrogen excretion, lower nitrogen losses in urine, and higher nitrogen retention. According to the cited authors, diets with the addition of yellow mealworm larvae meal can be fed to fattening rabbits with no adverse effect on the analyzed parameters.

STRYCHALSKI et al. (2021) investigated the gastrointestinal response and growth performance indicators of rabbits to the dietary replacement of SBM with insect meals. Three diets were tested: the first (control) diet contained 10% SBM, the second diet contained 5% SBM and 4% silkworm pupae meal, and the third diet contained 5% SBM and 4% mealworm larvae meal. Rabbits fed diets supplemented with insect meals were characterized by higher final body weights and higher daily body weight gains, as well as higher apparent total tract digestibility of crude fat, acid detergent fiber (ADF) and acid detergent lignin (ADL). Increased digesta viscosity, a decrease in the extracellular activity of cecal bacterial enzymes (α-glucosidase, β-glucosidase, α-arabinofuranosidase and β-xylosidase) and a decrease in the intracellular activity of β-glucuronidase were also noted in experimental groups. The addition of insect meals to rabbit diets enhanced the activity of N-acetyl-beta-D-glucosaminidase (an enzyme involved in chitin degradation) in the cecal digesta. Rabbits fed mealworm larvae meal had the lowest cecal concentrations of acetic acid, propionic

acid, and total short-chain fatty acids. Animals receiving silkworm pupae meal had a lower colonic concentration of isovaleric acid. The experiment revealed that the dietary inclusion of insect meals at 4% improved rabbit productivity, with no negative effect on nutrient digestibility.

In the work of GUGOŁEK et al. (2021), rabbit diets were supplemented with silkworm pupae meal to determine its effect on gastrointestinal function, nitrogen retention and selected blood parameters. Three groups of rabbits were fed a diet containing 10% SBM, a diet containing 5% SBM and 5% silkworm pupae meal, and a diet containing 10% silkworm pupae meal, respectively. It was found that both nutrient digestibility and nitrogen retention decreased as the dietary inclusion level of silkworm pupae meal increased. The analyzed additive significantly increased gastrointestinal pH values. Rabbits receiving 10% silkworm pupae meal were characterized by the highest weight of cecal tissue and digesta. Silkworm pupae meal also contributed to a decrease in the activity of numerous bacterial enzymes in the gut and to an increase in the activity of N-acetyl-ß-D-glucosaminidase. The total cecal concentration of volatile fatty acids was lowest in rabbits fed a diet with 10% silkworm pupae meal. Blood analyses revealed that the addition of silkworm pupae meal to rabbit diets increased the plasma levels of albumins and urea. The authors concluded that rabbit diets can be supplemented with silkworm pupae meal at up to 5% since its higher inclusion rate may disturb gastrointestinal physiology.

Another study by KOWALSKA et al. (2021) aimed to evaluate the effect of insect meals on the growth performance of rabbits. The composition of three diets formulated in this experiment was identical to that described by STRYCHALSKI et al. (2021). The study demonstrated that rabbits fed diets supplemented with silkworm pupae and mealworm larvae meals had higher final body weights, better carcass characteristics, and a higher crude fat content of meat than control group animals. Therefore, insect meals can be used as partial substitutes for SBM in diets for growing rabbits.

NOWAKOWICZ-DĘBEK et al. (2021) focused on the effect of fermented rapeseed meal on meat quality and gastrointestinal morphometry in rabbits. A control diet and experimental diets containing different proportions of fermented rapeseed meal (4%, 8% and 12%) were formulated. The addition of fermented rapeseed meal caused a significant increase in the length of the cecum and the large intestine as well as in the weight of the heart. The proximate chemical composition of rabbit meat was comparable in all groups, excluding collagen content which increased in the longissimus thoracis and longissimus lumborum muscles of animals fed diets supplemented with fermented rapeseed meal. The collagen content of the

biceps femoris muscle was highest in rabbits receiving 4% fermented rapeseed meal, and lowest in those receiving 12% fermented rapeseed meal. A decrease in the pH and water-holding capacity of the longissimus thoracis and longissimus lumborum muscles was also observed in animals fed diets with the addition of fermented rapeseed meal. The values of the above parameters did not decrease in the biceps femoris muscle. Changes in meat color, including increased yellowness, were also noted in experimental groups. The results of this study suggest that fermented rapeseed meal can be fed to rabbits with no negative influence on the quality of their meat.

Wlazło et al. (2021) examined the effect of fermented rapeseed meal on gut microbiota and the immune status of rabbits. The composition of experimental diets was identical to that described by Nowakowicz-Dębek et al. (2021). The counts of coliform bacteria, including *Escherichia coli,* and *Clostriduim perfringens* decreased, whereas the counts of beneficial lactic acid bacteria increased in the gastrointestinal tract of rabbits fed diets with the addition of fermented rapeseed meal. Immunoglobulin levels and the size of gut microbial populations increased with growing inclusion rates of fermented rapeseed meal. The authors reported that fermented rapeseed meal can be a valuable component of rabbit diets. It exerted a beneficial influence on their gut microbiota and immune status, thus contributing to reduced use of antibiotics in rabbit farming.

The results of studies conducted in Central Europe indicate that SBM in rabbit diets can be completely or partially replaced with other high-protein ingredients of plant and animal origin, such as oilseed meals, legume seeds, DDGS and insect meals, applied alone or in combinations, without compromising the growth rate or reproductive performance of animals.

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THERAPEUTIC POTENCY OF LEMON-SCENTED GUM (*EUCALYPTUS CITRIODORA*) LEAF EXTRACT FOR THE DEVELOPMENT OF ANTIBACTERIAL DRUG

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Key words: *Eucalyptus citriodora*, ethyl acetate and aqueous fractions, acute toxicity, antibacterial, alkane, compounds.

Abstract

Objectives: The antibacterial activity, phytochemical components and safe dose of the leaf part of *Eucalyptus citriodora* was determined using standard methods.

Results: Ten phytochemicals (flavonoid, phenols, alkaloids, tannins, steroids, cardiac glycosides, saponins, terpenes, anthraquinones and resins) were present in other solvent extract but tannins and resinswere absent in the aqueous extract. All test organisms were susceptible to ethyl acetate crude extract with mean inhibition zone diameter (IZD) range from 14.00±0.00 to 21.66±0.88 mm but were not susceptible to the aqueous extract; minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values from 7.5 to 60 mg/ml respectively were obtained. Eight fractions were obtained from column chromatography of which 5 were active; however, 3 shows significant (p<0.05) activity with IZD of 26.00±0.00 mm, 23.6±0.33 mm and 20.33±0.33 mm respectively while their corresponding MIC/MBC were 3.75, 1.88 and 3.25 mg/ml/60, 30 and 30 mg/ml. In the same vein, 28, 18 and 17 compounds were detected by gas chromatography in the 3 significantly active fractions. Generally, the alkane group of compounds was the most detected, but for emphasis, Tetracosane (14.03% of ECE1), Octadecanal (59.54% of ECE4) and Decane (13.70% of ECE5) were the most abundant in the three active fraction. The LD_{50} for the extract was 707.10 mg/kg bw while mild tissue necrosis and degenerative glomeruli were observed in the liver and kidney pathology of experimental rats administered with 1000 mg/ml of extract (Fig. 1).

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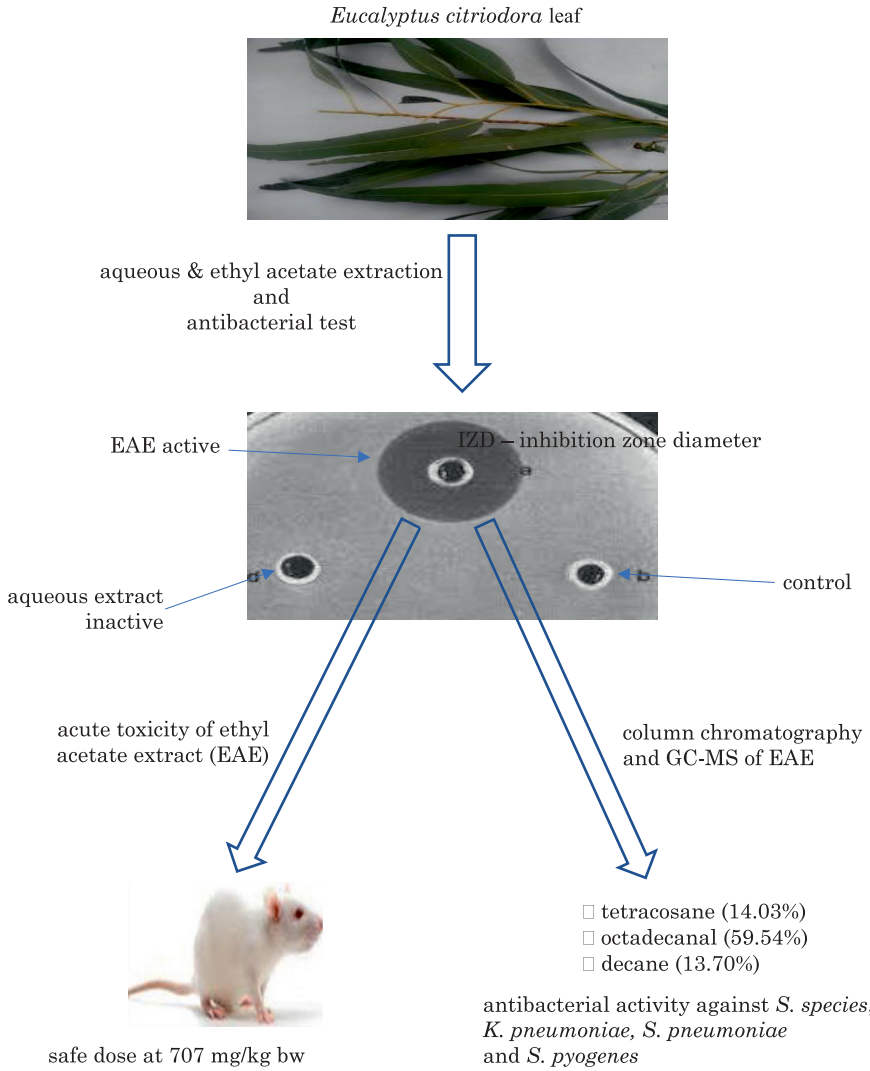


Fig. 1. Graphic abstract

Conclusion: Therefore, *Eucalyptus citriodora* leaf extract indicated antibacterial potency and therefore considered for drug development.

Introduction

Modern medicine in the 20th century was characterized by the introduction in the United Kingdom of the National Health Service (Hajir et al. 2016). Advances were also notable in the treatment of mental illness

through both psychotherapy and the administration of drugs (HAJIR et al. 2016) and plant-based medicines have been reported to have important therapeutic potency to cure human diseases (SARDAR et al. 2012). Plants have been seen as sources of medicine for thousands of years, since the creation of man. In Nigeria, almost all plants are medicinal and the application of medicinal plants especially in traditional medical practice has been well acknowledged and established as a viable profession (SARSWATI et al. 2013). Herbal medicines otherwise called herbal drugs are generally of natural plant parts such as stem, leaves, roots, flowers, stem bark, seeds, bulb (INNALEGWU et al. 2016). In addition to providing the animal kingdom it's food, fuel and shelter, plants accumulate other phytochemical constituents – the secondary metabolites which are produced as by-products and are sometimes not directly useful to them. These secondary metabolites give plants their medicinal values. Some of these metabolites include alkaloids, annins, phenols, steroids, saponins, flavonoids, anthraquinones, cardiac glycosides, terpenes, essential oils and resins (INNALEGWU et al. 2016). *Eucalyptus* (also called *Corymbia*) is a diverse genus of trees in the family *Myrtaceae*. Out of the more than 700 species that comprise this genus, most are endemic to Australia. A smaller number are also native to New Guinea, Indonesia and the Phillipines. *Eucalyptus* can be found in almost every region of the Australian continent. They have also been widely introduced into the subtropical and tropical regions in areas as diverse as Africa, the Middle East, India, USA and South America (EWANSIHA et al. 2017). Plant oils from some *Eucalyptus* species (e.g *Eucalyptus pulverulenta*) comprise up to 90% cineol (EWANSIHA et al. 2020)

Materials and Methods

Harvesting, identification and processing of plant material

Fresh leaves of Lemon scented gum (*Eucalyptus citriodora*) – Figure 2 – were collected from house-hold gardens in Bosso Local Government, Minna, Niger State in the month of August, 2015. The plant materials were identified by local herbal practitioners in Minna, Niger State (with longitude 6.546316 and latitude 9.583555) while authentication of the plant samples was done by Dr. Ugbabe Grace and Mr. John Atogwe in the Herbarium Department of the National Institute of Pharmaceutical Research, and Development, Idu, Abuja, Nigeria where voucher specimens were deposited with voucher number: NIPRD/H/6787. The plant materials

(leaves) were dried at room temperature, until a constant weight was obtained. The plant samples were pulverized with a milling machine (Lab world Navbhart, with serial No. R66902 by Motor MFG. CO. Mumbai – India), and sieved with a 150 µm pore size filter to obtain a fine powder-like texture. This was done to enhance the penetration of the extraction solvents into the plant cells, thus facilitating the release of the active principles (ACUNA 2015). The pulverized plant samples were then stored in amber bottles and kept in a cool and dry environment at room temperature (27ºC) until required for further use.

Fig. 2. Plate I – lemon scented gum *(Eucalyptus citriodora)*

Methods

Confirmation of test organisms identity

The test organisms, *Salmonella typhi, Salmonella paratyphi* A, B & C, *Klebsiella pneumoniae, Streptococcus pneumoniae* and *Streptococcus pyogenes* (the choice of these organisms was informed by the claims of local marketer/consumers of the use of extracts from the experiment plants to treat infections caused by the test organisms) obtained from stock cultures in the Microbiology laboratory Federal University of Technology, Minna. The bacteria were reconstituted by sub-culturing onto freshly prepared nutrient agar and then incubated at 37ºC for 24 hours, after which their identities were confirmed using gram staining and molecular identification.

Gram staining

An overnight culture of each test organisms was Gram stained according to the method described by (EWANSIHA et al. 2021, PRADHAN and TAMANG 2019). Gram-positive and Gram-negative organisms were recorded. A control smear of known Gram-positive organism (*Staphylococcus aureus*) and a known Gram-negative organism (*Escherichia coli*) were stained simultaneously for quality control.

Molecular identification of test organisms

The molecular confirmation of the identity of the test organisms was carried out according to the method stated in promega Technical Manual #TM050.

Treatment of Gram-positive cells with EDTA

One milliliter (1 ml) of an overnight culture of Gram-positive isolate was centrifuged for 2 minutes at 15000 x g and the supernatant was discarded. The pallet cells were suspended in 480 µl of 50 mM of ethylene diamine tetra acetic acid (EDTA), to aid the lysing process and 120 µl of lytic enzyme (lysozyme) was added. The mixture was incubated for 30 to 60 minutes at 37^{o}C. On completion of incubation, the mixture was centrifuged for 2 minutes at 15000 x g and the supernatant was decanted.

Lysing of gram-positive and gram-negative organisms

Six hundred microliter (600 µl) of nuclei lysis solution was added to the pallet cells separately and mixed gently by pipetting in and out of the tube. The mixture was incubated for 5 minutes at 80^{o}C and then allowed to cool to room temperature. Three microliters (3 µl) of RNase solution were added, mixed gently and incubated at 37^{o}C for 15 to 16 minutes after which they were allowed stand and attains room temperature at 27^{o}C.

Protein precipitation

Two hundred milliliters of protein precipitation solution was added to the mixture above and vortexed for 5 seconds after which it was incubated for 5 minutes. The mixture was centrifuged at 15000 g for 3 minutes. The supernatant was kept for DNA precipitation and rehydration.

DNA precipitation and rehydration

The supernatant from 3.3.2.3 was transferred into a clean tube containing 600 µl of isopropanol and gently mixed. The mixture was centrifuged for 2 minutes at 15000 g and the supernatant was decanted. Six hundred microliters (600 µl) of 70% ethanol was added and it was mixed and centrifuged for another 2 minutes at 15000 g. The ethanol was aspirated and the DNA pallet was rehydrated in 100 µl rehydration solution for 1 hour at 65ºC.

Polymerase chain reaction (PCR) reaction cocktail

Ten microliters (10 µl) of 5x Go Taq reagent, 3 µl of $MgCl_2$, 1 µl of 10 pmol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 3′-AAGGAGGTGATCCAGCC-5′ primers and 0.3 units of Taq DNA polymerase (Promega, USA) was made up to 42 µl with sterile distilled water and 8 µl DNA template. PCR was carried out in a Gene Amp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA) PCR profile, an initial denaturation of 30 cycles at 94°C for 5 min; 50°C for 60 s and 72°C for 1-minute 30 s and a final extension at 72°C for 10 mins. It was then allowed to cool down to 4ºC.

Integrity check on agarose gel

The integrity of the amplified gene fragment was checked on a 1% Agarose gel to confirm amplification. This was carried out by mixing 8 µl of amplified product to 4µl of loading dye and ran on the solidified Agarose gel at 110 V for about 1 hour. Also, the amplified product was checked on a Nano drop of model 2000 from thermo scientific to quantify the concentration of the amplified product.

Purification of amplified product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of sodium acetate 3 M and 240 µl of 95% ethanol were added to each fragments of the PCR amplified products in a new sterile Eppendorf tube, it was vortexed for 5 seconds and kept at -20°C for 30 min. The mixture was centrifuged for 10 min at 13000 g and 4°C followed by removal of the supernatant (by inverting the tube on trash once) after which the pellets were washed by adding 150 µl of 70% ethanol, mixed and then centrifuged for 15 min at 7500 x g and 4°C. Again, the supernatant was decanted and the tube was inverted on blotting paper

and was allowed to dry in the fume hood at room temperature for 10–15 min. It was then suspended with 20 µl of sterile distilled water and kept in a refrigerator at – 20ºC prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel, ran on a voltage of 110 V for about 1hr asdescribed above, to confirm the presence of the purified product before sequencing.

Sequencing and aligning

The amplified fragments were sequenced using a Genetic Analyser 3130 x l sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of Big Dye terminator v 3.1 cycle sequencing kit. Bio-Edit software and MEGA 6 were used for all genetic analysis. Identities and accession numbers of the test organisms were determined by BLAST from the GENE bank through the NCBI web site.

Extraction

Crude extraction by reflux and decoction

The pulverized plant samples were subjected to successive reflux extraction method according to (ABOSHORA et al. 2014) to obtain the crude leaf extract using ethyl acetate as extraction solvent and water. This was achieved by transferring 100 g of the plant samples into a round-bottom flask of 1000 ml capacity using a Soxhlet apparatus. Ethyl acetate (polarity index = 4.1 P'), was gradually added until a ratio of 1:3 of the pulverized plant sample to ethyl acetate was attained. The power source was then switched on and the temperature was adjusted to 35ºC. The mixture was allowed to reflux for 2 hours in a round bottom flask with a reflux condenser attached at the top end, after which it was then filtered through a Whatman filter paper (pore size of 20 µm). The solvent (ethyl acetate) in the filtrate (containing the solvent and plant extract) was then evaporated using a rotary evaporator while the unevaporated plant extract was collected and transferred into universal containers and stored in the refrigerator at 4ºC (39.2ºF) for further use. The residue (merc obtained after filteration) was dried at room temperature (26ºC) and 100 g was transferred into the round-bottom flask containing water until a mixture of the merc and solvent attained a ratio of 1:4 (i.e., 100 g to 400 ml). The aqueous extract was obtained by decoction method considering the fact that water does not reflux. A total of 500 g each of the plant material/merc was com-

pletely extracted. The percentage yield of the crude extracts was determined using the equation below:

Percentage Yield [g] = Weight of Extract or Oil [g]/Weight of Dry Plant Material [g] ·100.

Phytochemical screening

Phytochemical analysis was performed using the method as described by HAJIR et al. (2016) to screen the extracts (hexane, ethyl acetate, methanol and aqueous leaf extracts of *Eucalyptus citriodora*) for the presence of the following active principles: alkaloids, tannins, saponins, flavonoids, anthraquinones, cardiac glycosides, volatile oils, terpenoids, resins, steroids and phenol.

Test for alkaloids

One ml of Dragendorff reagent was added to 1ml of filtrate. The formation of cloudy orange was observed indicating the presence of alkaloids.

Test for tannins

One ml of each extracts was added (separately) to one ml of 3% $FeCl_3$. A greenish black precipitate is indicative of the presence of tannins.

Test for flavonoids

One ml of each extract was added (separately) to one ml of 10% KOH. It was gently shaken. Appearance of yellow colour is indicative of the presence of flavonoids.

Test for cardiac glycosides

Bontrager's test – To show the presence of free Anthraquinone, 0.5 g of the pulverized leaf extract was taken in dry test tubes. Ten milliliters of chloroform were added and the mixtures shaken for 5 minutes. The extracts were next filtered and an equal volume of ammonia was added to the filtrate and thoroughly shaken. A bright pink color in the upper aqueous layer indicates the presence of free anthraquinones.

Test for saponins

Approximately 0.2 ml of each extract was mixed seperately with 5 ml of distilled water. Mixture was shaken vigorously for 5 min. Persistence of foams indicated the presence of saponins.

Test for terpenes and sterols

Five grams of the extracts was extracted by maceration with 95% ethanol and the extracts was filtered and the filtrates evaporated to dryness. The residues were dissolved in 10 ml of anhydrous chloroform and filtered. The filtrate was then divided into two equal portions and the following tests were carried out on it. i. Liebermann-Burchard test for the presence of terpenes. One portion of the chloroform solution from above was mixed with 1 ml of acetic anhydride, which was followed by the addition of concentrated sulphuric acid down the wall of the test-tube to form a lower layer. The formation of a reddish violet colour in the chloroform layer indicated the presence of terpenes. ii. Salkowski's Test – To show the presence of sterols. The other portion of the solution was mixed with 2 ml of concentrated sulphuric acid carefully so that the acid forms a layer. A reddish-brown colour at the interface indicated the presence of a steroidal ring.

Test for resins

Solutions of 5 ml petroleum ether was made using 0.1 g of powdered leaf extract and was labelled appropriately. An equal volume of copper acetate solution was next added and shaken vigorously then allowed to separate. A green colour was indicative of the presence of resins.

Test for volatile oils

Volatile oils are characterized by their odour, oil-like appearance and ability to volatility at room temperature. The plant material was heated with water by steam distillation and the distillates were collected in a graduated tube. The aqueous portion which separates automatically was returned to the distillation flask. The formation of emulsion which floats on top of the aqueous phase owing to its low density is indicative of the presence of plant oils.

Test for anthraquinone

One milliliter of chloroform was added to 0.1 g of plant extract and shaken thoroughly for 5 min; it was filtered and the filtrate was mixed with 100% ammonia solution. Pink, violet or red colour in the ammoniacal layer (lower layer) indicated the presence of free anthraquinone.

Test for phenol

Two ml of extract was added to one ml of distilled water and warmed at 45–50ºC. Then 2 ml of 3% $FeCl_3$ was added. Appearance of green or blue color indicated the presence of phenols.

Antibacterial susceptibility assays of crude extracts

Standardization of inoculum

Two hundred microliter (200 µl) of overnight cultures of each test organism was transferred into 6 ml of sterile distilled water and it was thoroughly mixed. 100 µl (equivalent to 10^8 CFU/ml McFarland standardize) of the mixture was inoculated into freshly prepared Muller-Hinton agar, used for the antibacterial susceptibility assays.

Preparation of extracts concentration

One hundred milligram (150 mg) and 200 mg of the ethyl acetate and aqueous crude extract were weighed and dissolved in 5 ml each of 10% dimethyl sulfoxide (DMSO) (10 ml DMSO was made up to 100 ml with distilled water) to give 30mg/ml and 40 mg/ml concentrations respectively.

Determination of antibacterial activity of crude extracts

The antibacterial activity of the extracts was investigated using the agar-well diffusion method as described by (Clinical And Laboratory Standard Institute 2012) and as modified by (EWANSIHA et al. 2017). Muller-Hinton agar was prepared according to manufacturer instructions and inoculated with the standardized test organisms by the spread plate method using a sterile rod spreader to obtain homogenous microbial growth. Wells were made in the inoculated media using sterile cork-borer (6 mm diameter) after which molten medium was used to seal the base of the wells to prevent unwanted spread of the extracts. One hundred microliter (100 µl) each of the prepared extract was transferred into the wells with a sterile micropipette tip and it was well labelled, while 100 µl of 10% DMSO (free of extract) was transferred into wells to serve as the negative control. Ciprofloxacin (1 mg/ml) (a broad-spectrum antibiotic) was used as positive controls. This was done by transferring 100 µl of the prepared standard antibiotics into the well and the cultures were allowed to stand for 30 min after which it was incubated at 37°C for 24 hours. The experiment was carried out in triplicates and the mean values with the corresponding

standard deviation of the inhibition zone diameters (IZD) in millimeter were calculated.

Determination of the minimum inhibitory and minimum bactericidal concentration (MIC and MBC) of active crude extracts

Serial dilution of crude extracts

The tube dilution method as described by Clinical and Laboratory Standard Institute (CLSI 2012)with slight modification using spectrophotometer was used to determine the minimum inhibitory concentration. Two-fold serial dilutions of the crude plant extracts was prepared to give decreasing concentrations of 120, 60, 30, 15, 7.5, 3.75, 1.875 and 0.938 mg/ml. This was achieved by dissolving 480mg of the crude extract in 2 ml of 10% dimethyl sulfoxide and transferring the prepared 2 ml stock concentration (containing 240 mg/ml) of the plant leaf crude extract into a test tube containing 2 ml of nutrient broth labelled A (to give 120 mg/ml), from test tube A, 2 ml (containing 120 mg/ml) was next transferred into the next test tube containing 2 ml of sterile nutrient broth labelled B (to give 60 mg/ml). This process continued until a concentration of 0.938 mg/ml was obtained in the last test tube.

Assay for minimum inhibitory and minimum bactericidal concentration (MIC and MBC) of crude extracts

All the prepared extract dilutions were properly shaken to obtain homogenous mixtures and they were then inoculated with 100 µl of the test organisms appropriately. Positive and negative control tubes were also maintained for each test batch of extract concentration and test organisms respectively (ACEVEDO and STRONG 2012.). For the positive control, sterile nutrient broth was inoculated with 100 µl of the test organisms without the addition of the extract while for the negative control; serial dilutions of the extracts were prepared without the inoculation of the test organisms. The test tubes were all incubated at 37ºC for 24 h in a shaker incubator. At the end of the incubation period, the optical density of the cultures in the test tubes were read using spectrophotometer at a wavelength of 600 nm (this wavelength was used due to the fact that absorbance of light by other molecules in the microbial cells such as flavin's and carotenoids is minimal at this position (MOHANAPRIYA et al. 2013). Spectrophotometer was used due to the fact that the absorbance is directly proportional to the number of cells in the cultures and the colour intensity of the extract will

not allow an effective visual observation (GHADIR et al. 2014) while the spectrophotometer was adjusted to zero using sterile nutrient broth void of extracts and test organism. The MIC was determined by subtracting the absorbance of the negative control from the absorbance of the test and comparing the result with the absorbance of the positive control (see below formulae). The concentration/test tube where significant reduction in absorbance was first observed, was recorded as the MIC:

$$T\text{-}\ C_0 = C_1 \text{ (ACEVEDO and STRONG 2012)},$$

where:
T – absorbance of test
C_0 – absorbance of negative control
C_1 – absorbance of positive control.

Therefore, a deviation from the values of the positive control (C_1) is evidence of either inhibition (antibacterial activity) or resistance by the organism; by this, the MIC was determined. The minimum bactericidal concentration (MBC) was determined by subculturing the cultures with the lowest optical density beginning with the test tube containing the minimum inhibitory concentration and above onto a freshly prepared nutrient agar medium. The cultures were incubated for 24 hours at 37ºC, after incubation, the culture concentration without visible growth was regarded as the minimum bactericidal concentration.

Fractionation of crude extracts

Thin layer chromatography of crude extracts

The analytical thin layer chromatographic technique was done to spot, separate and determine the Rf (Retention factors) values and a suitable solvent system for fractionation of the phytochemical components by column chromatography on the crude extracts. This was achieved by using the pre-coated Thin Layer Chromatography silica gel (60 F254 Aluminum sheet, Merck KGaA, Millipore Corporation Germany) as the stationary phase for the active crude extracts of *Eucalyptus citriodora*. The mobile phase used for the Hexane extract was a mixture of Hexane and Chloroform in the ratio 4:1. The solvent for the separation was put in a glass tank and the tank was closed and allowed to stand for about 10 minutes so that the atmosphere in the tank becomes saturated with solvent vapor. A line was drawn on the pre-coated aluminum plates with a pencil at 1.5 cm from the origin and also 5 cm at the top of the plate from the 1.5 cm mark to mark the distance moved by the mobile phase. A capillary tube was used to place a drop of the extracts/fraction at the centre point of the drawn

line on the plates respectively and they were appropriately labelled and allowed to dry for 5 minutes. The plates were then inserted into the tank with the origin spot towards the bottom of the tank. The spots on the plate were higher than the solvent level in the tank and the glass tank was recapped while the ascension of the solvent was observed by capillary action. The plate was then removed from the tank as soon as the solvent got to the drawn line at the finishingspot (solvent front) and dried in an oven and were next sprayed with Vanillin in sulphuric acid (conc.), so as to locate the separated spots. The distance moved by the solvent and distance moved byeach spot were then measured in millimeter using a meter rule (TOJOLA et al. 2019). The solvent system that gave the best separation based on the Rf values were used to fractionate the crude extracts by column chromatography, while the number of spots seen was recorded. The Rf values were determined by dividing the distance moved by the substance by the distance moved by the solvent:

$$Rf = \text{Distance moved by substance [cm]/Distance moved by solvent [cm]},$$

where:
Rf – the retention factor.

Column chromatography (partial purification) of crude extract

The micro scale column chromatographic method described by (EWANSIHA 2020) was used to separate the fractions of the active crude extracts (ethyl acetate) of *Eucalyptus citriodora* that showed activity against the test organisms. The column (40 mm diameter width and 150 mm length) was prepared by packing; this was achieved by transferring prepared slurry of silica gel (150 g of 0.015–0.04 mm mesh size, dissolved in 500 ml n-hexane) into the column using the wet method. The column/silica gel was allowed to pack for about 1 hour and the excess n-hexane was drained off to almost dryness. A filter paper of 40 mm diameter was next inserted into the column at the top of the packed gel to further filter the extract into the column, this is to prevent blockage of the column. The extract was prepared by dissolving 3 g in 10 ml of chloroform (a moderately polar solvent) with the addition of 10 g of dried silica gel to aid adsorption and drying of the extract. The column was next loaded with the dried sample by the wet method after which a 40 mm diameter filter paper was placed on the extract. The extract was fractionated by the addition of eluting solvents, which include n-hexane, chloroform, ethyl acetate, methanol and water (as the mobile phases); beginning with the nonpolar solvent to the polar solvent systems until all the fractions were collected and they were bulked together according to their retention factor (Rf) values by TLC chromatography.

The fractions were collected in test tubes according to their color development and the eluting solvents were allowed to vaporize until a constant weight was obtained.

Antibacterial susceptibility test of fractions

The agar-well diffusion and the tube dilution methods as enumerated in the above section was used to determine the antibacterial activity, MIC and MBC of the active fractions obtained from the chromatographic procedure. The fractions obtained were designated as Eucalyptus citriodora and were numbered according to their number of elution (i.e., ECEn); where "n" is the number of fractions obtained.

Quantitative analysis and identification of compounds

The determination of the identity of active components in the fractions that shows considerable activity (ECO2 and ECO4) were done by gas chromatograph interface to a mass spectrometer (GC-MS) analysis using GC-MSQP 2010 Plus, Shimadzu system (SHIMADZU, JAPAN). The gas chromatograph interface to a mass spectrometer (GC-M5S) instrument was used while the Column elite-1 was fused with silica capillary column (30 m x 0.25 mm 1D x µldf, composed of 100% dimethyl polysiloxane). An electronic ionization system with ionization energy of 60 eV was used for the GC-MS detection while Helium gas (99.99%) was used as the carrier gas at a flow rate of 1ml/min and injection size of the fraction was 2 µl (0.002 ml with split ratio of 1:40 and film thickness of 0.20 µm). The GC oven temperature was set at 70°C for 3.00 min and then programmed to rise from 70 to 250°C at a rate of 3°C min-1 and held isothermally for 3.00 min at 200°C (Isothermal for 2 min.) with an increase of 10°C/min to 200°C then 5°C/min to 280°C/min, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da. Total GC running time was 28.00 minutes. Relative percentages and amount of each component were deduced by comparing individual average peaks area to the total areas. Turbomass was used for the mass spectra and chromatogram while the detection of compounds was done using the database from the library of National Institute of Standard and Technology (NIST) NIST Ver. 2.0-year 2009 (Sarswati et al. 2013).

Toxicity studies on the crude extract

Preparation and acclimatization of test animals

The rats used for the investigation were obtained from the animal house of the Department of Biological Sciences, at the Niger State Polytechnic, Zungeru, Nigeria. The rats were kept under observation for about 7 days before the onset of experiment to exclude any infection and for acclimatization. The temperature of the experimental animal room was maintained at 22ºC (+3ºC). The relative humidity was set at 30% and not allowed to exceed 70% with a fluctuating range of 50–60% during room cleaning. The lighting was made to be near artificial, with a sequence of 12 hours light and 12 hours darkness. The animals were fed with conventional laboratory diets with an unlimited supply of drinking water. All the experimental animals were group-caged by dose, and the number of animals per cage and not allowed to interfere with clear observations of each animal.

Acute oral toxicity studies

The acute toxicity studies were conducted to determine the safe dose of the extract. The studies were carried out using the Lorke's method and modified as per internationally accepted protocol drawn under OECD No 420 guidelines (ARAGE et al. 2022). This was achieved using 22 white female albino rats (120±20 g body weight) while the extracts were administered in two phases (phase I and phase II). In phase I, the overnight fasted rats (i.e. the rats were deprived of food for 12 hours) were divided into 4 groups, consisting of three test groups and one control group. The test animals were administered ethyl acetate extracts of the leaves of *Eucalyptus citriodora* in various doses (10, 100, 500 and 1000 mg/kg bw) while the control group was administered 1 ml each of normal saline by oral gavage route. After administration of the extract, the animals were observed for toxicity signs continuously for the first 30 minutes, then periodically for the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days in the case of delayed toxicities to detect any changes in the behavioral responses and also for tremors, convulsion, salivation, diarrhoea, lethargy, sleep and coma and monitored for mortality. The median lethal dose (LD_{50}) otherwise known as the safe dose were calculated as the geometric mean of the dose that causes 0% and 100% mortality according to Lorke's formula:

$$LD_{50} = \sqrt{a \cdot b},$$

where:

a – is the highest dose at which no death occurred

b – the least dosage at which death occurred.

Result and Discussion

Percentage yield of plant extracts

The extraction results as presented in Table 1 explains the details of the total and corresponding percentage yield respectively per five hundred grams of the dried plant leaves and or merc of *Eucalyptus citriodora*. The highest yield of 27.07 g (5.41%) was obtained with ethyl acetate while 20.09 g (4.02%) was obtained with water. The most widely-used solvent to obtain crude extract from plant sources are polar solvents which have fairly moderate to higher boiling point range ofapproximately 63–189°C and are excellent solvents in terms of solubility and ease of recovery as reported by GETANEH and GIRMA (2014) and this in part could explain the reason for a higher yield with ethyl acetate. Previous researches have shown that ethyl acetate is a good extraction solvent for medicinal plant-based drugs. WAZA (2015) reported that water also possess broad spectrum and relatively non-selective property for extraction and a high percentage of plant extracts from water have been observed to possess antibacterial activity; but in this case the result in terms of yield compared to ethyl acetate shows the opposite. The major factors that may be considered in the choice of suitable solvent for plant extraction, is the polarity of the solvent as reported by AMMAR et al. (2017) that like attracts like i.e. a polar solvent will extract polar constituents and vice vasa. Therefore, the fact that the extraction process in this research work was continuous i.e. the extraction was carried out in increasing order of polarity and more yields were obtained with the ethyl acetate solvents. This could mean that the moderately polar constituents might be more than the highly polar constituents in the plants extracts.

Table 1

Percentage yield of plant leaf extracts

Plant parts	Percentage yield		
	WPP/WtM [g]	ethyl acetate extract g [%]	AqEg [%]
Eucalyptus citriodora leaf	500	27.07 (5.41)	20.09 (4.02)

Abbreviations: WPP – weight of pulverized plant part; AqE – aqueous extracts; WtM – weight of merc

Qualitative phytochemical properties of plant crude extracts

The qualitative phytochemical analysis revealed the presence of 10 phytochemical constituents present in the ethyl acetate extract, while out of the 10,3 (tannins, volatile oil and resins) were absent in the aqueous extract (Table 2), while only volatile oil was absent in the ethyl acetate

extract. The phytochemical constituents present include alkaloids, flavonoids, tannins, phenols, anthraquinones, terpenes, resins, steroids, cardiac glycosides and saponins. Phytochemical constituents are the bases for the therapeutic potency of medicinal plants as reported by EWANSIHA et al. (2012), however, the types and the quantity of phytochemical constituents also determine the level, presence or absence of activity. The absence of some key constituents such as tannins, volatile oil and resins could give the ethyl acetate extract an edge over the aqueous extract. Secondly, this may also be attributed to the difference in the method of extraction (reflux and decoction) used; in the report of EWANSIHA et al. (2012), cold maceration extraction methodwas used which is void of heat and the solvent application was not successive unlike the extraction method used in this research studies. These differences confirm the fact that individual/separate use of solvents and the application of heat during extraction of medicinal plant might have some advantages over successive application of extraction solvent and cold maceration respectively (AMMAR et al. 2017) also reported on the effect of temperature on phytochemical components, that some of these components are thermo-stable in nature and they can be extracted with the application of heat without being destroyed.

Table 2

Phytochemical constituents of *Eucalyptus citriodora* extracts

Plant extracts	Phytochemical constituents										
	flavonoid	phenols	alkaloids	tannins	steroids	cardiac glycosides	saponins	terpenes	volatile oil	anthraquinones	resins
Ethyl acetate	+	+	+	+	+	+	+	+	-	+	+
Aqueous	+	+	+	-	+	+	+	+	-	+	-

Abbreviations: "+" = present, "-" = absent

Antibacterial activity of *Eucalyptus citriodora* crude plant extracts

The results of the antibacterial activity of *Eucalyptus citriodora* crude extracts are presented in Table 3. The most active extract was the ethyl acetate extracts which shows considerable activity at a concentration of 30 mg/ml compared to aqueous extracts (40 mg/ml). Result in Table 3 showed that ethyl acetate extract was most active with mean diameter zone of inhibition (DZI) of 21.66±0.88 mm (p<0.05) against *Salmonella paratyphi B*, followed by 20.66±1.20 mm DZI against *K. pneumoniae* while the least DZI of 14.00±0.00 mm (p<0.05) was obtained against *S. typhi*.

The aqueous extract showed no activity against all the test organisms while the positive control is significantly (p<0.05) higher than the extract. The results of the antibacterial activity revealed that all the crude extracts have antibacterial activity against the test organisms and this could be attributed to the presence of the phytochemical constituents' present. ALTEMIMI et al. (2015) reported that the presence of phytochemical constituents in medicinal plants is the reason for their therapeutic potency. Also, the absence of some vital constituents (tannins and resins) could also be responsible for the ethyl acetate extract to be more active than the aqueous extracts. The antibacterial activity of the plant extracts which is attributed to the abundant presence of phytochemicals constituents, authenticate the use of the leaf and the fruit parts of the plants under study by local herbal practitioners and consumers either singly or in combination for the treatment of typhoid fever and respiratory tract infections as reported by OKOKON et al. (2018), MAMMEN et al. (2012) and GRIFFITH and GINTER (2017) reported on a study implicating some of the phytochemical constituents as possessing inhibitory activity against organisms that causes plant diseases e.g. *Fusarium oxysporum*. The presence of particular secondary metabolite in medicinal plants may not necessarily guarantee its antimicrobial potency owing to the fact that there are different types of secondary metabolites and the presence of the active type may not be certain. A good example is the existence of different types of tannins such as the hydrolysable and the non-hydrolysable tannins in plant cells as reported by ODELEY and OBAMESO (2022).

Table 3

Mean diameter zones of inhibition of *Eucalyptus citriodora* crude leaf extract[mm]

Organisms	Plant extracts		Control	
	EtOAc (30 mg/ml)	AqE (40 mg/ml)	Cpx* (1 mg/ml)	DMSO (100 µl)
S. paratyphi A	18.00 ± 0.57^{b}	0.00^{a}	23.50 ± 1.50^{c}	0.00^{a}
S. paratyphi B	21.66 ± 0.88^{b}	0.00^{a}	26.66 ± 0.88^{c}	0.00^{a}
S. paratyphi C	14.00 ± 0.57^{b}	0.00^{a}	24.66 ± 1.45^{c}	0.00^{a}
S. typhi	14.00 ± 0.00^{b}	0.00^{a}	25.33 ± 0.33^{c}	0.00^{a}
K. pneumoniae	20.66 ± 1.20^{b}	0.00^{a}	25.33 ± 0.33^{c}	0.00^{a}
S. pneumoniae	19.33 ± 0.66^{b}	0.00^{a}	25.33 ± 0.88^{c}	0.00^{a}
S. pyogenes	20.33 ± 0.66^{b}	0.00^{a}	30.00 ± 1.00^{cd}	0.00^{a}

Abbreviations: EtOAc – ethyl acetate extract; AqE – aqueous extract; Cpx – ciprofloxacin; values on the same row with different superscript are significantly (p<0.05) different, n = 3

Minimum inhibitory concentrations and minimum bactericidal concentrations of *Eucalyptus citriodora* leaf crude ethyl acetate extract

The results of the MIC and MBC of the crude ethyl acetate extract against the test organisms at different concentrations of the ethyl acetate crude plant extract is presented in Table 4. Tubes with low optical density corresponding to 7.5 mg/ml was recorded as the lowest MIC value of the crude extract against *Salmonella paratyphi* A*, Salmonella paratyphi* B and *Streptococcus pyogenes* while 15 mg/ml was recorded as the highest MIC value of the crude extract against *Salmonella paratyphi* C, *Salmonella typhi, Klebsiella pneumoniae* and *Streptococcus pneumoniae*. The MBC values was 60 mg/ml against *Salmonella paratyphi* B and *Streptococcus pyogenes* and 120 mg/ml against *Salmonella paratyphi* A, *Salmonella paratyphi* C, *Salmonella Typhi, Klebsiella pneumoniae* and *Streptococcus pneumoniae*. MIC of drugs guides the choice of antimicrobial to be used against a given infection or in therapy by predicting efficacy (Rehman et al. 2022).

Table 4

Minimum inhibitory concentrations and minimum bactericidal concentrations (MIC and MBC) of *Eucalyptus citriodora* ethyl acetate crude extract

Organism	Concentrations (mg/ml)/(OD600 nm)									EA extract	
										MIC [mg/ml]	MBC [mg/ml]
	+ve C	120	60	30	15	7.5	3.25	1.625	0.938	NA	NA
	-ve C	0.368	0.188	0.095	0.048	0.026	0.014	0.005	0.003		
S. paratyphi A	0.590	0.516	0.538	0.550	0.571	**0.584**	0.592	0.591	0.593	7.5	120
S. paratyphi B	0.587	0.497	0.521	0.555	0.571	**0.582**	0.589	0.589	0.588	7.5	60
S. paratyphi C	0.563	0.479	0.508	0.539	**0.556**	0.562	0.563	0.563	0.562	15	120
S. typhi	0.535	0.451	0.480	0.505	**0.528**	0.535	0.536	0.538	0.537	15	120
K. pneumoniae	0.527	0.443	0.443	0.509	**0.520**	0.526	0.528	0.528	0.529	15	120
S. pneumoniae	0.690	0.623	0.652	0.670	**0.681**	0.689	0.690	0.690	0.690	15	120
S. pyogenes	0.669	0.582	0.614	0.642	0.663	**0.661**	0.669	0.669	0.669	7.5	60

Abbreviations: OD – optical density; NA – not applicable; +ve C – positive control; -ve C – negative control; EA – ethyl acetate

Retention factor of *Eucalyptus citriodora* leaf ethyl acetate crude extract revealed by thin layer chromatography (TLC)

The result of thin layer chromatography on the ethyl acetate crude extract reveals the possible number of fractions contained in the extract which may be deduced from the number of spot and retention factor.

In Table 5, the highest number of spot (that is the possible number of fraction) seen with absolute chloroform was 8 while the lowest number (1) was recorded with ethyl acetate: methanol and the highest retention factor recorded was 0.98 cm while the lowest retention factor recoded was 0.8 cm. The result also reveals an increased number of spots towards the middle of the table i.e., where the moderately polar solvents such as chloroform and ethyl acetate were used while fewer spots were obtained with the highly polar solvent. FAIR and KORMAS (2008) reported that substances that produces retention factors between 0.3 and 0.7 cm need not adjusted in terms of solvent system.

Table 5
Solvent system, number of spots and retention factors of *Eucalyptus citriodora* leaf ethyl acetate crude extract

Solvent system (2 ml)	NS	DMS [cm]	DMF [cm]	Rf [cm]
100% n-hexane	2	5.0	0.6, 0.9	0.12–0.18
9:1 hexane: chloroform	3	5.0	0.6, 2.1, 3.1	0.12–0.62
4:1 hexane: chloroform	4	5.0	0.8, 2.1, 2.8, 3.5	0.16–0.70
1:1 hexane: chloroform	5	5.0	0.6, 1.8, 3.4, 4.0, 4.6	0.12–0.92
1:4 hexane: chloroform	7	5.0	0.7, 1.0, 1.2, 1.4, 2.3, 3.0, 4.6	0.14–0.92
1:9 hexane: chloroform	7	5.0	0.8, 1.1, 1.7, 2.4, 2.7, 3.1, 4.7	0.16–0.94
100% chloroform	8	5.0	0.7,1.0, 1.5, 1.8, 3.1, 4.3, 4.6, 4.9	0.14–0.98
9:1 chloroform: ethyl acetate	4	5.0	0.8, 3.1, 4.2, 4.9	0.16–0.98
4:1 chloroform: ethyl acetate	4	5.0	1.0, 3.6, 4.1, 4.9	0.20–0.98
1:1 chloroform: ethyl acetate	6	5.0	0.8, 1.2, 1.7, 1.9, 2.3, 4.6	0.16–0.92
1:4 chloroform: ethyl acetate	5	5.0	0.7, 1.9, 2.8, 3.3, 4.6	0.14–0.92
1:9 chloroform: ethyl acetate	4	5.0	2.5, 3.0, 3.7, 4.2	0.25–0.84
100% ethyl acetate	3	5.0	2.8, 3.5, 4.2	0.56–0.84
9:1 ethyl acetate: methanol	3	5.0	3.0, 3.8, 4.6	0.6–0.92
4:1 ethyl acetate: methanol	1	5.0	4.6	0.92
1:1 ethyl acetate: methanol	1	5.0	4.9	0.98
1:4 ethyl acetate: methanol	NSp	5.0	NSp	NSp
1:9 ethyl acetate: methanol	NSp	5.0	NSp	NSp
100% methanol	NSp	5.0	NSp	NSp

Abbreviations: DMS – distance moved by the solvent (mobile phase); DMF – distance moved by fraction; Rf – retention factor; NS – number of spots; NSp – no spot seen

Column chromatogram and retention factor of *Eucalyptus citriodora* ethyl acetate fractions

A total of 9 fractions were eluted (ECE1 TO ECE9) from *Eucalyptus citriodora* ethyl acetate extract (Table 6). Fraction 9 (ECE9) had the highest yield of 11.4 (57%) followed by fraction 8 (ECE8) with 2.05 g (8.20%) while fraction 2 (ECE2) had the lowest yield of 0.39 g (1.56%). The highest retention factor was recorded for fraction ECE7 and fraction ECE8 followed by fraction ECE9 and ECE7 (spot number two) while fraction ECE3 had the lowest retention factor. Also, fraction ECE9 used up a higher volume of solvent (800 ml) for its elution followed by fractions ECE8, ECE6 and ECE5 (700 ml) while fraction ECE2 consumed less solvent (300 ml). All fractions had one spot except for fraction ECE2, ECE4, ECE5 and ECE7 which had two spots each.

Table 6

Percentage yield, solvent systems and retention factor of *Eucalyptus citriodora* ethyl acetate fractions (20 g)

Solvent systemand volume [ml]	Description	Percentage yield [%]	NS	Rf [cm]	Fraction eluted
100% n-hexane (500)	yellow and oily	0.7 (3.5)	1	0.48	ECE 1
100% n-hexane (300)	red and oily	0.39 (1.95)	1	0.44	ECE 2
100% n-hexane (400)	grey and oily	0.60 (3.0)	1	0.38	ECE 3
9:1 n-HEX: $CHCl_3$ (600)	light green	0.56 (2.80)	1	0.70	ECE 4
1:1 n-HEX: $CHCl_3$ (700)	dark green oily	0.86 (4.30)	1	0.46	ECE 5
1:9 $CHCl_3$: EtOAc (700)	dark green	0.91 (4.55)	1	0.58	ECE 6
9:1 $CHCl_3$: EtOAc (600)	light green	1.02 (5.10)	2	0.80; 0.76	ECE 7
100% EtOAc (700)	black	2.05 (10.25)	1	0.80	ECE 8
100% water (500)	off white	12.33 (61.65)	NSp	NSp	ECE 9

Abbreviations: n-HEX – N-hexane; $CHCl_3$ – chloroform; EtOAc – ethyl acetate; NSp – no spot seen; NS – number of spots; Rf – retention factor

Antimicrobial activity of *Eucalyptus citriodora* ethyl acetate leaf fractions

A total of 9 fractions were eluted by column chromatography from the ethyl acetate crude extract of *Eucalyptus citriodora*. Of the 9 fractions eluted, 5 were active against the test organisms while 4 were not active as shown in Table 7 and Table 8. The mean diameter zone of inhibition (DZI) of the active fraction's ranges from 13.33±0.33 mm (ECE5 against *Streptococcus pneumoniae*) to 26.00±0.00 mm (ECE1 against *Streptococcus pyogenes*). ECE1 shows a greater activity against the test organisms as com-

pared to the other fractions and also its activity is more comparable to the positive control drug. This was followed closely by fraction ECE4 (15.33±0.66 mm to 23.66±0.33 mm) and ECE5 (13.33±0.33 mm to 20.33±0.33 mm) while fraction ECE2 (11.33±0.33 mm to 24.33±0.33 mm) was the least in activity when compared to the positive control drugs. Streptococcus pyogenes was more susceptible to the fractions and standard drugs among all other test organisms while *S. paratyphi* C and *S. typhi* were the least susceptible. The antimicrobial activity of several *Eucalyptus* plants has been attributed to their possession of certain specific Terpenes and Phenolic compounds (BEN-ARFA et al. 2006) The majority of compounds in *Eucalyptus citriodora* ethyl acetate fractions was alkane hydrocarbons of which decane is their basic structure ($C_{10}H_2$). It is reported that decane is the basic structure of the novel antibiotic drug Aranorosin with molecular formula $C_{23}H_{33}NO_6$ (1-oxaspiro 4, 5 decane) isolated from the fungi *Pseudoarachniotus roseus* (MALIK et al. 2014). The result of this investigation revealed large qualitative and quantitative differences in the phytochemical composition of same plants grown in Algerian origins as reported by BAYNESAGNE (2017). In his report, vital phytochemical constituents with evidence of antibacterial activity were reported absent in the plant extracts. This may be as a result of environmental factors such as the, time of harvest, drying method, type of soil, plant part used, solvent used for extraction, methods of extraction and the amount of plant material used for extraction.

Table 7

Mean diameter zones of inhibition of *Eucalyptus citriodora* ethyl acetate leaf fractions [mm]

Organisms	*Eucalyptus citriodora* ethyl acetate leaf fractions				Control	
	ECE1 (40 mg/ml)	ECE2 (40 mg/ml)	ECE3 (40 mg/ml)	ECE4 (20 mg/ml)	Cpx (1 mg/ml)	DMSO (100 µL)
S. paratyphi A	23.66±0.33^{d}	8.33±0.33^{b}	9.66±0.33^{b}	15.33±0.66^{c}	23.50±1.50^{d}	0.00^{a}
S. paratyphi B	25.00±0.00^{d}	9.33±0.33^{b}	8.00±0.00^{b}	19.00±0.00^{cd}	26.66±0.88^{d}	0.00^{a}
S. paratyphi C	23.66±0.33^{d}	8.33±0.88^{b}	8.00±0.57^{b}	23.66±0.33^{d}	24.66±1.45^{d}	0.00^{a}
S. Typhi	25.66±0.33^{d}	5.66±0.33^{b}	5.33±0.33^{b}	22.00±0.00^{cd}	25.33±0.33^{d}	0.00^{a}
K. pneumonia	21.33±0.66^{cd}	8.00±0.57^{b}	7.33±0.33^{b}	19.00±0.57^{c}	25.33±0.33^{d}	0.00^{a}
S. pneumoniae	25.66±0.33^{d}	10.00±0.00^{b}	9.66±0.33^{b}	15.66±0.66^{c}	25.33±0.88^{d}	0.00^{a}
S. pyogenes	26.00±0.00^{d}	10.00±0.57^{b}	11.33±0.33^{b}	20.66±0.33^{cd}	30.00±1.00^{e}	0.00^{a}

Abbreviations: Org – organism; ECE 1–8 – *Eucalyptus citriodora* ethyl acetate fraction 1 to 8; Cpx – ciprofloxacin; DMSO – dimethyl sulfoxide. Values on the same row with different superscript are significantly different (p<0.05), n – 3

Table 8

Mean diameter zones of inhibition of *Eucalyptus citriodora* ethyl acetate leaf fractions [mm]. *Eucalyptus citriodora* ethyl acetate leaf fractions control

Organisms	*Eucalyptus citriodora* ethyl acetate leaf fractions				Control	
	ECE5 (40 mg/ml)	ECE6 (40 mg/ml)	ECE7 (40 mg/ml)	ECE8 (40 mg/ml	Cpx (1 mg/ml)	DMSO (100 µl)
S. paratyphi A	15.00 ± 0.57^{c}	0.00^{a}	0.00^{a}	0.00^{a}	23.50 ± 1.50^{d}	0.00^{a}
S. paratyphi B	18.66 ± 0.66^{cd}	0.00^{a}	0.00^{a}	0.00^{a}	26.66 ± 0.88^{d}	0.00^{a}
S. paratyphi C	18.33 ± 0.33^{c}	0.00^{a}	0.00^{a}	0.00^{a}	24.66 ± 1.45^{d}	0.00^{a}
S. Typhi	14.66 ± 0.33^{bc}	0.00^{a}	0.00^{a}	0.00^{a}	25.33 ± 0.33^{d}	0.00^{a}
K. pneumoniae	20.33 ± 0.33^{cd}	0.00^{a}	0.00^{a}	0.00^{a}	25.33 ± 0.33^{d}	0.00^{a}
S. pneumoniae	13.33 ± 0.33^{bc}	0.00^{a}	0.00^{a}	0.00^{a}	25.33 ± 0.88^{d}	0.00^{a}
S. pyogenes	16.66 ± 0.66^{c}	0.00^{a}	0.00^{a}	0.00^{a}	30.00 ± 1.00^{e}	0.00^{a}

Abbreviations: ECE1–8 – *Eucalyptus citriodora* ethyl acetate fraction 1 to 8; Cpx – ciprofloxacin; DMSO – dimethyl sulfoxide. Values on the same row with different superscript are significantly different ($p<0.05$), $n = 3$.

Minimum inhibitory and minimum bactericidal concentration (MIC and MBC) of *Eucalyptus citriodora* ethyl acetate fractions [mg/ml]

The results of the MIC and MBC for ECE1 (*Eucalyptus citriodora* leaf ethyl acetate fraction 1), ECE4 (*Eucalyptus citriodora* leaf ethyl acetate fraction 4) andECE5 (*Eucalyptus citriodora* leaf ethyl acetate fraction 5) are presented in Table 9 – Table 11. The lowest MIC recorded was 1.88 mg/ml for ECE1 and ECE4 against *Salmonella paratyphi* B and *Streptococcus pyogenes* (ECE1 and ECE4) and *Streptococcus pyogenes* (ECE4). This was followed by MIC of 3.75 mg/ml for ECE1 (against *Salmonella paratyphi* A) and ECE4 (against *Salmonella paratyphi* A and *Streptococcus pneumoniae*) while 15 mg/ml MIC was recorded for the 3 fractions (ECE1, ECE4 & ECE5) against *Salmonella paratyphi* C, *Salmonella typhi and Streptococcus pneumoniae* (ECE1), *Salmonella paratyphi* C*, Salmonella typhi, Klebsiella pneumoniae and Streptococcus pneumoniae (*ECE5)*, Salmonella typhi* (ECE4). The MBC of the fractions against the test organisms ranges from 30 mg/ml to 120 mg/ml.

Table 9

Minimum inhibitory and minimum bactericidal concentration (MIC and MBC) of *Eucalyptus citriodora* ethyl acetate fractions [mg/ml]

Organism	Concentrations [(mg/ml) /(OD600 nm)]									ECE1	
										MIC [mg/ml]	MBC [mg/ml]
	+ve C	120	60	30	15	7.5	3.75	1.88	0.938	NA	NA
	-ve C	0.300	0.160	0.082	0.045	0.024	0.011	0.006	0.003	NA	NA
S. paratyphi A	0.502	0.383	0.405	0.429	0.450	0.468	**0.480**	0.498	0.497	3.75	120
S. paratyphi B	0.524	0.401	0.422	0.442	0.471	0.494	0.510	**0.522**	0.530	1.88	60
S. paratyphi C	0.516	0.442	0.458	0.488	**0.504**	0.520	0.521	0.522	0.520	15	120
S. typhi	0.551	0.448	0.485	0.504	**0.521**	0.539	0.539	0.540	0.539	15	120
K. pneumonia	0.560	0.447	0.493	0.516	0.527	**0.549**	0.561	0.561	0.560	7.5	120
S. pneumonia	0.644	0.544	0.581	0.610	**0.630**	0.644	0.645	0.645	0.644	15	120
S. pyogenes	0.628	0.489	0.518	0.552	0.588	**0.611**	0.628	0.627	0.627	7.5	60

Abbreviations: OD – optical density; NA – not applicable; +ve C – positive control; -ve C – negative control; ECE1 – *Eucalyptus citriodora* ethyl acetate fractions 1

Table 10

Minimum inhibitory and minimum bactericidal concentration (MIC and MBC) of *Eucalyptus citriodora* ethyl acetate fractions [mg/ml]

Organism	Concentrations [(mg/ml) /(OD600 nm)]									ECE4	
										MIC [mg/ml]	MBC [mg/ml]
	+ve C	120	60	30	15	7.5	3.75	1.88	0.938	NA	NA
	-ve C	0.300	0.160	0.082	0.045	0.024	0.011	0.006	0.003	NA	NA
S. paratyphi A	0.502	0.383	0.405	0.429	0.450	0.468	**0.480**	0.498	0.497	3.75	120
S. paratyphi B	0.524	0.401	0.422	0.442	0.471	0.494	0.510	**0.522**	0.530	1.88	60
S. paratyphi C	0.516	0.442	0.458	0.488	**0.504**	0.520	0.521	0.522	0.520	15	120
S. typhi	0.551	0.448	0.485	0.504	**0.521**	0.539	0.539	0.540	0.539	15	120
K. pneumonia	0.560	0.447	0.493	0.516	0.527	**0.549**	0.561	0.561	0.560	7.5	120
S. pneumonia	0.644	0.544	0.581	0.610	**0.630**	0.644	0.645	0.645	0.644	15	120
S. pyogenes	0.628	0.489	0.518	0.552	0.588	**0.611**	0.628	0.627	0.627	7.5	60

Abbreviations: OD – optical density; NA – not applicable; +ve C – positive control; -ve C – negative control; ECE4 – *Eucalyptus citriodora* ethyl acetate fractions 4

Table 11

Minimum inhibitory and minimum bactericidal concentration (MIC and MBC) of *Eucalyptus citriodora* ethyl acetate fractions [mg/ml]

Organism	Concentrations [(mg/ml) /(OD600 nm)]									MIC [mg/m])	MBC [mg/ml]
	+ve C	120	60	30	15	7.5	3.75	1.88	0.938	NA	NA
	-ve C	0.297	0.146	0.080	0.039	0.019	0.009	0.005	0.003	NA	NA
S. paratyphi A	0.580	0.470	0.508	0.530	0.559	**0.571**	0.580	0.580	0.581	7.5	60
S. paratyphi B	0.530	0.301	0.436	0.488	0.512	**0.526**	0.531	0.530	0.530	7.5	60
S. paratyphi C	0.575	0.379	0.411	0.542	**0.568**	0.577	0.578	0.577	0.577	15	120
S. typhi	0.522	0.432	0.480	0.502	**0.515**	0.523	0.523	0.523	0.523	15	120
K. pneumonia	0.504	0.390	0.428	0.460	**0.498**	0.507	0.507	0.508	0.508	15	120
S. pneumonia	0.618	0.534	0.568	0.591	**0.610**	0.620	0.620	0.620	0.620	15	120
S. pyogenes	0.630	0.411	0.538	0.567	0.593	0.611	**0.625**	0.631	0.631	3.25	30

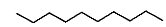
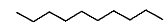
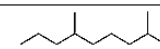
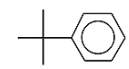
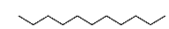
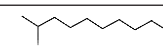
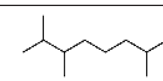
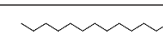
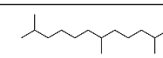
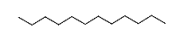
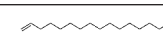
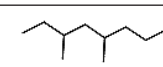
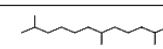
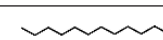
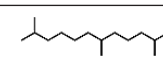
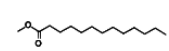
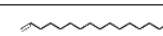
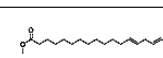
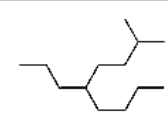
Abbreviations: OD – optical density; NA – not applicable; +ve C – positive control; -ve C – negative control; ECE5 – *Eucalyptus citriodora* ethyl acetate fractions 5

Compounds identified in *Eucalyptus citriodora* ethyl acetate fraction (ECE1)

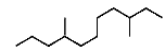
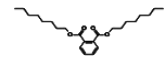
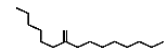
The result of the gas chromatography mass spectrometry (GC-MS) of ethyl acetate fraction (ECE1) of *Eucalyptus citriodora* is presented in Table 12. A total of 28 compounds were identified in the ethyl acetate fraction. The result shows the peak number, retention time, peak area in percentage, molecular weight, molecular formula and structure of each identified compounds. Peak number 28 corresponding to Tetracosane was the most abundant compound with peak area of 14.03% followed by peak number 2 that is Decane, with peak area of 11.49%; others were between the ranges of 1.17% to 5.06% while peak number 14 that is 1-Octanol, 2-butyl- was the least abundant with peak area of 0.6%. The most abundant compounds present in the ethyl acetate fractions ECE1 are Tetracosane (14.03%) and Decane (14.49%), some are in limited amount ranging from 1.17% to 6.59% while others are in trace amount. These group of compounds identified are reported to possess antibacterial activity against Gram negative and positive pathogens. The test organisms were more susceptible to fraction ECE1 with mean diameter zones of inhibition ranged from 15 mm to 26 mm compared to fraction ECE5 which exhibited intermediate activity with mean zones of inhibition ranging from 15 mm to 20 mm.

Table 12

Percentage composition and suspected structures of compounds identified in *Eucalyptus citriodora* ethyl acetate fraction (ECE1)

Peak No.	RT	P.A [%]	MW [g/mol]	MF	Compound name	Suspected structures
1	3.607	3.77	142.28	$C_{10}H_{22}$	decane (A)	
2	4.923	11.49	142.28	$C_{10}H_{22}$	decane (B)	
3	5.245	3.39	156.31	$C_{11}H_{24}$	nonane, 2,6-dimethyl-	
4	5.847	4.50	134.22	$C_{10}H_{14}$	benzene, tertbutyl-	
5	6.368	6.59	156.31	$C_{11}H_{24}$	undecane	
6	7.783	3.63	156.31	$C_{11}H_{24}$	decane, 2-methyl	
7	8.808	2.17	156.31	$C_{11}H_{24}$	octane, 2,3,7-trimethyl-	
8	9.251	3.01	184.36	$C_{13}H_{28}$	tridecane	
9	10.298	2.50	212.41	$C_{15}H_{32}$	2,6,11-trimethyldodecane	
10	10,607	5.38	142.28	$C_{10}H_{22}$	decane (C)	
11	11.412	3.78	170.33	$C_{12}H_{26}$	dodecane	
12	11.895	3.22	212.41	$C_{15}H_{32}$	n-Pentadecane	
13	11.117	1.27	224.43	$C_{16}H_{32}$	hexadecane	
14	13.682	0.71	142.26	$C_{10}H_{22}$	octane, 3,5-dimethyl-	
15	14.346	1.94	212.41	$C_{15}H_{32}$	2,6,11-trimethyldodecane	
16	15.619	1.60	170.33	$C_{12}H_{26}$	dodecane	
17	17.348	1.22	212.41	$C_{15}H_{32}$	2,6-dimethylheptadecane	
18	17.895	1.54	228.37	$C_{14}H_{28}O_2$	tridecanoic acid, methyl ester	
19	19.237	1.17	256.47	$C_{17}H_{36}O$	1-Heptadecanol	
20	19.344	1.69	224.43	$C_{16}H_{32}$	hexadecane	
21	20.987	4.36	294	$C_{19}H_{34}O_2$	13,16-octadecadienoic acid, methyl ester	
22	22.310	1.64	184	$C_{13}H_{28}$	nonane, 2-methyl-5-propyl-	

cont. Table 12

23	23.480	1.38	184	$C_{13}H_{28}$	undecane, 3,8-dimethyl-	
24	24.522	2.16	282.55	$C_{20}H_{42}$	eicosane	
25	25.470	4.90	282.55	$C_{20}H_{42}$	n-eicosane	
26	25.949	1.91	390.56	$C_{24}H_{38}O_4$	di-n-octyl phthalate	
27	26.354	5.06	310	$C_{22}H_{46}$	pentadecane, 8-heptyl-	
28	27.322	14.03	338.65	$C_{24}H_{50}$	tetracosane	

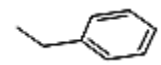
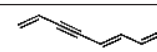
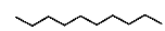
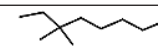
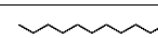
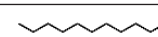
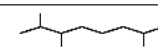
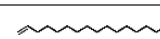
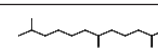
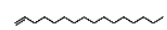
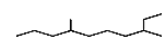
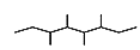
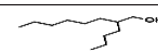
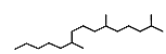
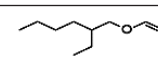
Abbreviations: RT – retention time; PA – peak area; MW – molecular weight; MF – molecular formula

Compounds identified in *Eucalyptus citriodora* ethyl acetate fraction (ECE4)

The result of the gas chromatography mass spectrometry (GC-MS) of ethyl acetate fraction (ECE4) of *Eucalyptus citriodora* is presented in Table 13. A total of 18 compounds were identified in fraction (ECE4). The result in Table 13 shows the peak number, retention time, peak area in percentage, molecular weight, molecular formula and structure of each identified compounds. Peak number 16 corresponding to Octadecanal was the most abundant compound with peak area of 59.54% followed by peak number 17 that is 1-Eicosanol, with peak area of 7.90%; others were between the ranges of 0.80% to 4.35% while peak number 13 that is 1-Octanol, 2-butyl- was the least abundant with peak area of 0.16%. The high activity of ECE1 and ECE4 could be attributed to the presence of Tetracosane, eicosane, n-eicosane, Decane and Octadecanal which are reported by BRINDA and MOHAN (2016) to be bactericidal in activity. Pentadecane, 2, 6, 10-trimethyl identified in ECE4, though in trace amount was also reported by BELAYNEH et al. (2019) to possess antibacterial activity against Gram-negative bacteria.

Table 13

Percentage composition and suspected structures of compounds identified in *Eucalyptus citriodora* ethyl acetate fraction (ECE4)

Peak No.	RT	PA [%]	MW [g/mol]	MF	Compound name	Suspected structures
1	3.351	1.44	106.17	C_8H_{10}	ethylbenzene	
2	3.616	1.86	104	C_8H_8	1,3,7-octatrien-5-yne	
3	4.934	4.35	142.28	$C_{10}H_{22}$	decane	
4	5.258	0.97	142.28	$C_{10}H_{22}$	3,3-dimethyloctane	
5	6.378	3.15	156.31	$C_{11}H_{24}$	undecane	
6	7.839	1.53	156.31	$C_{11}H_{24}$	undecane	
7	8.875	1.25	156	$C_{11}H_{24}$	octane, 2,3,7-trimethyl-	
8	9.257	1.77	226	$C_{16}H_{32}$	hexadecane (a)	
9	10.303	1.25	212.41	$C_{15}H_{32}$	2,6,11-trimethyldodecane	
10	10.613	3.01	226	$C_{16}H_{32}$	hexadecane (b)	
11	11.418	2.07	170	$C_{12}H_{26}$	3,7-dimethyldecane	
12	11.907	1.99	170.34	$C_{12}H_{26}$	octane, 3,4,5,6-tetramethyl	
13	13.128	0.61	186.33	$C_{12}H_{26}O$	1-octanol, 2-butyl-	
14	14.356	0.80	254.49	$C_{18}H_{38}$	pentadecane, 2,6,10-trimethyl-	
15	21.024	3.42	242	$C_{15}H_{30}O_2$	oxirane, [(dodecyloxy)methyl]-	
16	23.211	59.54	268.48	$C_{18}H_{36}O$	octadecanal	
17	25.576	7.90	298.55	$C_{20}H_{42}O$	1-eicosanol	
18	25.961	3.10	156.27	$C_{10}H_{20}O$	heptane, 3-[(ethenyloxy)methyl]-	
Total	–	100.0	–	–	–	–

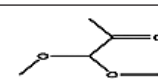
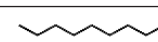
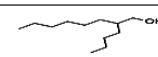
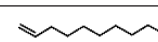
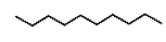
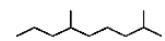
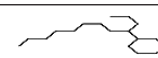
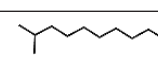
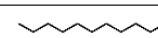
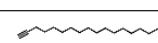
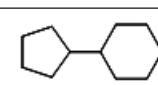
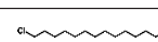
Abbreviations: RT – retention time; PA – peak area; MW – molecular weight; MF – molecular formula

Compounds identified in *Eucalyptus citriodora* ethyl acetate fraction (ECE5)

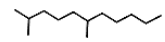
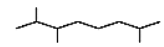
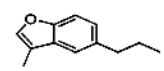
The result of the gas chromatography mass spectrometry (GC-MS) of ethyl acetate fraction (ECE5) of *Eucalyptus citriodora* is presented in Table 14. A total of 23 compounds were identified in fraction ECE5. The result in Table 14 shows the peak number, retention time, peak area in percentage, molecular weight, molecular formula and structure of each identified compounds. Peak number 5 corresponding to Decane was the most abundant compound with peak area of 13.70% followed by peak number 9 that is Undecane, with peak area of 11.68%; others were between the ranges of 0.42% to 8.98% while peak number 22 that is Nonanoic acid, methyl ester was the least abundant with peak area of 0.36%. Most of the compounds identified in the *Eucalyptus citriodora* fractions (ECE1, ECE4 and ECE5) belongs to the alkanes group, which are reported to exhibit antimicrobial activity in combination with other elements (LA et al. 2012).

Table 14

Percentage composition and suspected structures of compounds identified in *Eucalyptus citriodora* ethyl acetate fraction (ECE5)

Peak No.	RT	PA [%]	MW [g/mol]	MF	Compound name	Suspected structure
1	3.212	0.42	118.13	$C_5H_{10}O_3$	2-propanone, 1,1-dimethoxy-	
2	3.602	1.24	128.26	C_9H_{20}	nonane	
3	4.051	1.76	186.33	$C_{12}H_{26}O$	1-octanol, 2-butyl-	
4	4.811	5.01	140.27	$C_{10}H_{20}$	1-decene	
5	4.929	13.70	142.28	$C_{10}H_{22}$	decane	
6	5.252	4.90	156	$C_{11}H_{24}$	nonane, 2,6-dimethyl-	
7	5.484	5.18	364	$C_{18}H_{36}$	dodecane, 4-cyclohexyl-	
8	5.857	7.05	156.31	$C_{11}H_{24}$	decane, 2-methyl-	
9	6.380	11.68	156.31	$C_{11}H_{24}$	undecane	
10	6.723	2.51	250.46	$C_{18}H_{34}$	1-octadecyne	
11	6.971	3.57	152.28	$C_{11}H_{20}$	cyclopentylcyclohexane	
12	7.316	3.83	232.83	$C_{14}H_{29}Cl$	tetradecane, 1-chloro-	

cont. Table 14

13	7.840	8.98	170.33	$C_{12}H_{26}$	dodecane (a)	
14	8.093	3.34	184.36	$C_{13}H_{28}$	2,6-dimethylundecane	
15	8.875	4.86	156	$C_{11}H_{24}$	octane, 2,3,7-trimethyl-	
16	9.258	6.18	184.36	$C_{13}H_{28}$	tridecane	
17	10.302	3.86	174.23	$C_{12}H_{14}O$	nonane, 3-methyl-5-propyl	

Abbreviations: RT – retention time; PA – peak area; MW – molecular weight; MF – molecular formula

Toxicity of plant extracts

The result of the acute toxicity and LD_{50} of *Eucalyptus citriodora* (ethyl acetate) extracts and the corresponding histopathology of the kidney & liver of the dead rats is presented in Table 15 and Plate II & III respectively (Fig. 3 and Fig. 4). There were no observable signs of toxicity in the experimental rats in response to the first three doses administered (10 mg, 100 mg and 1000 mg/kg bw) but at 1000 mg/kg bw, the animals reacted with shivering within the first 1 to 3 h and 1 death after 48 h. The result shows that the minimum tolerated and maximum lethal doses for both *Eucalyptus citriodora* ethyl acetate extract was 500 mg/kg bw and 1000 mg/kg bw respectively therefore, the LD_{50} for the crude extract was 707.10 mg/kg bw which is far lower than the recommended lethal dose of 5000 mg/kg bw (ZHAO et al. 2019). Furthermore, the fact that the kidney and liver plays vial roles in the processing of drugs in the human system, makes it very important to determine the state of these vital organs in the dead animals. These changes on the organs could be as a result of toxic agents present in the plant extracts considering the fact that such changes were not observed in the control rats and this may affect the kidney and impair its physiological functions. But the result of this study reveals that the effect is dose dependent. This result is similar to the result of BIN ZHAO et al. (2019) who reported that the same pathological changes were observed in experimental rats' organs administered with oil extracts from *Eucalyptus viminalis*. ODELEY and OBAMESO (2022)· reported that physiological changes in kidney histology may not be sensitive enough in detecting renal toxicity or damage. OKOKON et al. (2018) who reported that degenerative glomeruli were observed in kidney section of male albino rats administered with *Eucalyptus citriodora* acetone extract but were less effective with lower doses.

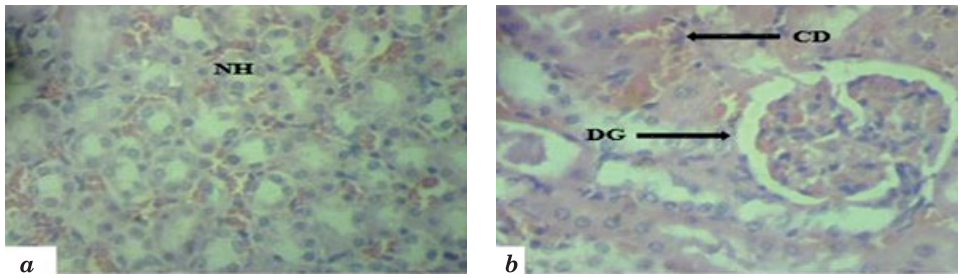


Fig. 3. Plate II. Photomicrographs of normal and experimental rat liver sections (*a*) section of kidney administered with normal saline showing normal histology (NH); (*b*) section of kidney administered with 1000 mg/kg bw *Eucalyptus citriodora* oil extract showing mild degenerative glomerulus & renal tubules and cytoplasmic distortion

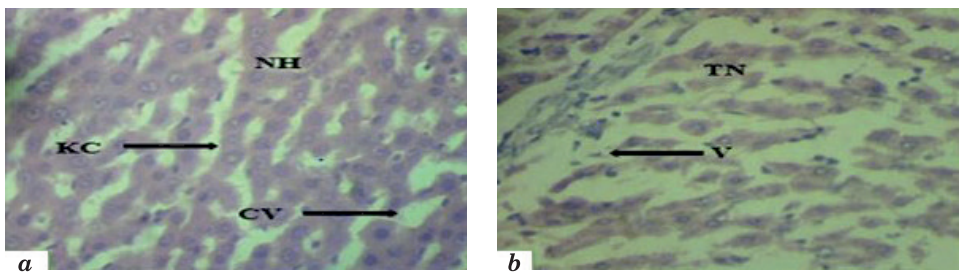


Fig. 4. Plate III. Photomicrographs of normal and experimental rat kidney sections (*a*) section of liver administered with normal saline showing normal hepatocyte (NH), Central vein (CV) and kupfer cell (KC); (*b*) section of liver administered with 1000 mg/kg bw *Eucalyptus citriodora* oil extract showing mild tissue necroses (TN) and vacuolation (V)

Table 15

Acute toxicity and LD_{50} of plant extracts

Extract	Number of animals	Dose [mg/kg bw]	Motality/ survival	Toxicity reactions
ECE	3	10	0/3	no sign of toxicity
	3	100	0/3	no sign of toxicity
	3	500	0/3	no sign of toxicity
	3	1000	1/2	shivering within the first 1–3 hrs and 1 death after 48 h
NS3	3	10	0/3	no sign of toxicity
	3	100	0/3	no sign of toxicity
	3	500	0/3	no sign of toxicity
	3	1000	0/3	no sign of toxicity

Abbreviations: ECE – *Eucalyptus citriodora* ethyl acetate fraction; NS – normal saline; mg/kg bw – milligram per kilogram body weigh N.B:LD_{50} – √(*minimum tolerated dose*) (*maximum lethal dose*), route of administration: oral, LD_{50} = √(500)(1000) = 707.10 mg/kg bw

Conflict of interest. The authors declare that there is no conflict of interest.

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MICROPLASTICS IN THE WATERS OF EUROPEAN LAKES AND THEIR POTENTIAL EFFECT ON FOOD WEBS: A REVIEW

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Key words: microplastics, freshwaters, lakes, Europe, zooplankton, fish.

Abstract

Microplastics are widely distributed in aquatic environments. Studies to date have focused mainly on marine environments, and there is a substantial body of work in this topic. Contamination in freshwater ecosystems is a new and growing problem that has received decidedly less attention; this includes lakes that are particularly vulnerable because of their close proximity to emission sources. The first studies on the problem of lake microplastic pollution were not published until 2011, but interest among researchers has increased in recent years. The aim of this review is to assess the current state of knowledge about levels of microplastic pollution in European lake waters and to identify the most urgent areas of research that are required. A review of available data indicated that the number of lakes that have been investigated remains small in light of the overall number of lakes in Europe. The pollution levels in European lakes are similar to those in other lakes worldwide, but they are usually lower than those in marine ecosystems. There is a near total lack of data on the topic of the pollution of microplastics < 300 μm. This is a particularly significant gap in knowledge since some studies indicate that the quantity of microplastics in lakes might rise substantially if increasingly smaller particles are analyzed. Little attention is paid to the fate of microplastics in the water column and the influence they have on lake food webs. There is still no evidence confirming whether freshwater zooplankton ingest microplastics in their natural environments. However, the trophic transfer of microplastics in lake food webs is highly likely since microplastics have been confirmed in a wide range of freshwater ichthyofauna. Furthermore, there is a near total lack of multi-dimensional models that would describe the primary factors responsible for the accumulation of microplastics in lakes. Based on the present review, the most relevant recommendations are: developing a coherent research methodology that will facilitate comparing research results; assessing microplastic concentrations at various water column depths; increasing research on fine fractions of microplastics; identifying and estimating microplastic consumption rates by natural populations of aquatic organisms and assessing the risk of microplastic accumulation in food webs.

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Introduction

Initially, global attention focused on environmental pollution with larger plastic debris, but in recent years, researchers have come to focus on microplastics (GESAMP 2015). These are small plastic particles of various size, chemical composition, and physical properties. There is a lack of full consensus among researchers on how to define the term microplastic. The vast majority of researchers agree that microplastics include all synthetic polymer particles smaller than 5 mm (DUIS and COORS 2016). Some authors have also determined a lower limit for particle size. CLAESSENS et al. (2011) defined microplastics as particles ranging in size from 1 to 5 mm. The lower particle size limit is also sometimes defined as 0.1 μm (LUSHER et al. 2017a). Smaller particles known as nanoplastics range in size from 0.1 μm to 0.001 μm (KLAINE et al. 2012).

The shapes of polymer particles polluting the environment are varied, and the literature lacks cohesion with regard to the morphological description of the particles analyzed. Researchers have distinguished up to ten different particle shapes. HIDALGO-RUZ et al. (2012) distinguished the following shape categories of microplastics in the aquatic environment: filaments, films, foamed plastic, granules, fragments, pellets, and styrofoam. In terms of type, literature data indicate that the most frequently identified microplastic particles in aquatic ecosystems are polyethylene, polyethylene terephthalate, polypropylene, polystyrene, polyvinyl chloride, and, less frequently, polyamide (nylon), polyester, and acrylic (HIDALGO-RUZ et al. 2012, WAGNER et al. 2014).

Microplastic particles in surface waters originate from either primary or secondary sources. Primary microplastics reach the environment in the form in which they were manufactured. Polymer microgranules are used, inter alia, in personal care products such as toothpastes, gels, exfoliants, and many others. An estimated 1,500 metric tons of microplastics from personal care products are released annually into the global aquatic environment (SUN et al. 2020). Another source of primary microplastics is industrial abrasives that contain polystyrene, acrylic, melamine, polyester, and poly allyl diglycol carbonate microplastics (DUIS and COORS 2016). Primary microplastics also include pre-production granulates or regranulates that can be released into the environment accidentally during production, processing, storage, and transport (BOUCHER and FRIOT 2017). Primary microplastics are released into the aquatic environment with industrial wastewater, municipal wastewater, and in runoff from surfaces that are insufficiently secured during production or application. KARLSSON et al. (2018) estimated that wastewaters discharged daily into a stream

near a Swedish plastic factory could carry from several to nearly one hundred thousand granules, which would mean a theoretical annual release of 73 to 730 kg of microplastics.

Although various sources of primary microplastics are identified in the literature, the annual global discharge of these microplastics has not yet been estimated. Thus, the significance of primary microplastic sources remains unknown as does the relative importance of primary and secondary sources (KOELMANS et al. 2014, BOUCHER and FRIOT 2017). It is presumed, however, that secondary microplastics are more widespread in the environment (COLE et al. 2011, EERKES-MEDRANO et al. 2015). Secondary microplastic environmental pollution is the product of macroplastic degradation that occurs mainly from mechanical factors and photodegradation. Microfibers generated by synthetic fabrics during daily use is currently the main source of microplastics in the environment (ACHARYA et al. 2021). Huge quantities of microfibers are released when laundering clothing in washing machines, and these are discharged with wastewaters from treatment facilities. The results of recent studies indicate that, depending on the type of clothing made of synthetic fibers that is laundered, one laundry cycle releases from 124 to 308 μg per kg of fabric laundered, and this corresponds to numbers of microfibers in the range of 640,000–1,500,000 (DE FALCO et al. 2019). Although the efficiency of retaining microfibers from wastewaters is currently high at treatment facilities, the sheer numbers of microfibers in wastewaters means that billions of these fibers are released into surface waters daily with discharged treated wastewaters.

Synthetic textile fibers are also released into the environment during the everyday use of fabrics (SUNDT et al. 2014). In addition to textiles, microplastics are generated during everyday household activities such as opening plastic packaging (SOBHANI et al. 2020) and by abrasion when using everyday plastic objects, and microplastics remain suspended in the air or settle as dust (MAGNUSSON et al. 2016). Secondary microplastics are generated outside, inter alia, by vehicle tire abrasion, which is a substantial source of environmental pollution (SOMMER et al. 2018). Other sources of secondary microplastics include synthetic polymer-based paints used in construction and road and ship paints that are released into the environment through mechanical abrasion or removal (DUIS and COORS 2016). Secondary microplastics form various sources are suspended in the atmosphere and deposited to aquatic ecosystems with precipitation. Secondary microplastics are also produced as the macroplastics littering surface waters and their vicinities degrade. Plastics can end up in aquatic environments from dumping and improper storage, but they can also be carried by winds and water runoff (MAGNUSSON et al. 2016). Water tourism

and costal recreation are also sources of plastic refuse reaching the aquatic environment (THUSHARI and SENEVIRATHNA 2020).

Many studies focus on the problem of marine and oceanic microplastic pollution (e.g., MOOR et al. 2001, 2002, COLLINGTON et al. 2012, HIDALGO-RUZ et al. 2012, ISOBE et al. 2015), and the first data on the topic of the microplastic pollution of waters were published in the 1970s (CARPENTER and SMITH 1972). Studies of inland waters have not been conducted for nearly as long, and data regarding these waters are relatively few in comparison to those for seas and oceans (EERKES-MEDRANO et al. 2015, DUIS and COORS 2016). The first data on the topic of lake microplastic pollution was published just a decade ago (ZBYSZEWSKI and CORCORAN 2011). In this context, European lakes deserve special attention. Europe has close to 500,000 natural lakes with a surface area of at least 0.01 km^2. The highest concentrations of lakes are in the boreal and arctic regions. Most lakes are located in Norway, Sweden, Finland, and the Karelo-Kola Region of Russia. Water bodies in this area are usually characterized by large areas and perimeters. Many lakes are also located around the Baltic Sea in areas that were affected by the glacial period and in the northwestern part of the United Kingdom, Ireland, and Iceland. In central Europe, many lakes lie in mountain regions, including larger ones, which are located in Alpine valleys (European Environment Agency, VERPOORTER et al. 2014). European lakes are characterized by great diversity in terms of their origin, morphometry, trophic status, thermal regime, stratification, and water balance (BENGTSSON 2012). A basin's topography and water depth determine the variety of living organism communities it hosts. All the major physical zones of lakes and their associated fauna can be exposed to microplastics (Figure 1).

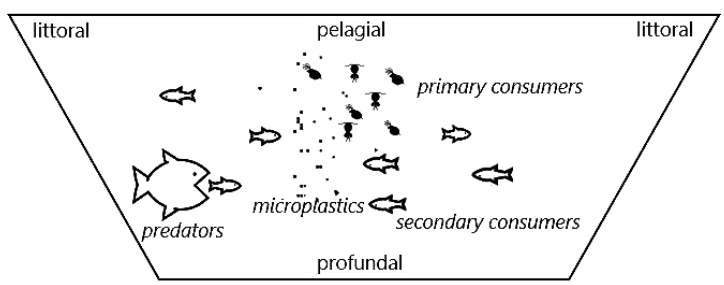

Fig. 1. Microplastics in a lake pelagial and their effect on local food webs

Microplastics in lake pelagic zones can be ingested intentionally or accidentally by primary consumers (zooplankton) or secondary consumers (fish) and transferred via the food web to higher trophic levels. In Europe

the likelihood that surface waters are polluted with microplastics is very high because of the high population density and the high degree of industrialization (LEBRETON and ANDRARY 2019). Lakes are aquatic ecosystems that are particularly exposed to the accumulation of microplastics, and lakes that are fed by river waters are especially exposed since rivers are considered important vectors for transporting plastic pollution from the land to waters (LEBRETON et al. 2017). River waters flowing into lakes carry all types of pollution found in their catchments. Microplastics that are transported with river waters can be retained in lakes and can pose potential threats to the organisms inhabiting them and to humans. Many European lakes have important functions and serve as potable water reservoirs, water sources for aquaculture, and also recreational areas.

Microplastics are freshwater pollutants of emerging concern. The magnitude of microplastic pollution in European lakes is still unknown, and environmental risks have not yet been evaluated. We need complex data on microplastic concentrations, sources, and fates in freshwater ecosystems to assess their effects on lacustrine food webs and the responses of individuals or communities to exposure. To solve this the problem, it is essential to analyze existing research and identify current gaps in knowledge. Therefore, the aim of this article is to review the current state of knowledge of microplastic pollution in lake waters in Europe, the potential influence microplastics have on food web organisms in the water column that might be accidental microplastic consumers, and to identify areas that most urgently require research. It is hypothesized that:

– European lakes are polluted with microplastics, regardless of where they are located;
– fibers are a common form of microplastic that occurs in lakes;
– consumers in the pelagic food web might ingest microplastics.

Materials and Methods

In this review, microplastics are particles smaller than 5 mm without a lower size limit. The search for the articles required to review the state of knowledge was done in December 2021 (first two weeks) and January 2022 (twice weekly throughout the month) by searching ISI Web of Knowledge and Google Scholar. The key words and word combinations used in the search included microplastic(s), plastic, lake(s), fresh water, Europe, zooplankton, and fish. Of the records obtained, all those that were thematically relevant were used in the review.

Results and Discussion

Microplastics in the waters of European lakes

Although there are more than a half million lakes in Europe, few of them have been studied. The microplastic concentrations in the lakes studies were varied, but the analysis of data published by different authors indicated that there is a lack of consistent study methodology that makes it very difficult to compare and interpret results. While some researchers collected samples with manta nets, which only collect samples from the surface water layer, some used plankton nets, and still others used pumps to collect samples from the water column and concentrated them through sieves. Water samples were also collected with small, graded containers. The concentrations of microplastic particles were reported per unit of water surface of lakes (the surface are covered by the trawl was known) or volume of water (based on the total volume of trawled or sampled water), and depended on the gear used to collect the microplastics (Table 1). Other researchers (DUSAUCY et al., 2021) also noted methodological inconsistencies and identified an urgent need to develop common standards for microplastic sampling, sample handling, analysis, and the presentation of research results.

Varying levels of lake microplastic pollution were confirmed in southern European Alpine countries (Table 1) ranging from a mean of 0.011 items m^{-2} in Lake Zürich to 0.220 items m^{-2} in lakes Maggiore and Geneva (Grand Lac) in Switzerland (FAURE et al. 2015) and from 0.004 items m^{-2} in Lake Garda to 0.057 items m^{-2} in Lake Iseo in Italy (SIGHICELLI et al. 2018).

Table 1

Microplastics concentrations in waters of European lakes

Lakes	Microplastic concentrations	Size of analyzed microparticles [µm]	Sampling method	Dominant types of microplastics (shape; polymer type)
Lake Kallavesi, (Finland)	a) 0.27±0.18 (mean ± SD) items m^{-3}	a) > 333	a) surface waters (manta trawl)	fibers, fragments; polyethylene, polypropylene,
	b) 1.8±2.3 (mean ± SD) items m^{-3}	b) > 300	b) surface waters (pump)	polyethylene, terephthalate
	c) 12±17 (mean ± SD) items m^{-3}	c) 100–300	c) surface waters (pump)	
	d) 155±73 (mean ± SD) items m^{-3}	d) 20–100	d) surface waters (pump)	
				UURASJÄRVI et al. (2020)

cont. table 1

67 European lakes (30° of latitude)	0–7.3 (median = 0.28) items m^{-3}	> 310	surface waters (plankton net)	fibers, TANENTZAP et al. (2021)
Lake Tollense (Germany)	19–22 items m^{-3} 29–50 items m^{-3}	> 63	water column 0–10 m (pump)	fibers irregular shape; mainly polyethylene, polypropylene, polyethylene, terephthalate, polyamide TAMMINGA and FISCHER (2020)
Lake Sassolo (Switzerland)	$2.6 \cdot 10^3$ items m^{-3} (excluding fibers) $2.6 \cdot 10^3$ items m^{-3} (excluding fibers)	> 125 > 125	surface waters (sampling by means of a jar) the outlet of the lake (sampling by means of a jar)	pellets, fragments, films; mainly polyethylene, polypropylene pellets, fragments, films; mainly polyethylene, polypropylene NEGRETE VELASCO et al. (2020)
Lake Bolsena (Italy) Lake Chiusi (Italy)	0.21-4.08 items m^{-3} 0.48–2.82 items m^{-3}	> 300	surface waters (manta trawl)	fragments, fibers FISCHER et al. (2016)
Lake Dzieka-nowskie (Poland) Lake Kalwa (Poland) Lake Majcz (Poland)	4.7 µg m^{-2} 4.4 µg m^{-2} 4.1 µg m^{-2}	> 20	surface waters (plankton net trawl)	fibers; mainly polyethylene terephthalate, polyurethane KALISZEWICZ et al. (2020)

cont. table 1

Lake Maggiore (Italy)	0.029±0.017–0.045 ± 0.013 (mean ± SD) items m^{-2}	> 300	surface waters (manta trawl)	fragments, balls, filaments; mainly polyethylene, polypropylene, expanded polystyrene
Lake Garda (Italy)	0.004 ± 0.0027 – 0.055 ± 0.029 (mean ± SD) items m^{-2}			fragments, balls, filaments; mainly polyethylene, polypropylene
Lake Iseo (Italy)	0.015 ± 0.011 – 0.057 ± 0.036 (mean ± SD) items m^{-2}			fragments, balls, filaments; mainly polyethylene, expanded polystyrene
				SIGHICELLI et al. (2018)
Lake Geneva (Grand Lac, Switzerland)	0.220 ± 0.160 (mean ± SD) items m^{-2}	> 300	surface waters (manta trawl)	fragments, foams, films, fibers; mainly polyethylene, polypropylene, polystyrene
Lake Geneva (Petit Lac, Switzerland)	0.033 ± 0.046 (mean ± SD) items m^{-2}			
Lake Constance, (Switzerland)	0.061 ± 0.012 (mean ± SD) items m^{-2}			
Lake Neuchâtel, (Switzerland)	0.061 ± 0.024 (mean ± SD) items m^{-2}			
Lake Maggiore (Switzerland)	0.220 ± 0.150 (mean ± SD) items m^{-2}			
Lake Zurich (Switzerland)	0.011 ± 0.002 (mean ± SD) items m^{-2}			
Lake Brienz (Switzerland)	0.036 ± 0.023 (mean ± SD) items m^{-2}			
				FAURE et al. (2015)
Lake Geneva, (Switzerland)	0.048 items m^{-2}	> 300	surface waters (manta trawl)	different; only rough data are available
				FAURE et al. (2012)

Samples were collected from these lakes with manta nets with a mesh size of 300 μm. Most of these lakes are in significantly urbanized catchments and are at high risk for microplastic pollution. In turn, FISCHER et al. (2016) determined the levels of microplastic pollution per cubic meter of water in the Italian lakes Bolsena and Chiusi that ranged from 0.21 to 4.08 items m^{-3} and from 0.48 to 2.82 items m^{-3}, respectively. Much higher microplastic concentrations of 2.6 10^3 items m^{-3} were confirmed in Lake Sassolo, in Switzerland (NEGRETE VELASCO et al. 2020). In contrast with the other Italian and Swiss lakes described above, Lake Sassolo is an isolated, alpine lake that is practically free of anthropogenic pressure. This suggests that the primary source of pollution could be the atmosphere, i.e., dust, aerosols, wet and dry atmospheric deposition, and snow. Synthetic fibers transported atmospherically are one of the most important sources of pollution in isolated areas (EVANGELIOU et al. 2020). Methodological differences must be borne in mind when interpreting the mean concentrations of microplastics in Lake Sassolo since the water samples were concentrated with sieves of a mesh size twice as small (125 μm) as those used in the study by FISCHER et al. (2016) – Table 1; thus, the results obtained for Lake Sassolo might have been partially the result of the greater selectivity of the microplastic sampling gear. Conversely, the results of certain studies suggest that lakes are dominated by a finer fraction of microplastics. This is illustrated well by UURASJÄRVI et al. (2020) who conducted research in Lake Kallavesi in Finland. When they concentrated water samples using a mesh of > 300 μm, the microplastic concentration was at a moderate value of 1.8 items m^{-3}, but when they used mesh with a selectivity range of 20–100 μm, the mean value was 155 items m^{-3}. This could indicate that microplastics that reach lakes degrade into increasingly smaller particles that accumulate. UURASJÄRVI et al. (2020) concluded that the risk of microplastic accumulation in Lake Kallavesi is highly likely since its surroundings are significantly urbanized.

The data available on the level of microplastic pollution in European lakes is supplemented by those from Central and Eastern Europe. KALISZEWICZ et al. (2020) studied Lake Dziekanowskie in central Poland and lakes Kalwa and Majcz in the northeast of the country with the assumption that they were exposed to different degrees or anthropogenic pressure. The first two lakes are in urban areas, while Lake Majcz is located far from human settlements. The levels of pollution in the three lakes were, nevertheless, similar and ranged from 4.1 μg m^{-2} in Lake Majcz to 4.7 μg m^{-2} in Lake Dziekanowskie. In turn, TAMMINGA and FISCHER (2020) determined the pollution level of Lake Tollense in Germany at 19–22 items m^{-3} for fibers and 29–50 items m^{-3} for other microplastics (Table 1).

The newest data from TANENTZAP et al. (2021) make an important contribution to research on the problem of the microplastic pollution of European lakes. These researchers studied 67 lakes in which microplastic particle concentrations ranged from 0 to 7.3 (median = 0.28) items m^{-3} (Table 1). The vast majority of the particles examined were of anthropogenic origin. The main research question in this study did not address monitoring pollution, but focused on the factors determining the levels of pollution, and this is the first study of this kind. The model showed that lakes in urban catchments are more exposed to microplastic accumulation since this is where there is high potential for microplastic emissions, high population density, and high waste production and wastewater discharge. In turn, lakes in forested catchments are less exposed to microplastic accumulation as are those with high biological degradation rates thanks to resident microbial communities that facilitate the degradation of inflowing pollution.

Attention should be drawn here to a fundamental gap in the existing data. In the vast majority of studies, water was sampled from lakes with nets of a mesh size of approximately 300 μm (Table 1), which means that the current state of knowledge is limited to the larger microplastic fraction. Studies conducted to date have omitted smaller particles that could be of particular significance in the functioning of inland aquatic ecosystems. Smaller-sized microplastic particles can, for example, be ingested by planktonic organisms, and this risk is greater the higher the concentrations of them are in waters. DUSAUCY et al. (2021) reached similar conclusions in their review of microplastic pollution of lakes worldwide.

In qualitative terms, the decided majority of microplastics in the lakes studied were fibers, mainly polyethylene or polypropylene. The primary source of surface water plastic fiber pollution is discharged wastewaters and precipitation. The latter factor could be responsible for polluting lakes that are distant from human settlements and not under direct anthropogenic pressure. The widespread detection of plastic microfibers in European lakes is also the result of the sampling methods researchers apply. In the decided majority of the studies cited in this review, the sampling methodology was based on collecting samples from the water surface layers of the lakes studied (Table 1). This means that the only synthetic polymers isolated from the environment were those that had a specific weight lower [<1 g cm^{-3}] than that of water (HIDALGO-RUZ et al. 2012), and that, consequently, floated on surface waters. This includes polyethylene and polypropylene polymers, which dominated the samples analyzed. Thus, these studies could have omitted synthetic polymer particles the specific weights of which were higher (>1 g cm^{-3}) than that of water (HIDALGO-RUZ et al.

2012), and could therefore be located at greater depths. This should be considered when interpreting the study results published in the literature, and this is why there is an urgent need to supplement existing studies with assessments of microplastic concentrations in the entire water column from the surface to the bottom.

Generally, the pollution levels in European lakes are similar to those in lakes in other parts of the world (Table 2). The average microplastic concentrations reported in the literature for lakes located outside of Europe range from 0.017 items m^{-2} in Lake Michigan (MASON et al. 2016) to 0.19 items m^{-2} in Lake Winnipeg (ANDERSON et al. 2017), while for the smaller fraction of microplastics (> 110 μm) it was 8.5 items m^{-2} in the Three Gorges Reservoir (ZHANG et al. 2015). The amount of pollution reported per cubic meter of water ranged from a mean of 0.9 items in lakes in the Pampean Region (ALFONSO et al. 2020) to $11 \cdot 10^3$ items in lakes from the West Siberian Plain (MALYGINA et al. 2021), while in this last study very high values were confirmed for nanoplastics, that are a very small size fraction ranging from 1 to 350 nm.

Table 2

Microplastic concentrations in lake water in selected locations worldwide

Study area	Mean concentration and analyzed particle size	Reference
America		
Laurentian Great Lakes (United States/Canada)	0.0425 items m^{-2} (> 330 μm)	ERIKSEN et al. (2013)
Lake Michigan (United States)	0.0173 items m^{-2} (> 330 μm)	MASON et al. (2016)
Lake Winnipeg (Canada)	0.1934 items m^{-2} (> 330 μm)	ANDERSON et al. (2017)
Nine lakes in the Pampean Region (Argentina)	0.9 ± 0.6 items m^{-3} (> 330 μm to 1 mm)	ALFONSO et al. (2020)
Asia		
Lake Hovsgol (Mongolia)	0.0203 items m^{-2} (> 330 μm)	FREE et al. (2014)
Three Gorges Reservoir (China)	8.4656 items m^{-2} (> 110 μm)	ZHANG et al. (2015)
Qinghai Lake (China)	0.1809 items m^{-2} (> 110 μm)	XIONG et al. (2018)
Six Lakes from West Siberian Plain (Russia)	$11 \cdot 10^3$ items m^{-3} (1–350nm)	MALYGINA et al. (2021)

Pollution concentrations in lakes, whether they are located in Europe or other places, are usually lower than those in seas and oceans (Table 3).

Table 3

Microplastic concentrations (examples) in marine water in different locations worldwide

Study area	Microplastic concentration	Reference
North Pacific Gyre	0.334 items m^{-2}	MOORE et al. (2001)
Northwestern Mediterranean Basin	0.116 items m^{-2}	COLLINGTON et al. (2012)
East Asian seas	1.7 items m^{-2}	ISOBE et al. (2015)
Pacific Ocean (California coast)	approx. 8 items m^{-3}	MOORE et al. (2002)
Different localities	0–8 (700 items m^{-3})	HIDALGO-RUZ et al. (2012)
Northeastern Pacific Ocean and coastal British Columbia	8–9 (810 items m^{-3}) (mean: 2 080)	DESFORGES et al. 2015
Nearshore and offshore Goeje Island, southern Korea	195 $\cdot 10^3$ items m^{-3} (mean)	SONG et al. (2015)
Southern Ocean	0.003–0.09 items m^{-3}	ISOBE et al. (2017)
Guanabara bay, Rio de Janeiro, Brazil	1.40–21.3 items m^{-3}	GLAUCIA et al. (2019)

Examples of mean values describing the level of microplastic pollution in the marine environment reported in the literature ranged from approximately 0.2 items m^{-2} in the Mediterranean Sea (COLLINGTON et al. 2012) to nearly 2.0 items m^{-2} in the area of the East Asian seas (ISOBE et al. 2015). In the North Pacific Gyre, within which drifts the great Pacific garbage patch, microplastic pollution reached a level of 0.334 items m^{-2} (MOORE et al. 2001). Results per cubic meter of water differed significantly in various locations; relatively low microplastic concentrations (0.003–0.09 items m^{-3}) were reported in the Southern Ocean near Antarctica (ISOBE et al. 2017), that were even lower than those of many lakes. In most cases, pollution in seas and oceans was higher than that of lakes and ranged from several to several tens of items m^{-3} (MOOR et al. 2002, OLIVATTO et al. 2019) to as much as several tens of thousands of items m^{-3} (SONG et al. 2015). As with the results of studies on lakes, care should be taken when comparing the results of various researchers because of differences in the methodologies applied in studies. Microplastic concentrations in seas and oceans are, however, higher than those in lakes since they are the final recipients of terrestrial pollutants, and deep marine waters are considered to be major sinks for microplastic debris (WOODALL et al. 2014).

Potential impact of microplastic accumulation in lake waters on food webs

Since microplastics have different weights, they occur at different depths in lake waters. Even polyethylene and polypropylene fibers, which are common in lakes, floating on the surface can become covered with plastic spheres, which are diverse microbial communities of heterotrophs, autotrophs, predators, and symbionts (ZETTLER et al. 2013). This increases the weight of particles and displaces them to deeper water layers. The different depths at which microplastic particles pollute the water column means that they can threaten a wide spectrum of living organisms. The heaviest microplastic particles sediment, which can eliminate them temporarily or permanently from the water column.

The current state of knowledge on microplastic concentrations in lake waters indicates that the quantity of microplastics in the water column increases substantially the smaller the particles analyzed are. This means that limnic environments are dominated by microplastic particles that, because of their small sizes, can directly threaten organisms of smaller body sizes, which are mainly aquatic invertebrates. In the water column, invertebrate zooplankton form assemblages with a wide spectrum of species that includes many filter feeders. Planktonic organisms are the primary consumer link in the food chain, and by the passive ingestion of microplastics suspended in the water, they can include them into the food web. While the literature lacks data that confirm freshwater species ingest microplastics in their natural environments, some studies document freshwater zooplankton species ingesting microplastics under laboratory conditions. In studies of the influence of microplastics on freshwater zooplankton, selected species were exposed to microplastic particles under laboratory conditions. IMHOFF et al. (2013) exposed the freshwater cladoceran *Daphnia magna* to microplastics (polymethyl methacrylate 29.5±26 μm) and reported that the plastic was present in the digestive tracts of all specimens exposed, which indicates there is a risk of bioaccumulation in the food web. JEMEC et al. (2016) documented *D. magna* ingesting microplastic fibers from a commercial polyethylene terephthalate (PET) fleece textile consisting of fibers 20 μm thick. Most of the fibers *D. magna* ingested were approximately 300 μm long, but very large, twisted synthetic polymer fibers of approximately 1,400 μm were found in the intestines of some specimens. Microplastic fiber ingestion caused higher mortality among *D. magna*.

However, fluorescent plastic granules have been used in many studies of the ecology of freshwater zooplankton to determine, inter alia, the filtration rates of different zooplankton species. These experiments are indirect

proof that spherical microplastics ranging in size from several to several tens of micrometers are ingested widely by various species of freshwater zooplankton mainly *Cladocera* and *Rotifera*, while some *Copepoda* avoided plastic microgranules (e.g., AGASILD and NÕGES 2005). Considering the possible selectivity of different species regarding varied microplastic forms and shapes in the environment, environmental studies must be continued. This is especially true given the inherent weakness in laboratory studies that prevents results from being extrapolated to natural conditions, which is that the microplastic concentrations to which zooplankton were exposed in the studies above exceeded the concentrations of synthetic polymers noted in lakes under natural conditions.

Some information on possible freshwater zooplankton ingestion of microplastics under natural conditions and the consequences of this is provided by studies of the marine environment, which are more advanced on this topic. Many studies have proved that under natural conditions various zooplankton species ingest microplastics that pollute marine waters (BOTTERELL et al. 2019). Zooplankton microplastic ingestion results in shorter or longer retentions times in organisms. Some particles are excreted without causing harm to organisms; however, even after excretion these feces tend to stick to carapaces meaning that organisms remain sources of microplastic contamination for potential consumers (COLE et al. 2013). Ingesting microplastic was fatal in some specimens, while others survived but ingested microplastics were retained in digestive tracts (BOTTERELL et al. 2019). Microplastic retention in the zooplankton trophic level could cause these pollutants to be incorporated into aquatic ecosystem food webs. Fish foraging on zooplankton are exposed to the indirect ingestion of synthetic polymer particles. DESFORGES et al. (2015) presented interesting results of their assessment of the microplastic ingestion of various zooplankton species in the marine environment concluding that synthetic polymers were present in 1 item per 34 copepods and 1 item per 17 euphausiids. Further, they estimated that zooplankton containing microplastics would lead to juvenile salmon and returning adult individuals in coastal British Columbia ingesting 2–7 microplastic items per day and approximately 90 microplastic particles per day, respectively. Thus, seemingly low zooplankton microplastic ingestion can cause significant accumulation of pollutants in subsequent trophic levels of the food web.

Ample evidence in the literature suggests that fishes ingest microplastics. CERA and SCALICI (2021) summarized the state of knowledge to date on this topic and reported that microplastics were confirmed in 135 freshwater ichthyofauna species and that microplastic contamination was also confirmed in fishes from lakes in Europe. KUŚMIEREK and POPIOŁEK (2020)

confirmed microplastics in the digestive tracts of approximately half of 400 common roach and gudgeon specimens caught upstream and downstream from Lake Michalice dam reservoir in southern Poland. ROCH et al. (2019) examined 22 fish species from lakes and rivers in southern Germany and reported that approximately a fifth of specimens examined had ingested microplastics. In turn, microplastics were confirmed in the digestive tracts of 7.5% of fishes examined from Lake Geneva (FAURE et al. 2015). These studies suggest that the degree of microplastic pollution in inland waters might determine the amount of pollution ichthyofauna ingest. PETERS and BRATTON (2016) confirmed that the microplastic content in the natural environment was positively correlated with fish ingesting it. In addition to the availability of microplastics in the environment, other factors can influence the quantities in which ichthyofauna ingest them including foraging strategies, trophic transfer (consumption of prey contaminated by microplastics), energy requirements, and individual specimen sizes. For example, larger roach were more likely to ingest the maximum possible number of available microplastic particles than were smaller fish (HORTON et al. 2018).

The effects of microplastic ingestion by fishes are varied. Negative effects of microplastic ingestion that have been observed in fishes include, inter alia, changes in swimming behavior (QIANG and CHENG 2019), limited growth and survival (NAIDOO and GLASSOM 2019), oxidative stress and changes in blood biochemistry (HATAMI et al. 2019), and disadvantageous endocrinological effects and reproductive disturbances (ROCHMAN et al. 2014), while nanoplastics, the smallest fraction, can cross the blood-brain barrier, which is highly selective in vertebrates, causing brain damage and behavioral changes (MATTSSON et al. 2017).

Microplastic migration through trophic levels of the food web could indirectly threaten humans through their diets. Not only could fish species sold commercially be contaminated (SANTILLO et al. 2017), so too could fish meal used in the manufacture of fodder for aquaculture and animal husbandry (THIELE et al. 2020). Inland waters are also often reservoirs of potable water and their contamination with microplastics should also be cause for concern with regard to human health (KOELMANS et al. 2019). Currently, according to the FAO (LUSHER et al. 2017b), the current state of the knowledge on microplastic particle toxicity indicates, that the risks associated with the consumption of fisheries and aquaculture products contaminated with microplastics is negligible in comparison with the benefits of consuming them. In their report, however, the FAO underscored the necessity of continuing research on polymer toxicity and that preventive and mitigation strategies must be implemented in the management of environmental plastic pollution.

Conclusions

Microplastics were recorded in all the European lakes that were investigated. However, considering the numerous lakes in Europe, their vast diversity, and the population density of the continent, the breadth of knowledge of European lake pollution remains insufficient. First, coherent research methodology must be developed that will foster comparing research results. Additionally, despite the growing number of studies of microplastics in European lakes, there is still insufficient information regarding the size fraction smaller than 300 μm, which is particularly important since the quantities of microplastics in water increase significantly as the size of these particles decreases. It is also necessary to supplement the state of the knowledge to date on microplastic concentrations in deeper water layers, because most studies focused only on surface water layers. Further, there are almost no multi-dimensional models describing the primary factors that are responsible for the accumulation of microplastics in lakes. To date, hydrological conditions, lake mixing regimes, and many other factors that could potentially affect microplastic retention levels in the water column have yet to be investigated. Information is also lacking on natural populations of zooplankton potentially ingesting microplastics and the effects of this, which is particularly important when these planktonic organisms are the foundation of the food web since any factor that poses a risk to this trophic level also threatens the functioning of entire lake ecosystems. Trophic transfer of microplastics in lake food webs is highly likely since microplastics were confirmed in wide range of freshwater ichthyofauna.

Considering the fact that only 85 lakes of over 500,000 European lakes (> 0.01 km^2) were investigated, further studies are necessary. Expanding research on microplastics in lakes will permit fully assessing the degree of environmental threat the presence of microplastics in lake waters poses to both the organisms inhabiting them and to humans. Based on the current state of the science, the following must be done:

– develop a consistent methodology for microplastic collection and sample analysis;

– increase the range of lakes monitored for microplastic pollution;

– assess microplastic concentrations in various layers of the water column from the surface to the bottom;

– increase research on the fine fraction of microplastics (< 300 um);

– identify and estimate microplastic consumption rates of natural populations of aquatic organisms and assess the risk of microplastic accumulation in food webs.

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DIVERSITY OF INDUSTRIALLY IMPORTANT HYDROLYTIC ENZYMES EXPLORED IN BACTERIA FROM AQUATIC ENVIRONMENT

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Key words: Arabian Sea, River Ravi, *Bacillus* sp., extracellular hydrolytic enzymes.

Abstract

Aquatic bacteria are famous for the production of bioactive metabolites and commercially important extracellular enzymes. Water samples of Arabian Sea (Karachi) and Ravi River (Lahore), Pakistan were analyzed for hydrolytic enzymes producing bacteria. A total of 23 bacterial strains were isolated and their *α*-amylase, DNase, gelatinase, *L*-glutaminase, pectinase, lecithinase and protease production potential was estimated. Following screening, 11 strains (48%) showed amylase activity whereas DNase and gelatinase was produced by 5 strains (22%) and 18 strains (78%), respectively. *L*-glutaminase and pectinase production was shown by 17 isolates (74%) while lecithinase and protease activity was only exhibited by 2 (13%) and 1 strain (4%), respectively. Ribotyping of five selected (S5, S6, H4, H5 and R8) isolates revealed their similarity to *B. tequilensis, B. pumilus, B. flexus*, *B. sonorensis* and *B. subtilis*. These isolates also showed multiple heavy metal and antibiotic resistance. These indigenous *Bacillus* spp. possess great potential to be utilized as commercial strains provided with optimum growth conditions. Extracellular enzymes of these bacterial strains can be used in different industries, agriculture and bioremediation purposes to replace synthetic components.

Introduction

Enzymes are biological catalysts that control the cellular metabolism of all organisms. Different types of enzymes can be obtained from plants, animals, algae, fungi and bacteria. They may be extracellular (exoenzymes) or intracellular (endoenzymes). Extracellular enzymes are secreted

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outside the living cell and act on the complex compounds and break them into simpler ones that are then absorbed by cell. Organisms obtain energy as well as play a major role in the cycling of organic matter with the help of extracellular enzymes. While, intracellular enzymes act inside the cell and carry out the essential biochemical reactions in the cell by forming new compounds or through assimilation.

Bacteria are considered as source for the isolation of both intracellular and extracellular enzymes. Commercially important extracellular enzymes produced by bacteria are amylases, gelatinases, proteases, pectinases, glutaminases, lipaseses, DNases, cellulases, coagulases, kinases, hemolysins, pullulanases, xylanases etc. They have various applications in different fields as additives, food and chemical industries and in biomedical research (PANDEY et al. 2000). Industrial processes are carried out under specific conditions which may not be feasible for enzymes' function. So, the enzymes that are capable of working at different conditions as temperature and pH values are of great importance for industries. Aquatic bacteria are source of such enzymes as they are able to survive under high pressure and salt conditions (DALMASO et al. 2015).

Aquatic environment has novel microorganisms which can produce a variety of viable secondary metabolites. Bacteria specifically *Bacillus* spp. have gained particular interest because of their crucial role in matter cycling in aquatic environments (TALLUR et al. 2016). Marine bacteria are more prominent due to production of thermo-stable extracellular enzymes and bioactive compounds while, less is known about freshwater bacteria (DEVI et al. 2008). It is important to explore the potential of aquatic bacteria to produce metabolic products and their particular role in the ecosystem.

Extracellular hydrolytic enzymes have a lot of applications in food and chemical industry and in biomedical sciences (SÁNCHEZ-PORRO et al. 2003). Aquatic extracellular enzymes may be same as their terrestrial counterparts or have completely distinctive properties (DEBASHISH et al. 2005).

Bacillus are ubiquitous in aquatic and terrestrial environments and consist of phylogenetically and phenotypically diverse species (BAL et al. 2009). Mostly extracellular hydrolytic enzymes producing bacterial species belong to the genus *Bacillus*. There are many reports on marine and freshwater bacterial diversity from different coastal areas of the world (NAWAZ and AHMED 2011). Mostly *Bacillus* systematic studies have focused on the terrestrial species, although marine *Bacilli* also have great potential for antibiotics, cyclic acylpeptides, and glucanases (OGUNTOYINBO 2007).

Materials and Methods

Sampling

Marine sample was collected from Arabian Sea, Karachi (24°51′36″N and 67°00′36″E) in June, 2017 while, fresh water was from Ravi River, Lahore (31°15′–31°45′ N and 74°01′–74°39′ E) in August, 2017 in sterilized screw capped bottles. Characteristics i.e., color, odor, temperature and pH of samples were recorded.

Isolation and purification of bacterial strains

To isolate bacterial strains, samples were serially diluted and spread on Nutrient-agar plates and incubated overnight at 37ºC. Total 23 morphologically different bacterial isolates were selected and further purified by quadrant streaking on Nutrient-agar (CAPPUCCINO and SHERMAN 2008).

Screening for extracellular hydrolytic enzyme production

Selected bacterial strains were screened for different extracellular hydrolytic enzymes as amylase, cellulase, DNase, gelatinase, *L*-glutaminse, lecithinase, pectinase, protease and tannase. Amylase activity was checked on starch agar and zones of clearance were observed by starch hydrolysis (SHANMUGASUNDARAM et al. 2015). Cellulase activity was determined on cellulose agar containing Congo-red as indicator (PATAGUNDI et al. 2014). Extracellular DNase activity was observed on DNase agar supplemented with methyl green as pH indicator (SMITH et al. 1969). Extracellular lecithinase production was determined on egg-yolk agar (SHARAFetal. 2014). Gelatinase production was checked in deep gelatin tubes supplemented with 12% gelatin (GERHARDT et al. 1994). *L*-glutaminase production was screened on Zobell's agar media containing *L*-glutamine as enzyme substrate and phenol red as pH indicator (SINHA and NIGAM 2016). The YEP medium supplemented with 2% agar was used to screen pectinase production and visualized zones of clearance through iodine flooding (SOARES et al. 1999). Protease activity was determined on milk Agar (ALNAHDI 2012). The tannase activity was tested on nutrient agar plates containing 2% tannic acid (KUMAR et al. 2010).

Morphological and biochemical characterization

Five bacterial isolates (S5, S6, H4, H5, R8) selected on the basis of maximum production of extracellular enzymes, were characterized morphologically and biochemically (CAPPUCCINO and SHERMAN 2008, HOLT et al. 1989).

Minimum inhibitory concentration (MIC)

MIC of different heavy metals (chromium-Cr, cobalt-Co, lead-Pb, nickel-Ni and zinc-Zn) and antibiotics (ampicillin-AMP, chloramphenicol-CHL, penicillin-PEN, tetracycline-TET and streptomycin-STP) was determined on Nutrient-agar media by progressively increasing the concentration of selected heavy metals and antibiotics by 50 µg/ml each time, until the bacterial strains were unable to give visible growth (SAMBROOK and RUSSELL 2001).

Growth kinetics

For growth kinetics, bacterial isolates were inoculated in Nutrient-broth (pH-7) and incubated at 37°C in shaking incubator (150 rpm) for 3 days. Samples were drawn aseptically and optical density (600 nm) was measured after every 3-hour interval (ZWIETERING et al. 1990).

Effect of different environmental conditions on bacterial growth

Impact of different temperatures (28°C, 37°C and 50°C) and pH (5, 7 and 9) on the growth of extracellular enzymes producing bacterial strains was determined. Effect of different carbon (fructose, glucose, galactose, maltose and sucrose) and nitrogen (ammonium chloride, beef extract, glycine, KNO_3 and peptone) sources on bacterial growth was also checked. For this purpose, bacterial cultures were inoculated in the Mineral salt medium (SHIRANE and HATTA 1987) and incubated at optimized conditions. Samples were drawn out aseptically after every three hours to measure optical density at 600 nm.

Exopolysaccharide (EPS) production

To determine EPS production, 100 ml Nutrient-broth was separately inoculated with respective bacterial strain and incubation was done at 37°C up to 48–72 hours at 150 rpm. Then, cultures were centrifuged and EPS production was determined by cold ethanol precipitation method (SHAHNAVAZ et al. 2015).

Slime production assay

To check slime production ability of *Bacillus* strains, they were streaked on Congo-red agar media and incubated at 37°C for 24 hours. Appearance of black colonies showed slime production (AN and FRIEDMAN, 1997).

Qualitative assay for biofilm formation

Biofilm formation was determined by staining method. Inoculum was given in Nutrient-broth and incubated for 24-hours at 37°C. Media in the test tubes was removed out and stained them with 0.01% crystal violet and let it stand for 20 minutes. Extra stain was washed and dried the test tubes. Biofilm formation was indicated by appearance of purple ring at bottom and walls of tubes (HASSAN et al. 2011).

16S rRNA gene sequencing of selected *Bacillus* strains

Five selected pure bacterial strains were sent to 1st Base Company and identified by 16S rRNA sequencing using Sanger dideoxy method. Thus, unassembled Chromas files were obtained and sequences were manually refined by cross checking them with raw data and assembled by Cap3. Then, refined sequences were analyzed at the BLASTn site (http://www.ncbi.nlm.nih.gov/BLAST, accessed on May 2018) and identified on the basis of maximum similarity index. The assembled sequences were submitted to NCBI GenBank to get accession numbers.

Results

Sample characteristics

Marine water was of grey color, odorless and temperature of sampling site was 28°C. Whereas, fresh water sample was of brown color, had muddy smell and temperature of sampling site was 25°C. The pH of both samples was 7.

Isolation and purification

Total 23 bacterial strains were isolated from marine (S1, S2, S3, S4, S5, S6, S7, S8, W1, W2, W3) and fresh water (H1, H2, H4, H5, H6, H7, H8, R1, R3, R4, R7, R8) samples.

Screening for extracellular hydrolytic enzyme production

Isolated bacterial strains showed positive results for extracellular production of various hydrolytic enzymes such as amylase, DNase, gelatinase, *L*-glutaminase, lecithinase, pectinase and protease as shown in Table 1 and Figure 1. Five bacterial strains (S5, S6, H4, H5, R8) were selected due to most diverse extracellular enzyme activities detected by mentioned enzyme assays.

Table 1

Extracellular enzyme producing activities of isolated bacterial strains

Sr. no	Bacterial strains	Amylase	Cellulose	DNase	Gelatinase	*L*-glutaminase	Lecithinase	Pectinase	Protease	Tannase
1	S1	+	–	–	+	+	–	–	–	–
2	S2	+	–	+	+	+	–	–	–	–
3	S3	–	–	+	+	+	–	–	–	–
4	S4	+	–	+	+	+	–	–	–	–
5	S5	+	–	–	+	+	–	+	–	–
6	S6	+	–	+	+	+	+	+	–	–
7	S7	–	–	–	+	–	–	+	–	–
8	S8	–	–	–	+	–	–	+	–	–
9	W1	–	–	–	–	+	–	+	–	–
10	W2	–	–	–	+	+	–	+	–	–
11	W3	–	–	–	–	+	–	+	+	–
12	H1	–	–	–	+	–	–	+	–	–
13	H2	–	–	+	+	–	+	+	–	–
14	H4	+	–	–	+	+	–	+	–	–
15	H5	+	–	–	+	+	–	+	–	–
16	H6	+	–	–	–	–	–	–	–	–
17	H7	–	–	–	–	+	–	+	–	–

cont. Table 1

18	H8	–	–	–	–	–	–	+	–	–
19	R1	+	–	–	+	+	–	+	–	–
20	R3	–	–	–	+	+	–	+	–	–
21	R4	–	–	–	+	+	–	–	–	–
22	R7	+	–	–	+	+	–	+	–	–
23	R8	+	–	+	+	+	–	+	–	–

+ = positive; – = negative

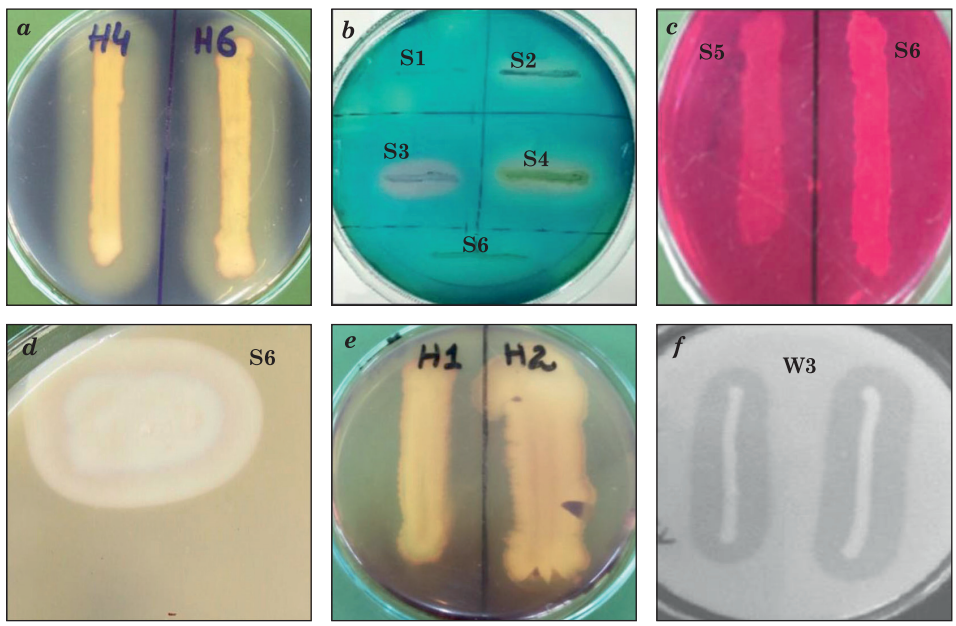

Fig. 1. Positive results of extracellular hydrolytic enzymes production by bacterial strains *a* – amylase activity; *b* – DNAse activity; *c* – *L*-glutaminase activity; *d* – lecithinase activity; *e* – pectinase activity; *f* – protease activity

Morphological and biochemical characterization

Selected bacterial strains were characterized morphologically and biochemically as shown in Table 2.

Table 2

Cultural, morphological and biochemical characteristics of hydrolytic enzyme producing bacterial strains

Characteristics	S5	S6	H4	H5	R8
	cultural and morphological characteristics				
Colony morphology	moderate, white, circular, entire, flat, transparent	small, yellow, circular, entire, convex, opaque	small, yellow, circular, entire, flat, opaque	large, white, irregular, undulate, convex, opaque	large, white, circular, entire, convex, translucent
Vegetative cells	rod	rod	rod	rod	rod
Motility	motile	motile	motile	motile	motile
Gram reaction	+	+	+	+	+
Endospores	+	+	+	+	+
Biochemical characteristics					
Catalase test	++	++	+	+++	+
Oxidase test	++	–	+	++	+++
Mannitol salt agar test	–	+++	+	+++	++
Oxidative/ fermentation glucose test	aerobe	aerobe	aerobe	facultative aerobe	facultative aerobe
Nitrate reduction test	+++	+++	–	++	+
Citrate utilization test	+	–	+	+	+
Voges-Proskauer (VP) test	+++	+	–	++	+++
Methyl red test	–	–	–	+	+
Pigment test	-	-	-	-	-
Novobiocin sensitivity test	+	+	+++	+	++

+ = positive; *++/ +++ = slightly positive/ strongly positive*; *– = negative*

Minimum inhibitory concentration (MIC)

Bacterial strains exhibited multiple heavy metals and antibiotics resistance. They were most resistant to Pb (200 µg/µl MIC) and less resistant to Cr, Ni, Co and Zn (100 µg/MIC) – Table 3. While, in case of tested antibiotics, maximum resistance was shown to TET (200 µg/ml MIC) and for STP, Amp, CHL, and PEN, MIC value was 100 µg/ml (Table 4).

Table 3

Minimum inhibitory concentration MIC for heavy metals shown by the hydrolytic enzyme producing bacterial strains

Bacterial strains	Heavy metals used [µg/ml]									
	chromium (Cr)		nickel (Ni)		zinc (Zn)		cobalt (Co)		lead (Pb)	
	200	100	200	100	200	100	200	100	200	100
S5	–	+	–	+	–	+	–	–	+	+
S6	–	+	–	+	–	+	–	–	+	+
H4	–	+	–	+	–	+	–	–	+	+
H5	–	+	–	+	–	+	–	–	+	+
R8	–	+	–	+	–	+	–	–	+	+

Table 4

Minimum inhibitory concentrations (MIC) for antibiotics shown by the hydrolytic enzyme producing bacterial strains

Bacterial strains	Antibiotics used [µg/ml]									
	penicillin (PEN)		chloramphenicol (CHL)		ampicillin (AMP)		streptomycin (STP)		tetracycline (TET)	
	200	100	200	100	200	100	200	100	200	100
S5	–	+	–	+	–	+	–	–	+	+
S6	–	+	–	+	–	+	–	–	+	+
H4	–	+	–	+	–	+	–	–	+	+
H5	–	+	–	+	–	+	–	–	+	+
R8	–	+	–	+	–	+	–	–	+	+

Growth kinetics

Growth kinetics showed that these aquatic bacteria grow very well under standard growth conditions as temperature 37°C and pH 7. The growth curves demonstrated longer stationary phases showing their stability that was directly related to enzyme production (Figure 2).

Effect of different environmental conditions on bacterial growth

Maximum growth was indicated at 37°C, pH 7, glucose as carbon source and peptone as nitrogen source as shown in Figures 2–6.

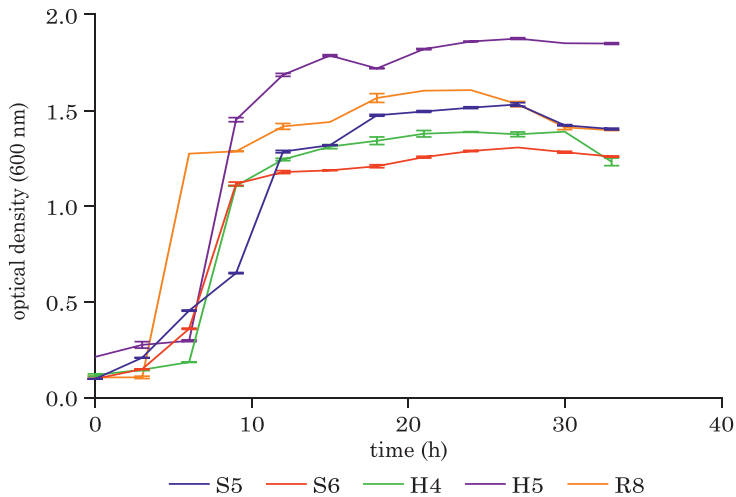

Fig. 2. Growth kinetics of bacterial strains (S5, S6, H4, H5, R8), showed longer stationary phases and stability directly related to enzyme production

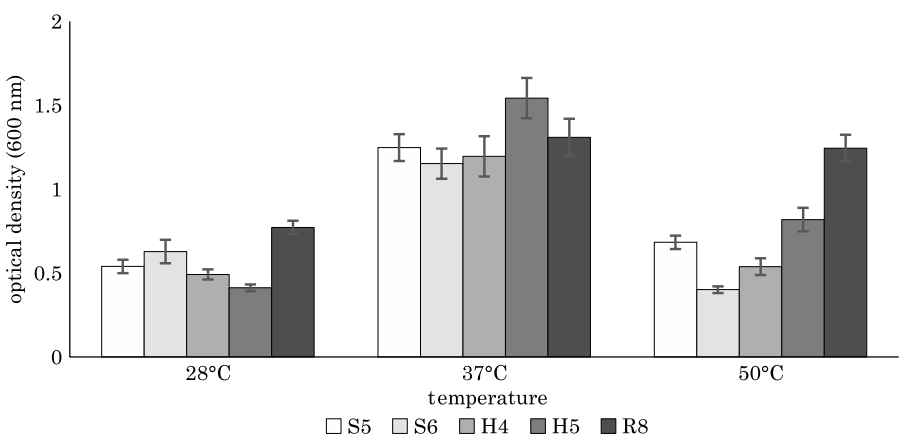

Fig. 3. Effect of variable temperatures on bacterial growth and showed best growth at temperature 37°C

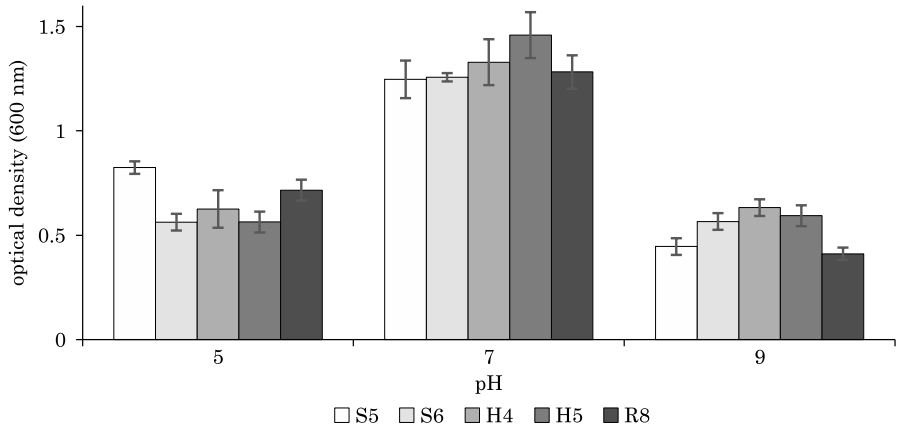

Fig. 4. Effect of variable pH on growth of bacterial strains showed pH 7 as optimum growth pH

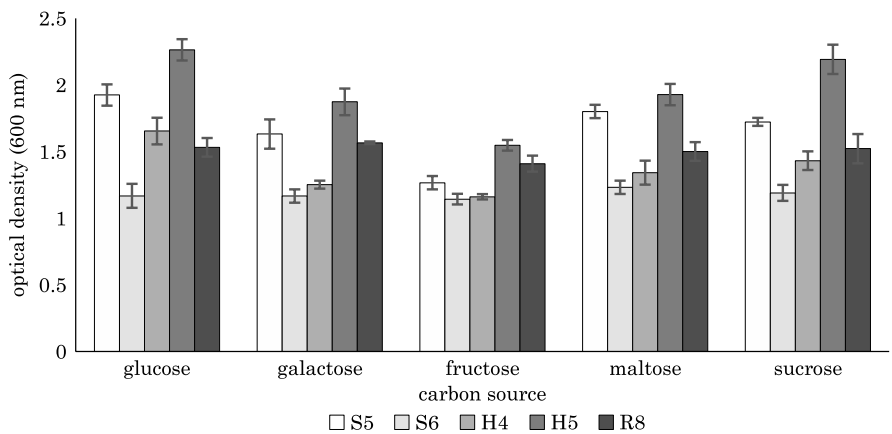

Fig. 5. Effect of different carbon sources on bacterial growth

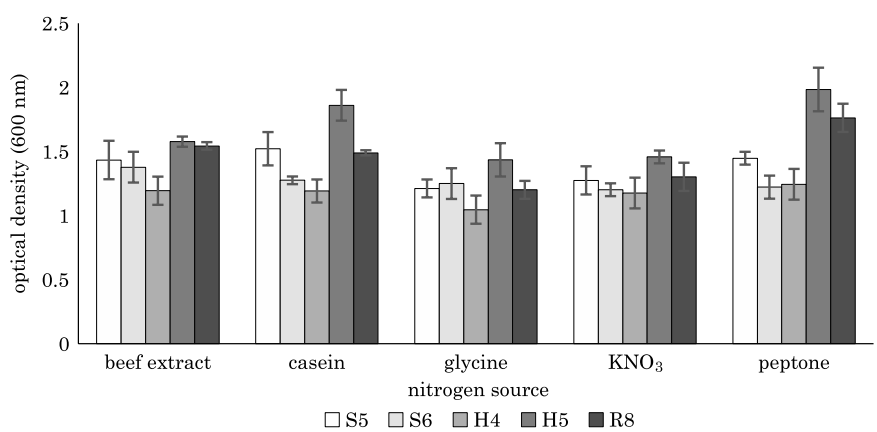

Fig. 6. Effect of different nitrogen sources on bacterial growth

EPS, slime production and biofilm formation

EPS production was demonstrated by S5 (0.08 mg/ml), S6 (0.14 mg/ml), and R8 (0.08 mg/ml). Bacterial strain S5 and S6 also showed slime production potential indicated by appearance of black colored colonies on Congo-red agar (Figure 7*a*). Biofilm formation was also estimated by purple ring formation (Figure 7*b*).

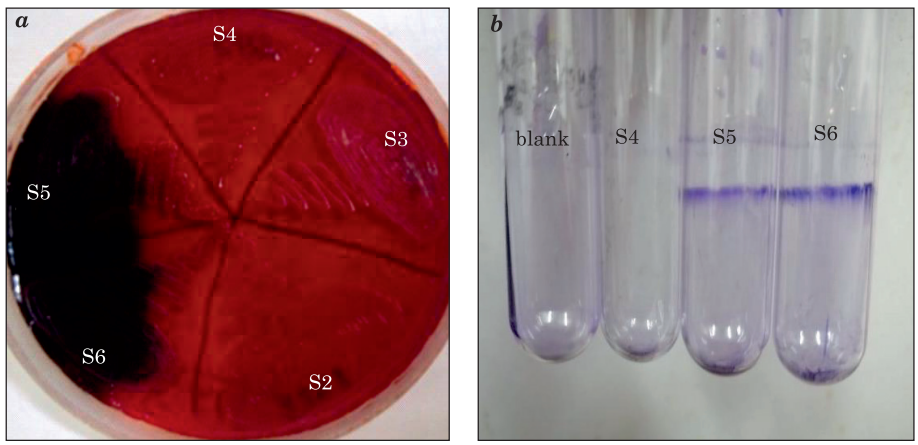

Fig. 7. Black coloration of Congo-red agar revealed slime production by S5 and S6 (*a*) and purple coloration of the walls and at bottom of the test tubes showed biofilm formation by S5 and S6 (*b*)

16S rRNA gene Sequencing

16S rRNA gene sequencing revealed similarity of S5 to *Bacillus tequilensis*, S6 to *B. pumilus*, H4 to *B. flexus*, H5 to *B. sonorensisa* and R8 to *B. subtilis*. These sequences were submitted to NCBI Genbank under the accession numbers MH371775, MH371776, MH371777, MH371778 and MH371779, respectively (Table 1.1, Appendix).

Discussion

Aquatic microbes produce highly thermo-stable extracellular enzymes making themselves different from their terrestrial counterparts with different adaptive characteristics (RAMESH and MATHIVANAN 2009). The main purpose of the current study was isolation and characterization of extracellular hydrolytic enzyme producing bacteria from aquatic environment as bacteria are reported to produce a variety of extracellular enzymes that are manipulated at industrial and commercial scale (KUMAR et al. 2013).

Isolated strains were found to produce amylase, DNAse, gelatinase, *L*-glutaminase, Lecithinase and Pectinase enzymes. Production of these enzymes has also been reported previously by many researchers. Marine and fresh water bacteria have been reported for extracellular amylase production (BAL et al. 2009, SURIBABU et al. 2015). In a past study, more than 72.4% marine isolates showed extracellular DNase activity (DANG et al. 2009). Findings of the present study are supported by several past workers as they reported that gelatinase is produced more frequently by fresh water bacteria than the isolates of soil and plant microenvironments (ALVES et al. 2014). Gelatinase production from marine *Bacillus* spp. was also described (BALAN et al. 2012). Marine and fresh water bacteria have been described for extracellular *L*-glutaminase production (KATIKALA et al. 2009, SALLIS and BURNS 1989). Lecithinase activity was determined in marine bacteria (GAUTHIER 1976). Lecithinase activity has also been reported in several *Bacillus* spp. (MASSOL-DEYA et al. 1995). Isolated *Bacillus* strains showed high potential for the production of extracellular pectinases. In a previous study, 28 bacterial strains from fresh water samples and 16 bacterial strains from sea water were isolated with pectinase activity that strengthen the outcomes of current research (ROHBAN et al. 2009, BAL et al. 2009).

Only one bacterial strain exhibited extracellular protease production whereas no strain showed tannase and cellulase activity. In accordance with this study, another group of researchers has reported that no tannase production potential was observed in the isolates of Arabian Sea whereas, cellulase production was observed in those strains (TALLUR et al. 2016).

These extracellular enzyme producing bacteria showed considerable resistance towards different heavy metals and antibiotics. These results have also been recorded by TALLUR et al. (2016) who reported the isolation of antibiotic resistant bacterial strains from the Arabian Sea simultaneously having extracellular enzymatic activity (TALLUR et al. 2016). Past studies described, that heavy metal resistant bacteria also have antibiotic resistance due to plasmids or transposons (DEVIKA et al. 2013). Various clinical and environmental bacterial cultures also revealed that antibiotic and heavy metal resistance are often closely related (JAFAR et al. 2013).

Enzymes work under optimum pH and temperature values and changes in them may denature their function. Our aquatic bacteria depicted that neutral conditions were best for their growth. Various past workers describe best optimized temperatures for aquatic bacteria in between 37°C to 50°C, and optimum pH was neutral and alkaline. In contrast to this study, researchers have reported optimum temperature between 45°C to 55°C and optimum pH 6.0–7.0 for extracellular hydrolytic

enzymes producing *Bacillus* spp. (SOARES et al. 1999). Previous studies also determined glucose as best carbon source for growth of extracellular enzyme producing bacteria (KRISHNAKUMAR et al. 2011).

Many bacteria are able to excrete extracellular polymeric substances (EPS) (VU et al. 2009). EPS production of microorganisms have fascinated researchers due to their versatile applications and advantages (SHAHNAVAZ et al. 2015). Marine and fresh water have extreme conditions due to contamination from different environmental sources and bacteria produce EPS to protect themselves. EPS production in marine and fresh water bacteria was determined by many workers and they also identified *Bacillus* as good EPS producers (KUMAR et al. 2011).

Exopolysaccharides are involved in bacterial attachment to the substrate and thus, in biofilm formation (COSTERTON et al. 1987). Therefore, EPS producing bacterial strains were further screened for biofilm formation and marine bacteria showed biofilm formation (HASSAN et al. 2011).

Conclusion

Five *Bacillus* marine and fresh water strains with multiple extracellular enzyme activities were identified that can be utilized for therapeutic, industrial, agricultural and bioremediation applications. This study signifies an emerging sight for prevalence of *Bacillus* in Arabian Sea, Karachi and Ravi River, Lahore. There are very limited number of reports about the extracellular enzyme producing *Bacillus* spp. from these specified areas of Pakistan up to our knowledge.

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Appendix

Table 1.1

16S rRNA gene sequencing of hydrolytic enzyme producing bacterial strains

Bacterial strains	Identity	Accession number
S5	*Bacillus tequilensis*	MH371775
S6	*Bacillus pumilus*	MH371776
H4	*Bacillus flexus*	MH371777
H5	*Bacillus sonorensis*	MH371778
R8	*Bacillus subtilis*	MH371779

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EFFECT OF NATURAL FEEDS ON THE GROWTH PERFORMANCE, SURVIVAL RATE AND FEED UTILIZATION OF THE TROPICAL SHORTFIN EEL *ANGUILLA BICOLOR* MCCLELLAND 1844 (PISCES: ANGUILLIDAE) LARVAE

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Key words: rebon-shrimp, bloodworm, golden apple snail, sardine fish, sidat.

Abstract

The high mortality of eels larvae usually occurs during the acclimatization process before growth, probably due to the unsuitable feed intake. Therefore, this study aimed to find suitable natural feed for tropical shortfin eel *Anguilla bicolor* larvae. The complete randomized experimental design with four treatments and four replications was used. Four natural feeds were tested, namely bloodworm *Tubifex* sp., golden apple snail *Pomacea canaliculata*, sardine fish *Decaptersus macarellus,* and sergestid shrimp *Acetes* sp. The initial total length and body weight of the samples were 5.0–7.0 cm and 0.15–1.78 g, respectively, while the experimental fish was reared at a density of 10 fish tank^{-1}. The eel larvae were raised in plastic containers volume 22 L, while natural feeds were given at a feeding ration of 10% body weight a day for 60 days. The results showed that the natural feeds produced significant effects ($P < 0.05$) on weight gain, specific growth and survival rate, as well as feed efficiency and conversion ratio. The sardine feed yielded a better result compared to other tested feeds, but the values were not significantly different from bloodworm. Based on the results, sardine fish and bloodworm feed are suitable for eel *A. bicolor* larvae.

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Introduction

Eels are commercial fish found worldwide with Indonesia having at least 7 species (SUGEHA et al. 2006, SYAIFULLAH et al. 2019) where 3 were reported from the Aceh waters, namely *Anguilla bicolor bicolor, A. marmorata,* and *A. bangelensis bangelensis* (MUCHLISIN and SITI-AZIZAH 2009, MUCHLISIN et al. 2015, MUCHLISIN et al. 2016a, MUCHLISIN et al. 2017). According to WATANABE et al. (2014), there are two subspecies of shortfin eel, namely *Anguilla bicolor bicolor* native to the Indian Ocean and distributed in the surrounding rivers, as well as *A. bicolor pacifica* from the Pacific ocean.

The bioecology and genetics of the eel *A. bicolor* from Indonesia especially Aceh waters have been reported by several studies (SIDQI et al. 2018, MUCHLISIN et al. 2017, MUCHLISIN et al. 2018, BATUBARA et al. 2021). Presently, this species has been cultured in Indonesia where the larvae were collected from the wild and acclimatized for several weeks indoors before rearing in the outdoor pond (THAMREN et al. 2018, MUCHLISIN et al. 2021). The fish fed on trashfish and commercial feed during this process, but the local farmers claimed that the eel larvae grow slowly with high mortality during acclimatization, this is probably due to the unsuitable stocking density and feeding. However, MUCHLISIN et al. (2021) has been examined that the maximum stocking density for eel larvae and found that the best value is 3 fish L^{-1}. However, the problem of the feeding eel larvae has not been solved. According to MUCHLISIN (2017), the alimentary tract of the larvae is underdeveloped and the activity of the digestive enzymes is low, hence, they are unable able to digest the feed optimally. At this phase, it is recommended that the fish should be given natural feeds (MUCHLISIN et al. 2003, FIRDUS and MUCHLISIN 2005).

Larvae is a critical phase of the fish, hence, they require intensive attention and proper handling, specifically in relation to feeding. Currently, there is no information on the best natural feed for eel larvae during the acclimatization process. This study was conducted to evaluate four potential natural feeds for eel larvae namely bloodworm (*Tubifex* sp.), golden apple snail (*Pomacea canaliculata*), sergestid shrimp (*Acetes* sp.) or locally known rebon-shrimp, and sardine *(Decaptersus macarellus).* These potential feeds have higher crude protein content, for example, the bloodworm has 57% crude protein and 13% lipid (TARIGAN 2014), the golden apple snail has 51% and 13.16%, respectively (ANDERSON et al. 2004), while sergestid shrimp and sardine have 52.35% and 57.48%, respectively (SHOLICHIN et al. 2012), indicating that these feeds are very promising for eel larvae. Therefore, this study aims to explore the suitable natural feed for eel *A. bicolor* larvae during the acclimatization process.

Materials and Methods

Experimental design

This study was conducted at the Faculty of Marine and Fisheries, Syiah Kuala University, Banda Aceh, Indonesia. The complete randomized experimental design method was used. Four types of natural food was tested, namely bloodworm (*Tubifex* sp.), golden apple snail (*Pomacea canaliculata*), sergestid shrimp (*Acetes* sp.), and sardine (*Decapterus macarellus*) with four replications.

Experimental fish

A total of 16 plastic containers (Vol. 22 L) were used, each was filled with 10 L and aerated continuously. The initial size of the experimental fish ranged from 0.15 g to 0.178 g and the total length of 5.0 cm to 7.0 cm. There is no commercial breeding technology for eels in Indonesia, hence, the fish larvae were collected from Beurenut River in Aceh Besar District. The larvae collection was performed at high tide from 06.00 PM to 05.00 AM at the water temperature regime of 26–28°C. Acclimatization was then performed for five days before the administration of the experimental diet, and no feed was given during this process.

Feed preparation

The frozen silk worms were purchased from ornamental fish vendors in Banda Aceh, immersed for 5–10 min in warm water, and given to the experimental fish when the ice was melted. The golden snails were collected from rice fields in the Aceh Besar District, the snail meat was removed from the shell and the innards were removed, then the meat was washed with salt water to remove the mucus and finely chopped. Meanwhile, sardine and sergestid shrimp were purchased from local suppliers in Banda Aceh City, and further washed and chopped into small pieces. The feeds were given to the experimental fish in wet conditions.

Stocking and feeding

The experimental fish was measured and weighed for initial body length [cm] and total body weight [g]. A total of 10 larvae were stocked into every container. The experimental fish was fed on natural feed twice a day on 08.00 AM and 16.00 PM at a feeding ration of 10% body weight for 60 days. The unconsumed feed was discharged by siphoning 30 minutes

after feeding, while the water quality was monitored every day to maintain the optimum condition for eel larvae. Approximately 25% of the waters were discharged and replaced every two days interval.

Parameters and data analysis

The weight gain was calculated using the formula:

$$WG = Wt - Wo,$$

where:
WG – weight gains [g]
Wt – the weight of the fish at the end of the experiment [g]
Wo – the body weight at the start of the experiment [g].

The specific growth rate was calculated based on MUCHLISIN et al. (2016*b*; 2016*c*) as follows:

$$SGR = [(Ln\ Wt - Ln\ Wo)/\ t] \cdot 100,$$

where:
SGR – the specific growth rate [% day^{-1}]
Wt – the body weight at the end of the experiment [g]
Wo – the body weight at the start of the experiment [g]
t – the feeding duration (days).

The feed conversion ratio and efficiency were calculated based on TACON (1987):

$$FCR = F/(Wt - Wo),$$

where:
FCR – feed conversion ratio
F – the total of feed taken during the experiment [g]
Wt – the body weight at the end of the experiment [g]
Wo – the body weight of the fish at the start of the experiment [g].

Feed efficiency [%] = 1/FCR · 100. Meanwhile, the survival rate was examined based on MUCHLISIN et al. (2016*b*; 2016*c*):

$$SR = [(No - Nt)/No] \cdot 100,$$

where:
SR – survival rate [%]
No – total fish at the start of the experiment
Nt – the total fish mortal during the experiment.

The data were subjected to One-way Analysis of Variant (ANOVA) followed by Duncan's multiple ranges test using SPSS ver. 20.0 software. Percentage data were transformed before analysis (MUCHLISIN et al. 2004).

Results

The results showed that the weight gain, specific growth rate, and the survival rate ranged from 0.08 to 0.13 g, 0.64% day^{-1} to 1.02% day^{-1}, and 63.33% to 80.00%, respectively. Meanwhile, the feed conversion ratio and efficiency ranged from 4.65 to 7.19 and 13.98% to 21.52%, respectively, as shown in Table 1.

Table 1

Growth performance, survival rate and feed utilization of Tropical shortfin eel *Anguilla bicolor* larvae fed on several types of natural feeds for 60 days. The average±SD value in the same row with different superscripts are significantly different ($P < 0.05$)

Parameters	Natural feed			
	bloodworm (*Tubifex* sp.)	apple golden snail (*Pomacea canaliculata*)	sardine fish (*Decaptersus macarellus*)	sergestid shrimp (*Acetes* sp.)
Average of the initial body weight [g]	0.163±0.009	0.150±0.005	0.155±0.005	0.178±0.006
Average of body weight at the end of the experiment [g]	0.261±0.014	0.249±0.022	0.281±0.009	0.243±0.028
Weight gain [g]	$0.09±0.022^{ab}$	$0.08±0.023^{a}$	$0.13±0.008^{b}$	$0.08±0.034^{a}$
Specific growth rate [% day^{-1}]	$0.77±0.19^{a}$	$0.71±0.15^{a}$	$1.02±0.03^{b}$	$0.64±0.20^{a}$
Survival rate [%]	$80.00±8.16^{b}$	$72.50±9.57^{ab}$	$77.50±5.00^{b}$	$63.33±5.77^{a}$
Feed conversion ratio (FCR)	$4.65±0.18^{a}$	$5.84±0.73^{b}$	$5.39±0.35^{ab}$	$7.19±0.73^{c}$
Feed efficiency [%]	$21.52±0.86^{c}$	$17.30±1.95^{b}$	$18.59±1.28^{b}$	$13.98±1.34^{a}$

The One-way ANOVA test revealed that the natural feed produced a significant effect on the body weight, specific growth rate, survival rate, feed conversion ratio and efficiency with $P < 0.05$. Furthermore, the Duncans test showed that the highest weight gain was recorded in fish fed with sardine, but the value was not different significantly from those fed using bloodworm. The specific growth rate in those fed with sardine was different significantly from other feeds. A high survival rate was also found in this group, but this value was not different significantly from those fed with bloodworm and golden apple snail. The better feed conversion ratio was recorded in eel fed with bloodworm (FCR 4.65), but this value was not significantly different from those fed using sardine (FCR 5.39). A good feed efficiency was also recorded with bloodworm (21.52%), and this value was significantly different from other tested feeds. Moreover, the results revealed that the body weight increased after 10-days of feeding and a high body weight gain was found on fish fed with sardine. The weight gain exceeded the other feed treatment starting from 20-days feeding (Figure 1).

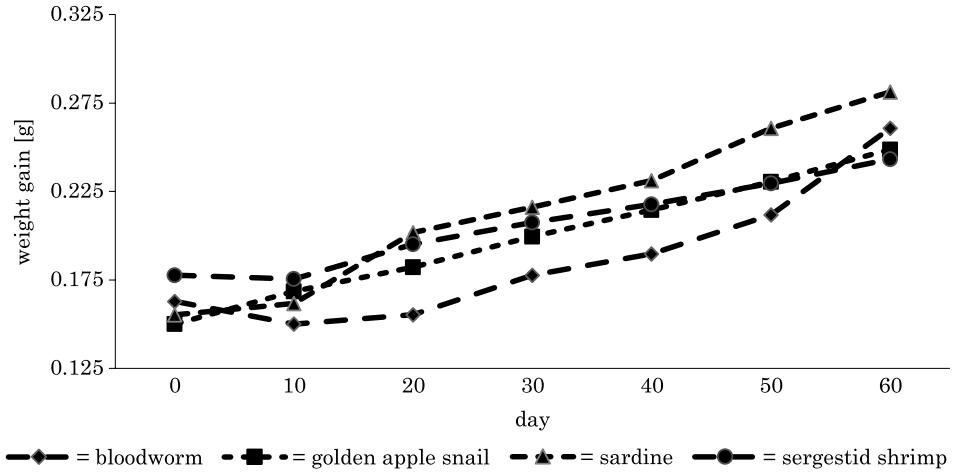

Fig. 1. The body weight gain trend of Tropical shortfin eel *Anguilla bicolor* larvae fed on natural feed for 60 days

The main water quality data showed that the temperature, pH, and Dissolved oxygen ranged from 27°C to 28.3°C, 7 to 8, 4.27 ppm to 6.50 ppm, respectively.

Discussion

Based on the results, the best weight gain and specific growth rate were recorded on fish fed with sardine, whe a good feed conversion ratio and efficiency were found in the treatment with the bloodworm feed. In addition, the highest survival was recorded on fish fed with bloodworm, but these values were not significantly different with sardine feed. In general, sardine produced faster growth compared to other natural feeds.

The growth performance of the fish is strongly related to the protein, lipid, and vitamin contents in the feed, as well as the fatty acid profile (Prusińska et al. 2020, Muchlisin 2017) where protein and lipid are the important energy source. However, the growth performance is not only influenced by protein levels but also affected by the amino acid compositions that build the protein. In general, fish requires protein ranging 40% to 77% (Cuzon 1988, Maigualema and Gernat 2003, Muchlisin 2017), where carnivorous fish require higher levels than herbivores or omnivores fishes (Krogdahl et al. 2005, Panserat et al. 2009). The presence of fatty acids is also crucial in the feed (Ridwanudin et al. 2021) because fish are quite effective in digesting lipids, but their content must not exceed the

maximum need. In general, fish requires lipids ranging from 4.90% to 16% (CUZON 1988, GIRI 2003). The results showed that sardine provided a better growth rate for eel larvae. This might be related to the nutritional contents of sardine, comprising 57.48% of proteins, 4.90% lipids, 0.45% fiber and 5.39% ash (MUNTAZIANA et al. 2013). Besides, sardine emits a fishy odor typical of the favored fish and stimulates the appetite of eel (HANY 2015).

The bloodworm feed produced a high growth performance close to sardine. This is because the bloodworm has high protein content 57% almost equivalent to sardine, but this feed has higher lipid and fiber contents of 13% and 2.9%, respectively (SUBEKTI et al. 2011). Therefore, it is presumed that eel larvae can not digest high lipid and fiber effectively. This is because the digestive tract of the larvae is underdeveloped (MUCHLISIN 2017). In general, sardine and bloodworms gave a better feed utilization compared to golden apple snail and sergestid shrimp. This indicates that sardine and bloodworm are suitable as natural feed for the eel larvae.

A low feed utilization was recorded in fish fed with sergestid shrimp compared to other natural tested feeds. This is probably because the sergestid shrimp has chitin (MATHUR and NARANG 1990), which is difficult to digest by fish, particularly during the larvae stage (GUTOWSKA et al. 2004). In general, the feed digestibility is strong depending on the balance in nutrition contents (AMIRKOLAIE 2005, AGUSTONO 2014), fish age or size (MUCHLISIN 2018), enzymes activities (KLEIN et al. 1988, GRISHAM and REGINALD 1999) and environmental factors such as temperature, pH, and dissolved oxygen (SETIAWATI et al. 2014, OOI and CHONG 2011).

Conclusions

The natural feed produced significant effects on weight gain, specific growth rate, survival, feed conversion ratio and efficiency. Based on the results, sardine and bloodworms are the most suitable feed for the eel larvae.

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FUNCTIONAL AND NUTRITIONAL CHARACTERIZATION OF CUPCAKES PRODUCED FROM BLENDS OF MUSHROOM, ORANGE-FLESHED SWEET POTATO AND WHEAT FLOUR

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Key words: wheat, mushroom, orange-fleshed sweet potato, cupcake, proximate composition, functional properties.

Abstract

The increased awareness of healthy eating has necessitated the need for producing healthy and functional foods. This study aimed at producing a healthy nutritious snack containing essential nutrients that are lacking in most conventional cupcakes. This research investigated the quality of cupcakes produced from wheat, orange-fleshed sweet potato (OFSP) and mushroom flourin the ratio 90:5:5; 80:10:10, and 70:15:15, respectively.

The control sample was a cupcake produced from 100% wheat flour. The proximate composition, total phenolic, vitamin A content, and sensory properties of the cupcakes, as well as some functional properties of the flour blend, were determined. The protein, ash and crude fibre of cupcakes increased as levels of mushroom and OFSP flours in the cupcake increased. Phenolic content of the cupcakes ranged from 0.13 to 0.16 mg/g and vitamin A content ranged from 0.64 to 1.06 mg/100 g. Functional properties of the flour sample ranged from 72.00–94.67%, 6.97–8.19% and 0.70–0.67 g/ml for water absorption capacity, swelling capacity and bulk density, respectively. The developed cupcakes had improved nutritional composition.

Introduction

A cupcake is a delicious snack baked in a thin paper cup purposely to serve only one person. The basic ingredients include flour, fat, sugar, raising agents and eggs.The production of cupcakes originated in the United States in the 19th century because it saves time and ingredients during preparation than the larger cakes. It was opined that the name 'cupcake'

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was adopted because cakes were originally cooked in cups and two, the recipes used in making cupcakes were measured out by the cup (DARNELL 2015). It is a good source of nutrients such as macronutrients; carbohydrates, proteins, and fats. Cupcakes also consist of vitamins and mineral compounds (RAHUT et al. 2012). Cupcakes are taken as desserts and used to mark special occasions. There is increased awareness of the importance of composite flour to prepare more nutritious food with an effort to reduce the calorie load which as a result will reduce the product's glycemic index, and increase the fibre and protein levels through the incorporation of protein-rich foods such as mushroom.

Over 2000 species of mushroom (*Pleurotus plumonarius)* exist in nature: despite that, less than 25 species are broadly accepted as important in food (LINDEQUIST et al. 2005, LILIAN et al. 2007). Historically, mushroom has both medicinal and culinary functions in many countries (IBRAHIM and HEGAZY 2014) which include lowering blood pressure and strengthening the immune system against disease (REGULA and SIWULSKI 2007). Besides, mushroom provides the body with important nutrients such as potassium, selenium, riboflavin, niacin, fibreand proteins. Mushrooms are consumed for their special aroma and flavour (VALVERDE et al. 2015).

Orange-fleshed sweet potato (OFSP) belongs to the group of tubers that has an excellent amount of beta carotene which is highly bioavailable (JAARSVELD et al. 2005). The high percentage of beta carotene in OFSP may help to reduce the incidence of vitamin A deficiency since beta carotene is a provitamin A (POBEE et al. 2017). Orange-fleshed sweet potato is rich in vitamin A, C, E, thiamin, riboflavin, and niacin (PADMAJA 2009). The crop can be used in nutrition intervention programs, especially in areas where vitamin A deficiency is prevalent (IBRAHIM and HEGAZY 2014). It can also contribute to the nutritional content of the developed product, lower gluten levels, and consequently, reduce the risk of celiac disease in people with gluten intolerance (TILMA et al. 2003). Additionally, orange-fleshed sweet potato flour enhances the sensory attributes by adding natural sweetness, colour, flavour and dietary fibre to the processed products (YADAV et al. 2006).

Wheat is one of the most cultivated crops produced in temperate regions, and one of the most important food crops consumed all over the world (SHIFERAW et al. 2013).Wheat grain and flour contain mainly carbohydrates and a considerable amount of proteins, mineral compounds and vitamins (SHEWRY and Hey 2015).

Several efforts have been made to increase the potential of underutilized crops by using them as composite flours in the production of baked products and other confectioneries. Composite flours are blends of flours from tubers such as cassava, yam, and potato, protein-rich flours and other

major or minor cereals which in some cases may include wheat flour (BUGUSU et al. 2001). SCHMELTER et al. (2021) advocated for the replacement of a substantial amount of wheat flour with flour from pulses which can increase the protein content of biscuits or cookies. In this study, however, partial replacement of wheat flour with a blend of orange-fleshed sweet potato and dried mushroom flour has the potential to improve the fibre, protein and vitamin contents of cupcake. This can benefit the body by reducing food cravings whilst offering required energy not just for children but serving as healthy snacks for adults who are health conscious. This study aimed at partially replacing wheat flour with a mixture of orange-fleshed sweet potato and mushroom flour to produce more nutritious and healthy snacks (cupcakes).

Materials and Methods

Samples procurement

The orange-fleshed sweet potatoes (OFSP) and mushrooms (*Pleurotus ostreatus*) were procured from a farmer in Osogbo and Ladoke Akintola University of Technology (LAUTECH) Research Farm, Nigeria, respectively. The cupcakes were processed in the Food Processing Laboratory, LAUTECH, Ogbomosho, Nigeria.

Production of high-quality dried orange-fleshed sweet potato flour

High-quality-OFSP flour was produced as described by SAEED et al. (2012). The tuber was weighed, peeled, washed, weighed and sliced manually into 2 mm thickness. It was dried in a solar drier at 45±2°C. OFSP was cooled, milled into flour using a Model SK-24-MS attrition mill (USA) and sieved using a 250 µm screen to obtain fine particles. The resulted (1.55 kg, 8% moisture content) flour was packed in an air-tight container for further use and stored at ambient conditions (26±2°C).

Preparation of mushroom flour

The procured mushrooms were sorted, cleaned, shredded and dried at 50°C in a cabinet drier for 48 h, milled into flour using a laboratory micro mill (Landersand CIA, USA). The flour (1.60 kg, 10% moisture content dry basis) was sieved into fine flour of 150 µm particle size to obtain a finer mushroom flour, packaged in a transparent polyethene bag and stored in a desiccator as described by OJO et al. (2017).

Formulation of wheat, mushroom and orange-fleshed sweet potato flours

Four different flour samples were formulated for cupcake production. The first batch was the control which was made up of 100% wheat flour (8.40% moisture content, Golden penny confectionery soft flour, Flour Mills of Nigeria); batch 2 which was tagged S1 comprised 90% wheat flour, 5% mushroom, and 5% orange-fleshed sweet potato flours (8.80% moisture content); batch 3 which was tagged S2 comprised 80% wheat flour, 10% mushroom and 10% orange-fleshed sweet potato flours (9.00% moisture content) and batch 4 which was tagged S3 comprised 70% wheat flour, 15% of mushroom, and 15% orange-fleshed sweet potato (9.50% moisture content) flours.

Functional properties of flours from wheat, mushroom and orange-fleshed sweet potato mixes

Functional properties such as bulk density, water absorption capacity and swelling capacity were analyzed according to methods described by SOSULSKI et al. (1976). The bulk density of the varying levels of wheat flour, mushroom and orange-fleshed sweet potato flour blends was determined by weighing 50 g of the sample into the 100 ml graduated cylinder, then, the bottom was trapped gently several times on a laboratory bench, until no further diminution of the sample level. After this, the final volume was expressed as g/ml. The swelling capacity of the varying levels of wheat flour, mushroom and orange-fleshed sweet potato flours was determined by filling 100 ml graduated cylinder up to 10 ml mark. Distilled water was added to make it up to 50 ml. The graduated cylinder was covered and inverted to ensure rigorous mixing of both flour and water. The mixture was mixed again after 2 min then left to stand for another 8 min. The volume was taken and swelling capacity determined). Water absorption was estimated as per cent water-bound per gram of flour. The method of CHANDRA (2015) was used for estimating oil absorption capacity.

Production of cupcakes

The cupcakes were prepared by replacing wheat flour with different levels of composite flour (Table 1) according to the method of RAHUT et al. (2012). The production of cake is presented in Figure 1. The wheat flour, orange-fleshed sweet potato, mushroom, and other ingredients for each cupcake were measured accurately after which sugar and shortening (Golden Penny Margarine, Flour Mills of Nigeria) were mixed in a bowl using a mixer (Kenwood, A901) till the mixture become fluffy and creamy.

Table 1
Ingredients formulation of cupcakes from blends of wheat, orange-fleshed sweet potato (OFSP) and mushroom flour

Ingredient	Composition [g]			
Wheat flour	100	90	80	70
Orange-fleshed sweet potato	0.00	5	10	15
Mushroom	0.00	5	10	15
Sugar	84	84	84	84
Shortening	84	84	84	84
Baking powder	3.6	3.6	3.6	3.6
Whole eggs	2	2	2	2

Source: own study based on RAHUT et al. (2012).

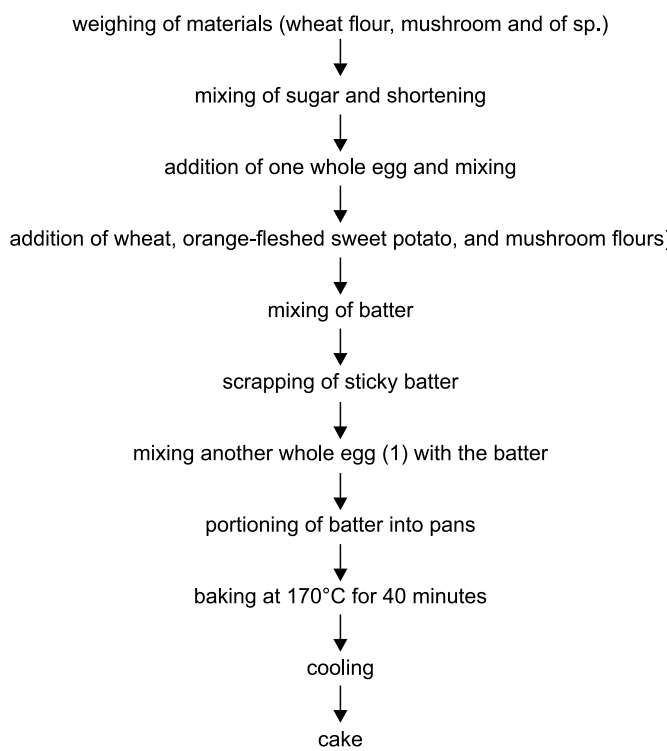

Fig. 1. Flow chart for production of cupcakes

In the later stage, one whole egg and other ingredients were added to the mixture and mixed using a Kenwood mixer (A901) at a low speed of 145 rpm for 10 minutes to ensure homogeneity. After scraping the batter at

the edge of the bowl, it was mixed for another two minutes at medium speed 250 rpm, then another whole egg was added and the batter was mixed. The batter was scooped into pre-greased cake cups and baked in a Thermocool oven (MY DIVA 604G OG-6840 INX) for 40 minutes at 170°C. The cupcakes were removed from the oven and cooled. The cupcakes were baked in three series.

Proximate composition of cupcake samples

The proximate composition of each sample was determined using the method of AOAC (2005). The moisture content was estimated by air-oven (Model DHG 9101. Changzhou, China) drying to a constant weight at 103 ± 2°C; ash content was obtained by incinerating the sample in a muffle furnace (STXMF115, STERICOX) at 550°C for 3–4 hours after partial oxidation of organic matter. The crude fat content was determined using the Soxhlet extraction method using petroleum ether while fibre was obtained by dilute acid and alkali hydrolysis. Crude protein content was estimated by the Kjeldahl method and % protein was obtained by multiplying nitrogen with a conversion factor of 6.25.

Vitamin A determination

Vitamin A in the cupcake was determined spectrophotometrically using a modified standard method of AOAC (2005). The cake sample (0.5 g) was homogenized and saponified with 2.5 ml of 12% alcoholic KOH in a water bath at 60°C for 30 min. The saponified extract was separated in a separating funnel containing 10–15 ml of petroleum ether. The lower aqueous layer was dispensed in a separating funnel and the upper petroleum ether layer containing the carotenoids was collected. The extraction was repeated until the aqueous layer became colourless. One ml of anhydrous sodium sulphate was mixed with the ether extract to remove excess moisture. The final volume of the petroleum ether extract was noted. The absorbance of the yellow colour was read in a visible Spectrophotometer (Beckman Instrument DK-2A) at 450 nm using petroleum ether as a blank.

Total phenolic content determination

The total phenolic content (TPC) of the cupcake sample was analyzed using Folin-Ciocalteu (FC) reagent. At first, 250 µl sample solution was mixed with 1 ml diluted (1:9) FC reagent. It was incubated for 5 min after which 750 µl 1% Na_2CO_3 solution was added. The sample was gently mixed and incubated for 2 h at 30°C. The absorbance of the solvent extract was

later measured at 760 nm on a Spectronic 21DUV spectrophotometer using the modified method of AYUSMAN et al. (2020).The TPC was measured as equivalents of gallic acid (mg GAE/g crude extract).

Sensory evaluation of cupcakes

The sensory evaluation of cupcakes sobtained from wheat, mushroom, orange-fleshed sweet potato flour mixtures at different levels, and control (100% wheat cupcake) was performed by 50 semi-trained panellists representing the staff and students (both) of Ladoke Akintola University of Technology Ogbomosho, Nigeria. The samples were scored for the appearance, flavour, texture, and overall acceptability using a nine-point hedonic scale where 9 indicated like extremely, 5 indicated like nor disliked, and 1 indicated dislike extremely as described by BABARINDE et al. (2020).

Statistical analysis

All data obtained were replicated three times ($n=3$) while the sensory attributes were carried out by fifty ($n=50$) panellists. Data obtained were subjected to a one-way analysis of variance and means were separated using the new Duncan multiple-range test (SPSS version 20).

Results and Discussion

Functional properties of flour samples of wheat, orange-fleshed sweet potato and mushroom mixes

The functional property of food determines the application and the use of food materials for various food products (ADELEKE and ODEDEJI 2010). The results of water absorption capacity, oil capacity, swelling capacity, and bulk density results are presented in Table 2.

The bulk density values were 0.69 g/ml, 0.70 g/ml, 0.70 g/ml and 0.68 g/ml for flour samples containing 100% of wheat, 90% wheat, 5% OFSP and 5% mushroom; 80% wheat, 10% OFSP and 10% mushroom and 70% wheat, 15% OFSP and 15% mushroom, respectively. The bulk density of composite flour ranged from 0.69 to 0.70. The lower densities observed in composite flour may be attributed to the difference in the particle size or total partial gelatinization of the flour during drying (FALADE and OLUGBIYI 2010). However, flour sample S1 and control flour had the same bulk density value (0.70) as control flour (0.70). This can be due to the low levels of OFSP and mushroom flour in the sample.

Table 2

Functional properties of flour samples

Samples	Water absorption capacity [%]	Oil absorption capacity [%]	Swelling capacity [%]	Bulk density [g/ml]
Control	57.67±5.13^{a}	69.00±3.61^{a}	6.90±0.22^{a}	0.70±0.03^{b}
S1	72.00±9.54^{b}	71.00±1.00^{ab}	6.97±0.24^{a}	0.70±0.03^{b}
S2	78.67±1.53^{b}	72.67±1.16^{ab}	7.00±0.31^{a}	0.68±0.03^{a}
S3	94.67±1.53^{c}	74.67±3.06^{b}	8.19±0.26^{b}	069±0.02^{a}

Values represent means of triplicate reading. Means within the same column with different alphabets are significantly different ($p < 0.05$)
S1 – 90% wheat, 5% mushroom, 5% OFSP flours
S2 – 80% wheat, 10% mushroom, 10% OFSP flours
S3 – 70% wheat, 15% mushroom, 15% OFSP flours
Control – 100% wheat flour

The water absorption capacity (WAC) of the samples increased with an increase in the inclusion of OFSP and mushroom, the WAC value of composite value ranged from 72 to 94%, while the control sample had the least WAC value being 57.67%. This confirmed the report of SAEED et al. (2012) who reported that the water absorption capacity of composite flours is higher than wheat flour. Water absorption is an attribute that has implications for viscosity. It aids bulking and ensures consistency in some products during baking (NIBA et al. 2001). A very low water absorption capacity can negatively influence the quality of the food products by reducing the volume of the product and increasing staling activity in baked foods (AWUCHI et al. 2019).

There was an increase in oil absorption capacity (OAC) of the flour samples with increased levels of OFSP flour and mushroom powder. The higher OAC observed in the flour samples may be attributed to the higher protein content of the flour sample containing mushroom and OFSP mixes. This has been reported to contribute to the oil retaining properties of food materials. The OAC of flour plays a major role in the storage stability of food products (FALADE and KOLAWOLE 2011). The oil absorption capacity is an important attribute that enhances flavour and mouth feel in food preparation. A flour with moderately high OAC is desirable and used as a functional ingredient in some baked products, especially cakes and desserts (SURESH and SAMSHER 2013).

The swelling capacity of the flours ranged from 6.90–8.19% for samples S1, S2 and S3. The higher swelling capacity observed in S1, S2 and S3 may be attributed to a higher level of orange-fleshed sweet potato and mushroom. This confirmed the report of AWUCHI et al. (2019) who similarly observed that the swelling capacity is often related to their protein

and starch content. The amylopectin is primarily responsible for granule swelling, the higher the amylopectin content in composite flour with a higher level of potato flour would increase the swelling power of composite flour (TESTER and MORISSON 1990).

Proximate composition of cupcakes

The results of the proximate composition of cupcakes produced from the blend of wheat, mushroom, and orange-fleshed sweet potato (OFSP) flours are presented in Table 3. The protein content of cupcake samples increased from 7.76 % in the control sample to 17.75% in samples containing 15% OFSP and 15% mushroom flour. Protein content increased with an increased level of mushroom substitution. VALVERDE et al. (2015) confirmed that mushrooms are high in important nutrients such as proteins, selenium, riboflavin, and fibre. Mushrooms can be incorporated into foods to enrich their protein contents and reduce the risk of protein energy malnutrition (IBRAHIM and HEGAZY 2014).

Table 3

Proximate composition of cupcake samples

Sample	Protein [%]	Moisture [%]	Fat [%]	Ash [%]	Crude fibre [%]	Carbohydrate [%]
Control	7.76 ± 0.25^{a}	12.19 ± 0.32^{a}	18.56 ± 0.51^{b}	0.67 ± 0.15	0.44 ± 0.10^{a}	59.87 ± 0.31^{ab}
S1	9.30 ± 0.25^{b}	13.00 ± 0.67^{a}	16.89 ± 1.02^{b}	$1.17\pm29a^{b}$	0.78 ± 0.19^{ab}	59.13 ± 0.91^{a}
S2	13.42 ± 0.07^{c}	17.26 ± 0.23^{b}	13.67 ± 0.58^{a}	1.43 ± 0.51^{b}	0.96 ± 0.34^{b}	58.27 ± 1.35^{ab}
S3	17.75 ± 0.44^{e}	17.67 ± 0.58^{b}	12.00 ± 1.33^{a}	1.47 ± 0.06^{b}	0.98 ± 0.01^{b}	50.13 ± 0.35^{b}

Values represent means of triplicate reading. Means within the same column with different alphabets are significantly different ($p < 0.05$)
S1 – 90% wheat, 5% mushroom, 5% OFSP flours
S2 – 80% wheat, 10% mushroom, 10% OFSP flours
S3 – 70% wheat, 15% mushroom, 15% OFSP flours
Control – 100% wheat flour

The incorporation of mushrooms into diets may reduce protein deficiency in developing countries, especially in areas where quality proteins from animal sources are either expensive or unacceptable due to ethnic bias or religious beliefs (DUNKWAL et al. 2007). Usually, roots and tubers arepoor sources of protein as observed in OFSP. NEELA and FANTA (2019) observed that the protein content of OFSP is similar to that of potatoes (2%). Orange fleshed sweet potato can be partially replaced with wheat flour because of its protein content and the significantly little visco-elastic property in the preparation of various bakery products (IBRAHIM and HEGAZY 2014).

An increase in ash and crude fibre contents was also observed in the cupcake samples. The values obtained were in the ranges of 0.67–1.47% and 0.44– 0.98% (Table 3). The increase in ash and fibre could be due to the incorporation of OFSP and mushroom flours. VALVERDE et al. (2015) reported that mushroom provides the body with important nutrients including fibre and ash. Ash and fibre contents of cake samples with different levels of mushroom flour were higher than that of control cakes.The ash content of OFSP as reported by MOHAMMAD et al. (2016) ranged from 1.17 to 4.33%. TOLERA and ABERA (2017) reported that oven-dried mushrooms contained 11.6% ash content. The values obtained in the cupcake samples were lower than the values reported by TOLERA and ABERA (2017) for mushrooms. The difference in the fibre could be due to varietal differences.

Crude fibre provides faecal bulkiness and plays a vital role in cholesterol reduction. It aids the entrapment of dangerous substances like cancer-causing agents and the growth of natural beneficial microbial floral in the gut (DHINGRA et al. 2012). ENDRIAS et al. (2016) reported that the dietary fibre of OFSP varieties ranged from 0.35 to 3.6%.

The moisture content of cupcake samples increased with an increase in mushroom and OFSP. The moisture content of cupcake samples containing various levels of OFSP and mushroom flours ranged from 13.00 to 17.67%. The sample containing 15% OFSP and 15% mushroom had the highest value as compared to the control sample. The increase in moisture could be related to the high water absorption capacity of the composite flour. Moisture is the major component in OFSP, which accounts for about 75% of the entire tuber (ENDRIAS et al. 2016).

The fat content of cupcake samples decreased with an increase in mushroom and OFSP flours. The sample containing 15% OFSP and 15% mushroom had the lowest fat content 12.00% while the control sample (100% wheat flour) had the highest fat content (18.56%). RODRIGUES et al. (2016) reported that OFSP is low in fat (< 1%), which is the usual trend for roots and tubers. The fat concentration of the OFSP is even lesser than other roots and tubers such as potato (0.09%), cassava (0.28%) and other staple tuber crops that contain less fat (0.15–1.00%) and this is a positive nutritional aspect of the OFSP (NEELA and FANTA 2019). Significant ($P < 0.05$) differences were noted among the values obtained for fat contents of cake samples.

The carbohydrate content of cupcake samples decreased with the inclusion of OFSP and increased in mushroom flour (Table 2). SHEIKH et al. (2010) reported that the carbohydrate content of the control cake was higher than other samples containing varying levels of OFSP and mush-

room flour. MOHANRAJ and SIVASANKAR (2014) reported that the carbohydrate from OFSP does not result in a blood sugar spike. The World Health Food Organization also acknowledged OFSP as the root crop with anti-diabetic activity (ANBUSELVI et al. 2012).

Total phenolic content and vitamin A

The results of the phenolic compound and vitamin A are shown in Table 4. The vitamin A content of cake samples containing various levels of OFSP and mushroom powder ranged from 0.62 mg/100 g to 1.06 mg/100 g. Sample containing the highest levels of OFSP and mushroom had the highest value (0.16 mg GAE/g) of total phenols that was significant at $p < 0.05$. Vitamin A and total phenol contents increased with an increase in mushroom and OFSP flour. The total phenolic contents of the cake samples were higher than the values 0.06–0.23 mg GAE/g fresh weight reported by TEOW et al. (2007) for various sweet potato cultivars. OFSP and mushrooms could have contributed to the higher values of total phenols since they are rich sources of total phenols (ELMASTAS et al. 2007) which contribute to their antioxidative and anti-tumour (ADEBAYO et al. 2014; LINDEQUIST et al. 2005) activities. Phenolic compounds are the major antioxidants in mushroom and which play a protective role in human health due to their ability to scavenge and inhibit free radicals (GĄSECKA et al. 2016).

Table 4

Total phenol and vitamin A contents of cupcakes

Samples	Total phenol [mg GAE/g]	Vitamin A [mg/100 g]
Control	0.13 ± 0.00^a	0.62 ± 0.05^a
S1	0.13 ± 0.00^a	0.64 ± 0.03^a
S2	0.15 ± 0.02^b	0.94 ± 0.05^b
S3	0.16 ± 0.01^b	1.06 ± 0.09^c

Values represent means of triplicate reading. Means within the same column with different alphabets are significantly different ($p < 0.05$)
S1 – cupcake baked from 90% wheat, 5% mushroom, 5% OFSP flours
S2 – cupcake baked 80% wheat, 10% mushroom, 10% OFSP flours
S3 – cupcake baked 70% wheat, 15% mushroom, 15% OFSP flours
Control – cupcake baked 100% wheat flour

On vitamin A content of the cake samples, KURABACHEW et al. (2015) reported that OFSP has a high potential to address vitamin A deficiency (due to the high concentrations of beta-carotene) using food-based intervention programs in areas where vitamin A deficiency is prevalent.

Sensory evaluation of cupcake samples

The cupcake samples produced from S1, S2 and S3 were subjected to sensory evaluation by a panel of 50 tasters. The mean scores for colour, aroma, texture, and overall acceptability of the cupcake are presented in Table 5. The cupcake samples showed varying degrees of acceptability of the analysed indices.

Cupcakes produced from 15% mushroom, 15% OFSP, and 70% of wheat flour had the highest rating for taste, appearance and colour, and overall acceptability, while S1 (90% wheat, 5% OFSP, 5% mushroom) had the highest rating of aroma. This indicates that both mushroom and OFSP flour had a strong impact on the taste, aroma, appearance and texture. This confirmed the report of SHEIKH et al. (2010) who reported that the increasing percentage of mushroom flour gave the highest score for colour, flavour and texture. Additionally, orange-fleshed sweet potato flour enhanced the sensory attributes by adding natural sweetness, colour, flavour and dietary fibre to the processed products (YADAV et al. 2006). Wheat flour had a higher rating in taste appearance and aroma. As shown in Table 5, the sensory scores revealed that cake containing 15% mushroom powder and 15% of OFSP was the most preferred in terms of colour than other cake samples containing various levels of mushroom powder and OFSP.

Table 5

Sensory evaluation of cupcake samples

Samples	Taste	Appearance	Texture	Aroma	Colour	Overall acceptability
Control	8.03 ± 0.72^{b}	7.80 ± 0.76^{bc}	8.07 ± 0.69^{b}	8.40 ± 14.30^{c}	7.40 ± 0.77^{b}	7.87 ± 0.73^{b}
S1	7.67 ± 0.93^{ab}	7.27 ± 1.02^{ab}	7.17 ± 1.18^{a}	7.73 ± 0.94^{b}	7.07 ± 14.56^{b}	7.83 ± 0.79^{b}
S2	7.13 ± 1.17^{a}	6.83 ± 1.26^{a}	6.63 ± 1.56^{a}	7.13 ± 1.43^{a}	6.47 ± 1.46^{a}	6.90 ± 1.32^{a}
S3	8.30 ± 1.06^{b}	8.03 ± 0.81^{c}	8.17 ± 0.91^{b}	7.90 ± 1.06^{b}	8.00 ± 0.87^{c}	8.30 ± 0.92^{b}

Values represent means of triplicate reading. Means within the same column with different alphabets are significantly different ($p < 0.05$)
S1 – 90% wheat, 5% mushroom, 5% OFSP flours
S2 – 80% wheat, 10% mushroom, 10% OFSP flours
S3 – 70% wheat, 15% mushroom, 15% OFSP flours
Control – 100% wheat flour

Conclusions

The protein, crude fibre and ash contents of the formulated cupcakes (S1, S2, S3) were higher than the control. The swelling capacities of the formulated S1, S2 and S3 flours showed higher values than 100% wheat

(control). Cupcakes produced from S3 (samples with 15% mushroom and 15% OFSP flour) revealed the highest total phenolic and vitamin A contents. The overall sensory quality of cupcakes formulated from S3 was higher than those prepared from S1, S2 and control. The result of this study suggests that mushroom and OFSP flours should be used for the production of nutritious and acceptable quality cupcakes. Our findings could be adopted in the development of novel and health-beneficial cupcakes with improved nutritional and antioxidant properties.

Conflict of interest. The authors declare no known competing interests that could influence the work reported in this paper.

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ANGLING IN CULTURAL AND PROVISIONING ECOSYSTEM SERVICES

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Key words: recreational fishing, social-ecological system, socio-economic factors, social geography.

Abstract

Recreational fishing is one of the most common recreational activities in the aquatic environment and a very complex social-ecological system (SES). It provides real benefits to anglers and as such, is considered an ecosystem service (ES). This article seeks to identify the scale and nature of cultural and provisioning ES in angling concerning socio-economic and engagement indicators. It also focuses on affiliation and preferred company of other anglers and preference for fishing in different waterbodies. Cultural service anglers were most numerous (68.5%) in this context and were clearer in their environmental and social preferences and characteristics. Anglers expecting to provision are harder to classify, making their behavior in the environment less certain. Association in organizations/clubs proves to be a key social factor that can influence anglers in the context of final ES choice. Despite uneven distribution, the lakes are the most frequently preferred by anglers.

Introduction

Angling is a very popular recreational activity practiced by many people worldwide (ARLINGHAUS et al. 2015, 2021). It is reported that currently in industrialized countries, recreational inland angling (the most frequently manifested and accessible way of recreational fishing) is subject to more pressure than commercial and subsistence fishing altogether (ARLINGHAUS et al. 2002). However, regardless of the level of development, recreational fisheries takes place in different legal and political (e.g. KARPIŃSKI 2017, KARPIŃSKI and SKRZYPCZAK 2019, MORTON et al. 2016, RADOMSKI et al. 2001), economic (e.g. FEDLER 2009, HUGHES 2015,

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Terluin 2003), historical (e.g. Near and Rechner 1993), sociocultural (e.g. Hughes 2015, Near and Rechner 1993, Skrzypczak and Karpiński 2020) and environmental (e.g. Cowx 2015, Dill 1993) domains, which makes angling an interdisciplinary activity. Recreational fishing generates great economic benefits. For example, spending in the United States in 2011 accounted for $41-48 billion and its economic impact equals around $115 billion (Arlinghaus et al. 2015, Tufts et al. 2015). It is also connected to social life, both in terms of jobs in many industry sectors (Arlinghaus, et al. 2017, Organa 2017, Tufts et al. 2015), and an increase in the quality of life of societies in the terms of well-being (e.g. Bracken and Oughton 2014, Fenichel et al. 2013, Liu et al. 2019, Skrzypczak et al. 2022).

Review of social-ecological research in angling

Angling also has an impact on the environment. However the positive and negative impacts are inconclusive both for fresh and marine waters (Furgała-Selezniow et al. 2012, Hughes 2015, Karpiński and Skrzypczak 2021, Lewin et al. 2019, Schafft et al. 2021). Based on its above mentioned characteristics and current research (Arlinghaus et al. 2016, 2017), angling is a strongly coupled social-ecological system (hereinafter SES) with a strong impact on sustainable development (e.g. Arlinghaus et al. 2016, Taylor and Suthers 2021, Ward et al. 2016). In the literature, a social-ecological system is characterized as: "a system of closely interconnected and interdependent elements of ecological and social subsystems influenced by political, cultural, economic or historical factors" (Dumieński et al. 2019) or "a set of critical resources (natural, socioeconomic and cultural) whose flow and use is regulated by a combination of ecological and social systems" (Redman et al. 2004). SES thus indicates that social and ecological systems are linked through feedback mechanisms. This concept is very meaningful in the sustainable development context and is gaining more and more attention in social geography. As assumed in the basis of the SES according to Ostrom (2007) and Ostrom et al. (2007) SES are very complex and difficult to interpret easily. They are also resistant to rapid adaptation. Every system, even the smallest, is very complex, multivariate, nonlinear, multi-scale, and changing. It requires a vast amount of interdisciplinary work to learn as much as possible about all its components. And this requires an integrated approach by all users and participants in the system.

The SES concept emphasizes humans as an inseparable part of nature interacting with it and getting something in return from nature

(BOULANGEAT et al. 2022). And as such, the concept is inevitably linked to ecosystem services (hereinafter ES), which constitutes everything that humans receive from the environment (WALLACE 2007). Recreational fishing is also such a service, and according to The Common International Classification of Ecosystem Services (CICES) it is within the cultural (biotic) section service (code 3.1.1.1.) which is aggregating services "using the environment for sport and recreation; using nature to help stay fit" (HAINES-YOUNG and POTSCHIN 2012, 2018). Placing angling as a cultural service is also indicated in other studies (e.g. HIRONS et al. 2016, HOSSU et al. 2019, JOBSTVOGT et al. 2014). Most researchers, because of its recreational character, classify it as a cultural, recreational service (HERNÁNDEZ-MORCILLO et al. 2013, KULCZYK et al. 2018). It is very lucrative service with a total valuation of about a $815 billion (COSTANZA et al. 1997, de GROOT et al. 2002). Recreation ES is the most known cultural ES (ABUALHAGAG and VALÁNSZKI 2021). Generally, it is the main ecosystem service produced by aquatic ecosystems and thus contributes significantly to human well-being (ARLINGHAUS et al. 2002, 2017, FAO 2012, LIU et al. 2019, REYNAUD and LANZANOVA 2017).

However, angling is not only a cultural service. Given that the phenomenon of recreational fishing itself is very complex and its perception from both the cultural and legal side varies around the world, it would be wrong to assume that it only has a cultural side (LIU et al. 2019, WINFIELD 2016). In practice, the potential benefits of angling are also of a provisioning nature (the fish and its value after catching) which contrasts with cultural benefits, where the fish is merely an add-on and a way to achieve the main goal of angling - pursuing a hobby (LIU et al. 2019). However, it should be noted that this division, although often based on internal beliefs, is not given permanently. The choice of the final ES depends on the individual angler and may change over time depending on the situation. Even if anglers' main motive has never been nutrition, eventually some of the fish caught may become so. Research now indicates that the trend is changing and that the distinction between recreational and subsistence fishing is slowly blurring (NYBOER et al. 2022). The Common International Classification of Ecosystem Services (CICES) indicates in the provisioning services section that there are "animals reared for nutrition, materials or energy" as well as "Wild animals (terrestrial and aquatic) used for nutritional purposes". Anglers catching fish mainly for above mentioned purposes should be treated as using provisioning final ES. Other anglers should be counted as using cultural ES (HAINES-YOUNG and POTSCHIN 2012, 2018). But we should remember that there are also anglers seeking, to varying degrees, both of these benefits of recreational fishing.

Research objectives

A more precise framework for angling ES based on the identification of the final ecosystem service from the individual angler's point of view is necessary. The purpose of this article is to indicate to what extent, in freshwater environments, anglers in their preferences and behaviors are more connected to cultural services and to what extent to provisioning services. It also aims to find out what preferences are exhibited by anglers who are not clear in their indications of using cultural or provisioning angling ES. The present study sought to determine the preliminary characteristics of groups with different attitudes toward the use of angling ES and whether they have different characteristics in terms of socioeconomic background and angling engagement. Finally, it is also the intention of the author to provide evidence of what other factors may influence the use of various angling ES.

In this regard, the following research questions were formulated: (1) identify the scale and nature of the angling recreation background in the context of cultural and provisioning ecosystem services concerning socio-economic and engagement indicators; (2) the impact of association on the choice of different ES in angling (3) connecting the cultural and provisioning background of angling to the preference for angling in the different aquatic environments (lakes, rivers and artificial water bodies) and the preferred social relationships while angling.

A better understanding of anglers in this regard and their behavior will result in: a better understanding of the impact of anglers on the economy and the environment; a more precise framework for valuing the overall capitalization of recreational fisheries; identifying the characteristics, causes, and effects of environmental and social behavior of anglers in particular aquatic spaces; an important point of reference, for a better understanding of human-environment relations and the effective development of environmental policy.

Materials and Methods

Design of the questionnaire and data collection

The data set for this article consisted of two surveys widespread in 2019 (February to November) and 2021 (July to September). These surveys were completed by a total of 1292 people, of which the first survey was completed by 722 and the second by 570 anglers. These two data series

were designed to examine whether there have been changes in the angling community during a period of dynamic changes in societies in recent years (including pandemics). Since socioeconomic and demographic issues, as well as engagement, can change over time for each angler, it was determined that if an angler completed both surveys in both years, he or she was considered a separate respondent.

Both questionnaires were anonymous and limited only by age (more than 14 y/o) and the time required to answer questions took about 5 minutes. The questionnaires were distributed through Internet websites, forums, and social media platforms (e.g. angling clubs and associations, Facebook groups and fan pages, anglers' discussion groups, and Internet forums) in English and Polish. Therefore, the sample consists of people present on angling media. Considering the spatial impact of the sites, groups, and profiles, it should be assumed that the survey covered mainly European residents with special attention to anglers who speak the Polish language (which does not mean that they fish in Poland) who constituted 96.0% in the first survey and 99.5% in the second survey (questionnaires completed in Polish). Due to the inability to conclude how many people saw the survey, response rate seems to be impossible to obtain. Rough estimates based on followers of social media groups used indicate that, it could have been up to 500 000 people who saw the survey.

Both surveys were constructed in the Google Forms platform (https://docs.google.com/forms) in two languages (English and Polish) and were composed of 22 questions. Those questions were related to socioeconomic status, demographics, engagement in angling, preferences for choice of the aquatic environment, and angling with companionship. There were also two questions designed to indicate whether the angler surveyed was willing to use its hobby as a provisioning service ("The possibility of keeping each caught fish is important to me"), or whether this need was not the leading one ("I release caught fish according to the 'no-kill' principle"). Socioeconomic, demographic, and engagement questions were formulated to answer one of the categories indicated earlier or to indicate correct to the facts, value.

The geographic location of anglers' domicile and favorite fishing grounds was also the subject of the survey, however, due to the sensitivity of this data in combination with other questions, for fear of respondents quitting the survey too soon, it was not mandatory. However, even so, some anglers answered in an incomplete or elusive manner, such as: lake, pond, river, secret, or I don't have favorite fishing ground, etc. Of the 965 people who responded to this question, 685 surveys were useful and were used to show the geographic location of anglers' domiciles and 680 of their

favorite fishing sites. According to the Polish central statistical office, inland waters explored by anglers in Poland account for about 2% of the country's area, of which about 49.1% are lakes, about 40.6% are rivers and 10.3% is water in artificial reservoirs and standing water (GUS 2022). The geographical distribution of respondents in this matter was entered by the author into Google My Maps tool and presented with the use of Google Maps (https://www.google.com/maps).

Questions about all preferences (place, companions, fish handling) were measured on a typical 5-point Likert scale. The "1" was used to express "I strongly disagree" while "5" meant "I strongly agree" with "3" meant "I have no opinion or it is difficult to determine it" (neutral opinion). This scale is widely used in social sciences to express preferences and opinions (e.g., NAVRÁTIL et al. 2009, NORMAN 2010, SKRZYPCZAK and KARPIŃSKI 2020). All questions, the exact method of answering, and possible choices can be seen in Appendix 1 (Table 1.1).

A small portion of paper surveys were used (3.6% of the second survey). This portion was widespread in older people angling communities that prefer this type of survey. Traditional surveys were collected fully anonymously and the data was manually added to the automatically generated web-assisted interviews (WAI) survey data. WAI is a completely anonymous type of survey and less error-prone in comparison to traditional questionnaires (BRADBURN et al. 2004). It also allows faster access and analysis of the data. Non-probability sampling methods were chosen, because of their lower costs in money, time, and resources as well as their simplicity in recruiting scattered populations. Additionally, survey respondents were encouraged to distribute it among their well-known angling communities involving non-random snowball sampling with the information to complete the survey only once (MILLER 2003, PARKER et al. 2019, VEHOVAR et al. 2016). It was assumed that it should prevent issues of "double filling" the survey. Surveys that looked like they were filled out without commitment (e.g. same answer to all questions on a Likert scale, mutually exclusive answers) were removed from further analyses.

Considering the smallest possible set of assumed respondents, 11.6% of Poles declaring that they have angling skills (GUS 2012), and assuming a 95% level of confidence and 5% margin of error, a minimum of 385 survey responses was required. Questionnaires used in the following study combined had the margin of sampling error calculated at a 95% confidence level indicating MoE ±2.73% for the whole sample and between ±1.1% and ±2.7% for each extracted sociodemographic subgroup. The smaller the error, the more confident the results are (DILLMAN 2014). Most researchers as a rule of thumb, accept MoE up to 8% at a 95% confidence level (DATA STAR 2008).

Respondents were classified into different groups based on cross-questions to indicate how they handled the fish they caught: 1)"The possibility of keeping each caught fish is important to me" and 2)"I release caught fish according to the 'no-kill' principle". The responses using a 5-point Likert scale were analyzed through a classification matrix which can be seen in Table 1. Three following groups were extracted: *Provisioning*, *Cultural*, and *Mixed* (provisioning-cultural). *Provisioning* was the group of anglers for whom it, was important when angling, to be able to supply fish and this need seems to be predominant. This group consists of anglers who were rather, or strongly disagreed with releasing the fish and, at the same time, were at least neutral in keeping them. Also, anglers neutral in releasing, but positive in keeping fish were included in this group. The second group was *Cultural*. It was made of anglers for whom the desire to release the fish after catching it, was dominant, while the need to keep fish was absent or neutral. This group also consisted of anglers speaking negatively about keeping fish but neutral in releasing them. The last group, *Mixed*, consisted of anglers who do not have a clear opinion on how they were addressing these issues (both questions on the Likert scale were "3") or their opinion was of a mixed, provisioning-cultural nature (both questions "1" and "2"; or both questions "4" and "5").

Table 1

The procedure of dividing surveyed anglers into angling ES groups

Specification	Likert scale	The possibility of keeping each caught fish is important to me				
		1	2	3	4	5
I release caught fish according to the 'no-kill' principle	1	**4**	**4**	**6**	**23**	**35**
	2	**7**	**5**	**23**	**33**	**17**
	3	**42**	**35**	**118**	**37**	**27**
	4	**84**	**132**	**59**	**20**	**17**
	5	**447**	**52**	**34**	**14**	**17**

Notes: Numbers represent the number of anglers giving answers to both questions in an exact manner. ■ *mixed* anglers, ■ *provisioning*, and □ *cultural*

Statistical data analysis

To highlight socioeconomic, demographic, and angling engagement differences in a deeper way the percentage difference index (PDI) was used. It is a useful indirect source of information showing whether any groups were over- or underrepresented in any question. The further the size of this index is away from 1.0, the further this group is away from the

average proportions calculated for the population under study. In other words, the more this index approaches zero, the less characteristic is the presence of a given group in each aspect, and when it is greater than 1 the more characteristic is the presence of a given group in a given aspect. Behaviors indicating more than 0.1 (10%) dynamics were indicated.

The statistical significance of differences between datasets and between groups was examined based on the t-test for dependent samples ($p < 0.05$). To analyze it a one-way ANOVA and a Tukey HSD for unequal N post hoc test were used. All statistical significance tests were performed using STATISTICA version 13.3 software. To find the variable that most differentiates the three anglers' groups surveyed, I-Trees classification pre-analysis was performed using STATISTICA version 13.3 software. The goal of this analysis was the construction of a model to obtain subsets that are maximally homogeneous from the point of view of the value of the dependent variable.

Preferences for fishing spots and social relationships while angling among anglers identified with different types of ecosystem services and association status were tested with the non-metric multidimensional scaling (NMDS) ordination analysis. The Bray-Curtis distance measure, two axes, and stress formula type 2 were applied for log-transformed variables (Ter Braak and Šmilauer 2018). The analysis was conducted using CANOCO 5.11.

Redundancy analysis (RDA), as a canonical form of principal component analysis and one of the linear techniques used in socio-economic research, has been used to the identification of the relationship between anglers' fishing spot preferences as well as social preferences and sociodemographic factors and engagement indices. The usefulness of this linear ordering method is determined by the size of the standard deviation in the dataset, i.e. when the largest gradient in the dataset does not exceed 3.0 (Ter Braak and Šmilauer 2018). The RDA space was used to explain the preferences of six groups of anglers identified with different kinds of ecosystem services and various affiliation statuses. Anglers' responses were compositional and had a gradient of 0.2 SD unit lengths, so a linear method better explained the data. Each variable that explained anglers' preferences was tested for statistical significance using Monte Carlo tests (499 random permutations). Data were normalized using the log $(x + 1)$ transformation (Ter Braak and Šmilauer 2018). All variables explained a significant amount of variation and were statistically significant ($p < 0.05$).

The explanatory variables (sociodemographic factors and engagement indicators) were selected based on a variance inflation factor (VIF) of less than 10. During the RDA analysis, the numbers of response data (prefer-

ence for the environment of the fishing spots and social relations while angling) and explanatory variables were verified each time based on the values of the correlation coefficients of the explanatory variables and VIF. The purpose of this verification was to obtain the maximum value of the percentage of the explained total variance of response data (TER BRAAK and ŠMILAUER 2018). Finally, the eight explanatory variables were implemented into the ordinal space, including age; educational level; annual income; place of residence expressed in the number of inhabitants; distance to the most visited fishing spot; avidity expressed by the frequency of angling; experience expressed by years of engagement in angling; average annual spending on angling. RDA was performed using the Canoco version 5.11 software.

Results

All respondents were classified under the 3 groups with different perceptions of ES use (Figure 1), i.e., *Provisioning* ($N = 201$); *Cultural* ($N = 885$), and provisioning-cultural *Mixed* ($N = 206$). All groups were predominantly male (93.3%) which can be seen in Table 2. Women in *Provisioning* and *Mixed* groups were represented more frequently than in the *Cultural* group with PDI respectively 1.11 and 1.30. The age category that has the most participants was 25–40 y/o with about 45.3% of all anglers under study.

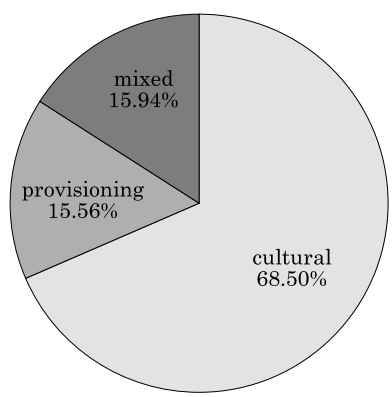

Fig. 1. Results of the classification matrix (Table 1) with the division of anglers into three groups with different perceptions of ES use

The Mixed group in this regard was characterized by high dynamics of proportions concerning the oldest and youngest group, where there was an overrepresentation of younger (up to 18 y/o) anglers (PDI = 1.48) and an

underrepresentation of oldest (more than 65 y/o) anglers with PDI = 0.75. It was slightly different in the *Provisioning* group, where the youngest anglers were less than the average in the surveyed population (PDI = 0.76), but this was at the expense of not the oldest group, but the group at the beginning of their careers (19–25 years; PDI = 1.29). In terms of earnings, proportionally, the largest number of highest earners (more than €24 000 per year) were in the *Mixed* group (PDI = 1.30) and accounted for 8.3% of this group, while the largest number of lowest earners (less than €5 000 per year) were in the *Provisioning* group (PDI = 1.19) and accounted for as much as 31.3% of this group. There were few noteworthy differences in educational status between the groups. It is noticeable that the *Cultural* group had the largest number with secondary education, while in the other two groups higher education was most represented. However, across the surveyed population, these two educational statuses were very close to each other (secondary 42.6% and higher 41.9%). Also, marital and employment status also do not indicate any intra-group variability. With regard to marital status, married anglers predominate (54.8%), while the employment status showed that there were far more employed (76.9%) in the population under study. Anglers were most often recruited from large cities with more than 100 000 residents (30.2%), from where anglers from the *Mixed* group (34.9% with PDI = 1.16) were especially common. In addition, this group had proportionally the fewest anglers from rural centers (PDI = 0.74). These, on the other hand, were relatively most often found in the *Provisioning* group (PDI = 1.11).

Angling engagement matters at first glance seem to reveal a bit more variability. Anglers with up to 30 years of experience predominate (23.8–25% for anglers with up to 10 years of experience; 10–20 and 20–30, respectively). The smallest number of the most experienced was evident in the *Mixed* group while the *Provisioning* group has the largest amounts of least (PDI = 1.11) and most (PDI = 1.19) experienced anglers. *Mixed* anglers in the largest proportion were occasional, as 18.4% (PDI = 1.48) fish only a few times a year. At the same time, this group shows an underrepresentation of anglers who fish most often, i.e. several times a week (PDI = 0.79). Most surveyed anglers practice their hobby with a frequency of approximately once a week (32.8%). When it comes to spending, anglers typically spend at least the equivalent of €100 per year (81.2%). Anglers from the *Cultural* group relatively spending the least (less than 25 euros per year) on their hobby are more represented (PDI = 1.13), although it was only 4.6% of those surveyed in this group, but only 1.9% of those who pay the least were in the *Mixed* group (PDI = 0.47). In contrast, there were relatively few anglers in the *Provisioning* group who spend the amount of more

than 500 euros per year on their hobby (PDI = 0.88). The largest percentage of surveyed anglers travel between 5 and 30 kilometers to their favorite fishing spot (46.4%). Anglers in the *Provisioning* group were more likely to visit closer fishing grounds (up to 5 km) – PDI = 1.15, and less likely to visit fishing grounds farther than 30 km (PDI = 0.88). Anglers in the *Mixed* group were opposite – they had their favorite fisheries closer less often (PDI = 0.89). They do, however, have their favorite fishery more than 30 km from their place of residence (31.1% and PDI = 1.31). The affiliation question seems to be perceived quite differently by all groups. Although in each group, more than two-thirds confirm that they were affiliated, but this varies widely. Anglers in the *Cultural* group were most often affiliated and only less than 1 in 6 were not. In the *Provisioning* group, 30.8% were unaffiliated (PDI = 1.61). The *Mixed* group seems to be in between the *Provisioning* and the *Cultural* group in this regard.

Table 2

Sociodemographic, economic, and engagement characteristics of groups of anglers divided based on their preference to choose the predominant angling ES (N = 1292)

Characteristics		Cultural (N = 885)		Provisioning (N = 201)		Mixed (N = 206)	
		N	%	N	%	N	%
I. Sociodemographic and economic							
1. Gender	male	831	93.9	186	92.5	188	91.3
	female	54	6.1	15	7.5	18	8.7
2. Age	less than 19	33	3.7	6	3.0	12	5.8
	19–25	84	9.5	26	12.9	20	9.7
	26–40	393	44.4	93	46.3	99	48.1
	41–65	321	36.3	64	31.8	66	32.0
	66–75	54	6.1	12	6.0	9	4.4
3. Earnings per year [€]	less than 5 000	223	25.2	63	31.3	54	26.2
	5 000–12 000	454	51.3	100	49.8	96	46.6
	11 000–24 000	155	17.5	26	12.9	39	18.9
	more than 24 000	53	6.0	12	6.0	17	8.3
4. Education	primary/vocational school	132	14.9	34	16.9	35	17.0
	secondary	390	44.1	80	39.8	80	38.8
	higher	363	41.0	87	43.3	91	44.2
5. Marital status	married	484	54.7	104	51.7	120	58.2
	not married (also widows/ widowers and single)	234	26.4	58	28.9	51	24.8
	in partnership	167	18.9	39	19.4	35	17.0

cont. Table 2

6. Place of residence	Village	222	25.1	54	26.9	37	18.0
	town to 25 thousand inhabitants	226	25.5	51	25.4	54	26.2
	a city with 25 to 100 thousand of inhabitants	176	19.9	39	19.4	43	20.9
	a city with over 100 thousand of inhabitants	261	29.5	57	28.3	72	34.9
7. Employment status	working	676	76.4	154	76.6	163	79.1
	unemployed (including student, pensioner, and retiree)	209	23.6	47	23.4	43	20.9
II. Engagement in angling							
1. How long have you been angling?	less than 10 years	207	23.4	53	26.4	48	23.3
	10–20 years	216	24.4	51	25.4	56	27.2
	20–30 years	228	25.8	43	21.4	52	25.2
	30–40 years	123	13.9	24	11.9	29	14.1
	more than 40 years	111	12.5	30	14.9	21	10.2
2. How often do you fish?	a few times a year	101	11.4	22	11.0	38	18.4
	a dozen or so times a year	67	7.6	22	11.0	16	7.8
	about 2–3 times a month	198	22.4	31	15.4	40	19.4
	about once a week	284	32.1	70	34.8	70	34.0
	a few times a week	235	26.5	56	27.8	42	20.4
3. How much money do you spend on your hobby per year?	up to 25 €	41	4.6	8	4.0	4	1.9
	25–100 €	128	14.5	29	14.4	33	16.0
	100–250 €	223	25.2	64	31.8	63	30.6
	251–500 €	239	27.0	54	26.9	55	26.7
	more than 500 €	254	28.7	46	22.9	51	24.7
4. What is the distance you most often travel to fish?	less than 5 km	263	29.7	69	34.3	55	26.7
	5–30 km	422	47.7	90	44.8	87	42.2
	more than 30 km	200	22.6	42	20.9	64	31.1
5. Affiliation in angling association/club	yes	746	84.3	139	69.2	160	77.7
	no	139	15.7	62	30.8	46	22.3

Two data series (2019 and 2021) were subjected to a test of statistical differences between responses to the same questions about angling behavior and preferences during and before the pandemic. Only preferences for

angling in rivers and streams and angling with the presence of family changed in a statistically significant way during the pandemic time regarding earlier period (Table 3). During the pandemic, the preference for angling with family declined, it should be noted, however, that anglers tended to have a negative attitude toward it regardless of the year of the survey (averages below 3 on the Likert scale). The preference for angling in rivers and streams increased significantly. In addition, it should be noted that this was not at the expense of a decline in preference for angling in lakes. Nevertheless, it is worth mentioning that all statistically significant differences have a significant variance (high SD).

Table 3

Differences between datasets used in the study (2019 and 2021)

Anglers' behavior and preferences	N = 722 2019	N = 570 2021
I release caught fish according to the 'no-kill' principle	3.99±1.19	3.88±1.17
The possibility of keeping each caught fish is important to me	2.18±1.34	2.21±1.32
I prefer to fish in lakes	3.91±1.28	3.94±1.25
I prefer to fish in rivers and streams*	3.55±1.43^{A}	3.79±1.39^{B}
I prefer to fish in artificial water bodies (ponds, artificial lakes, etc.)	2.48±1.29	2.35±1.38
I fish alone	3.01±1.32	3.11±1.36
I fish with my family**	2.69±1.43^{A}	2.44±1.31^{B}
I fish with my friends	3.35±1.33	3.40±1.41

The questions were measured by a 5-point Likert scale

Values with various superscripts (A; B) are significantly different using a one-way ANOVA and a Tukey HSD post hoc test for unequal N (df = 1290). * p = 0.0040; ** p = 0.0021

Based on the selection of anglers into the groups from the methodology section (Table 1), naturally, the two cross-questions were statistically significantly different and characterized by great between-group discrepancies which can be seen in Table 4. However, the other following observations were made in this regard. There was a very high number of undecideds in the *Mixed* group (57.3%) in both questions (Table 5). It was also relatively high in the *Provisioning* group (31.8%) for no-kill reference. The anglers in the *Cultural* group were the most confident in their answers, as indicated by the low percentage of "3" answers given on these questions on a Likert scale and also by averages closer to the mean extremes on a 5-point scale.

Table 4

Differences between different anglers' ES preference groups. Results are presented using the mean score ±SD

Anglers' behavior and preferences	Cultural	Provisioning	Mixed
I release caught fish according to the 'no-kill' principle*	4.52±0.65^{A}	2.00±0.80^{B}	3.34±0.94^{C}
The possibility of keeping each caught fish is important to me**	1.46±0.68^{A}	4.25±0.69^{B}	3.34±0.98^{C}
I prefer to fish in lakes	3.93±1.22	3.78±1.42	4.04±1.29
I prefer to fish in rivers and streams***	3.72±1.43^{A}	3.30±1.42^{B}	3.69±1.31^{A}
I prefer to fish in artificial water bodies (ponds, artificial lakes, etc.)	2.50±1.33	2.25±1.37	2.28±1.31
I fish alone	3.04±1.33	3.08±1.40	3.10±1.30
I fish with my family	2.53±1.36	2.68±1.47	2.66±1.39
I fish with my friends♦	3.48±1.35^{A}	3.17±1.43^{b}	3.12±1.31^{B}

$^{A, B, C}$ values with various superscripts are significantly different between angling ecosystem service choice groups different using a one-way ANOVA and a Tukey HSD post hoc test for unequal N (df = 1289): $^{*; **}$ $p = 0.0000$; *** $p = 0.0069$ for C-P and $p = 0.0136$ for M-P; ♦ $p = 0.0195$ for C-M, and ^{b} $p = 0.0603$ for C-P

Table 5

Anglers' ES preference groups. Frequencies of anglers agreeing with (agree and strongly agree) and denying (disagree and strongly disagree) in the environment and social-related questions (in percent)

Anglers' characteristics and behavior	Cultural		Provisioning		Mixed	
	agreeing	denying	agreeing	denying	agreeing	denying
I release caught fish according to the 'no-kill' principle	91.3	–	–	68.2	33.0	9.7
The possibility of keeping each caught fish is important to me	–	89.5	85.6	–	33.0	9.7
I prefer to fish in lakes	66.2	15.3	64.2	24.4	69.4	12.1
I prefer to fish in rivers and streams	60.2	21.4	48.8	32.3	51.5	14.6
I prefer to fish in artificial water bodies (ponds, artificial lakes, etc.)	21.9	51.9	18.9	61.7	16.5	56.3
I fish alone	34.8	38.9	42.3	28.4	34.0	26.2
I fish with my family	24.1	53.1	49.8	25.7	25.7	51.0
I fish with my friends	51.8	25.1	29.9	37.9	37.9	32.0

Respondents from the *Provisioning* group showed significantly less keenness on angling in rivers and streams than the two other groups. It should be also noted that they were more temperate when it came to indicate a preference for angling in any specified type of water (lakes, rivers, or artificial). This group had the highest percentage of anglers indicating that they do not prefer to fish in all the indicated aquatic environments.

However, only 33 anglers indicated that they do not prefer any of the indicated environments while 65.5% of this amount were *Provisioning* anglers. The results indicate that the anglers surveyed were evenly spread across the country (Figure 2) and likewise their favorite fishing grounds (Figure 3) indicating that angling is popular regardless of the location of residence and the number of water bodies in the community. The location of any indicated places of residence and favorite fishing grounds in Europe can be found in Appendix 2 (Figure 2.1, Figure 2.2).

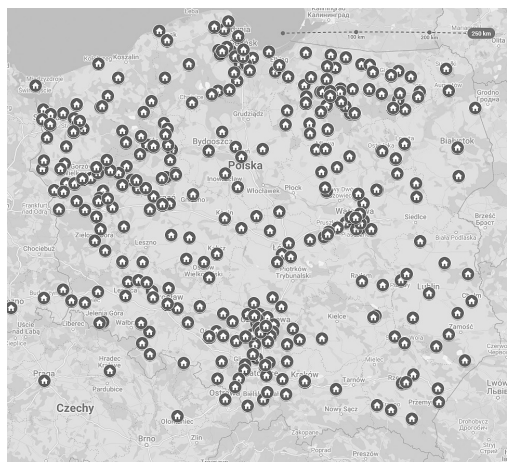

Fig. 2. Location of the communities of origin of the surveyed anglers (N = 678)
Source: own elaboration based on Google My Maps (Maps data: ©2023 GeoBasis-DE/BKG (©2009), Google, Inst. Geogr. National

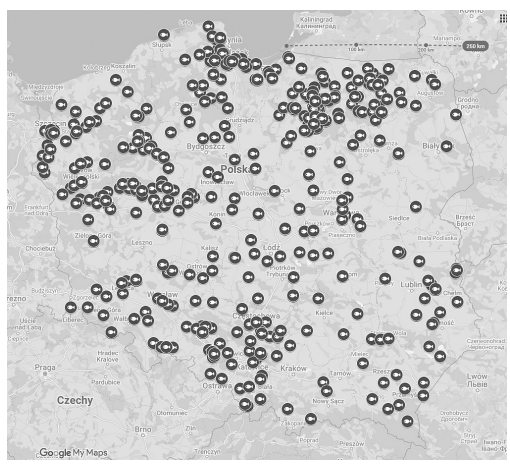

Fig. 3. Location of the favorite fishing grounds of the surveyed anglers (N = 670)
Source: own elaboration based on Google My Maps (Maps data: ©2023 GeoBasis-DE/BKG (©2009), Google, Inst. Geogr. National

All the groups showed no differences in attitudes toward angling alone (a rather neutral attitude) and angling with family (a moderately negative attitude). In terms of fishing in the presence of friends, anglers in the *Cultural* group show a moderately positive attitude towards this type of activity, while the other two groups were rather neutral on this issue. They most often indicate that they fish with friends (51.8%, which was 9 percentage points higher than *Provisioning* and 13.9 percentage points higher than *Mixed*). They were also the most likely to indicate that they do not fish alone or with family.

In the results of the interaction trees pre-analysis (Appendix 3, Figure 3.1, Figure 3.2), it was shown that the factor that most differentiated all groups internally was their affiliation status. In this regard, all groups were significantly different. The results of this separation can be seen in Figures 4 and 5. Consequently, follow-up analyses were carried out based on the assignment of groups of anglers to 6 distinct subgroups separating the 3 main groups according to the affiliated-unaffiliated dividing line.

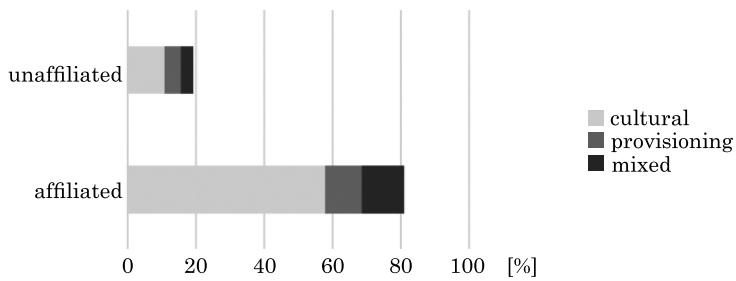

Fig. 4. Proportion of affiliated and unaffiliated anglers in three identified groups choosing different angling ES approach: cultural provisioning and mixed

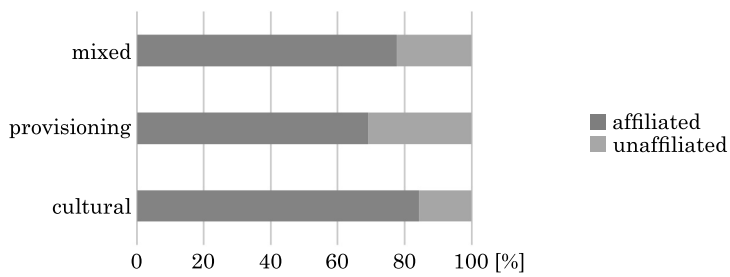

Fig. 5. Proportion of cultural, provisioning, and mixed groups in two affiliation groups: affiliated and unaffiliated

All preferences toward fisheries and social relationships were tested in a reduced ordination space using NMDS (Figure 6). With a stress value of 0.0003, this analysis visualized similarities and dissimilarities among anglers of different ecosystem service use and affiliation status. Most of the indexes included in the analysis were negatively correlated with the NMDS 1 axis (Appendix 4, Table 4.1). This axis explains 74.6% of the total variation. The separation of the affiliated *Cultural* ES group was most strongly determined by their preference for fishing in artificial reservoirs and in the company of friends. In turn, the separation in the ordination space of anglers from the *Provisioning*-affiliated group (PA) and *Mixed*-affiliated (MA) groups was due to this group's preference for river fisheries and angling in the company of family. The preference for angling alone especially in lake fisheries had the greatest influence on the separation of *Mixed*-unaffiliated group. In contrast, environmental-sociological preferences proved least useful in characterizing unaffiliated anglers with identified cultural or provisioning behavioral backgrounds.

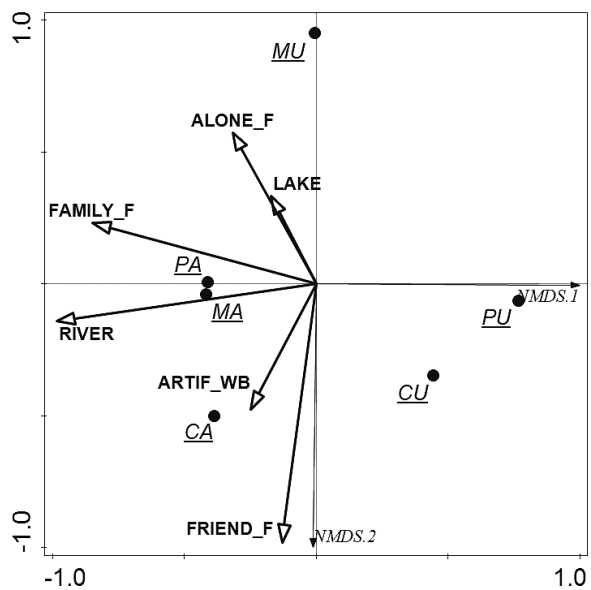

Fig. 6. NMDS triplot based on preferences toward different waterbodies and the sociological aspect of angling among anglers identified with different types of ecosystem services use
Abbreviations: LAKE – preference for fishing in lake; RIVER – preference for fishing in rivers and streams; ARTIF_WB – preference for fishing in artificial waterbodies; ALONE_F – preference for fishing alone; FAMILY_F – preference for fishing with family; FRIEND_F – preference for fishing with friend; CA – Cultural affiliated; PA – Provisioning affiliated; MA – Mixed, provisioning-cultural, affiliated; CU – Cultural unaffiliated; PU – Provisioning unaffiliated; MU – Mixed, provisioning-cultural, unaffiliated.

In ordination space, correlations were determined between preferred fishery and accompanied angling in anglers with different affiliation statuses and connection to ES and economic-demographic variables (Figure 7).

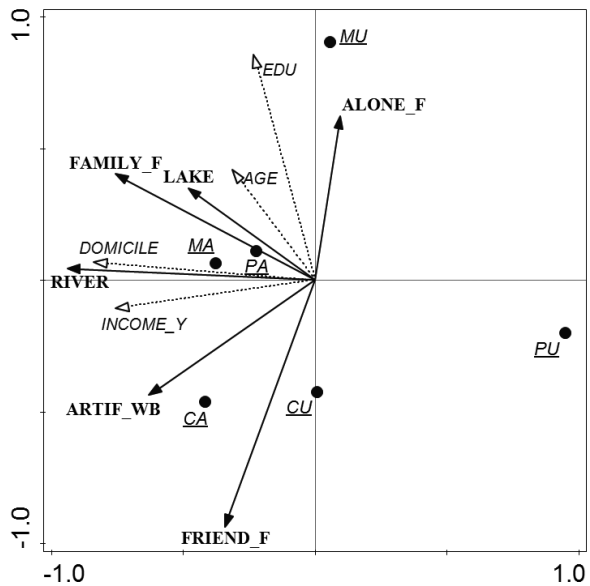

Fig. 7. Triplot of ordinal redundancy analysis (RDA) of environmental and sociological preferences among anglers with different kinds of ecosystem services use and various association statuses (response data, arrows) versus demographic-economic factors (explanatory variables, dotted arrows). Abbreviations: LAKE – preference for fishing in a lake; RIVER – preference for fishing in rivers and streams; ARTIF_WB – preference for fishing in artificial ponds; ALONE_F – preference for fishing alone; FAMILY_F – preference for fishing with family; FRIEND_F – preference for fishing with a friend; INCOME_Y – annual income; DOMICILE – place of residence expressed in a number of inhabitants; AGE – age expressed in a number of years; EDU – educational level; CA – Cultural affiliated; PA – Provisioning affiliated; MA – Mixed, provisioning-cultural, affiliated; CU – Cultural unaffiliated; PU – Provisioning unaffiliated; MU – Mixed, provisioning-cultural, unaffiliated.

For each group of anglers, the correlation of all axes was significant by Monte Carlo permutation test ($F = 3.31$, $p = 0.044$) with a total variance of 17.47 and explanatory variables accounting for 83.8% of the variance. The sum of all canonical eigenvalues was 0.8384 (Appendix 5, Table 5.1). The first two components of RDA explained 96.86% of the total variance in the response data, with the first axis accounting for 75.62%. The preference for fishing alone, a characteristic of MU anglers, was most positively correlated with educational level. In contrast, among PA and CA anglers, the preference for lake angling and for fishing with family correlated most positively with age. At the same time, among these anglers, the preference

for river angling increased with their average annual earnings and residence in larger urban areas. Among *Cultural* anglers of different association statuses, the preference for fishing in artificial reservoirs and in the company of friends correlated in varying degrees with average earnings (positively) and with age and level of education (negatively).

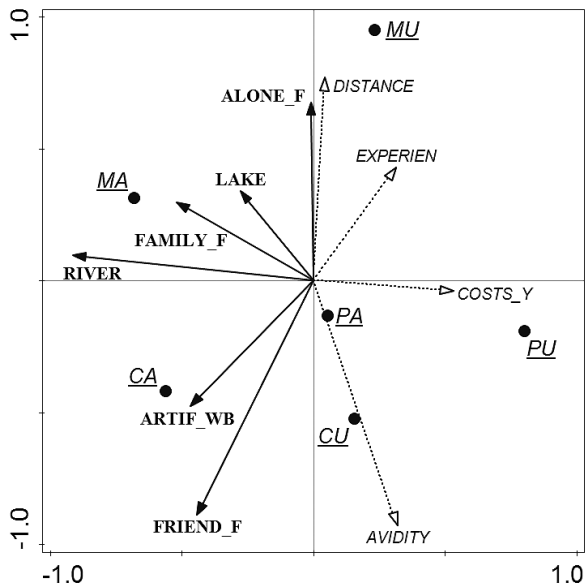

Fig. 8. Triplot ordinal redundancy analysis (RDA) of environmental and sociological preferences among anglers with different kinds of ecosystem services and various association statuses (response data, arrows) versus engagement indices (explanatory variables, dotted arrows). Abbreviations: LAKE – preference for fishing in a lake; RIVER – preference for fishing in rivers and streams; ARTIF_WB – preference for fishing in artificial water bodies; ALONE_F – preference for fishing alone; FAMILY_F – preference for fishing with family; FRIEND_F – preference for fishing with a friend; DISTANCE – distance to the most visited angling spot; AVIDITY – avidity expressed by the frequency of angling; EXPERIEN – experience expressed by years of engagement in angling; COSTS_Y – average annual spending on angling; CA – Cultural affiliated; PA – Provisioning affiliated; MA – Mixed, provisioning-cultural, affiliated; CU – Cultural unaffiliated; PU – Provisioning unaffiliated; MU – Mixed, provisioning-cultural, unaffiliated.

For RDA analysis of the relationship between respondents' preferences and indicators of their engagement in angling (Figure 8), for each group of respondents, the correlation of all axes was significant in a Monte Carlo permutation test ($F = 3.68$, $p = 0.038$) with a total variance of 24.77 and explanatory variables accounting for 86.4% of the variance. The sum of all canonical eigenvalues was 0.8636 (Appendix 6, Table 6.1). The first two components of RDA explained 84.74% of the total variance in the

response data, with the first axis accounting for 65.63%. The preferences of MA anglers were negatively correlated with spending and angling frequency in varying degrees of intensity. Increasing the frequency of angling had the strongest effect on separating CN anglers in the ordination space. In contrast, the preference of CA anglers showed the strongest negative correlations with angling experience and distance from the most frequently visited fishing grounds. The preference of MU anglers for angling alone was most strongly positively correlated with distance traveled to fishing grounds and somewhat less strongly with experience. At the same time, the behavior of these anglers was negatively correlated with the frequency of angling. They were also least keen on angling in a group of friends. The placement of the PA group close to the axis intersection indicates that they were the most difficult to identify under the influence of the indicated factors and have the least connection with them. In contrast, the PU group show a positive correlation with angling expenditures however, they were found to be the least correlated with the other indicators of both engagement and environmental-sociological components of analysis.

Discussion

Duality of angling ES in the social-ecological system

The results of the study show that angling in terms of ecosystem services demonstrates some kind of duality in its basis. The anglers in the groups, named in this work, *Provisioning*, and *Cultural* are people who indicate quite clearly the nature of the final service they expect from angling recreation. For the first, provisioning is important, while for the second it is not the most important part of angling. However, it should be borne in mind that this distinction is not certain and unchangeable. As Ostrom (2007) and Ostrom et al. (2007) wrote, in the case of social-ecological systems and the internal connections of these systems, one cannot think that it will be easy to find solutions to the problems of human use of the environment. And this use brings great benefits to the whole world year after year and should be managed wisely (Organa 2017, Tufts et al. 2015). SES management is very difficult, as they are very complex systems and the same behaviors can give different outcomes in different places (Ostrom 2007, Ostrom et al. 2007). It would be foolish to think that scientists, or even more so, politicians, will be able to effectively manage the space at the human-environment interface in a simple way. It, therefore, requires, a colossal amount of interdisciplinary, social geography, work, of which this article is a part, and as much knowledge as possible about all

the components of this system. It also requires the development of good practices that will be implemented by all users and participants of the system and, finally, educational programs.

Social-ecological systems, among them, recreational fisheries as a highly coupled SES (ARLINGHAUS et al. 2016, 2017), can depend on local factors, change non-linearly over time and operate differently in different types of space depending on their adaptive capacity and resilience. Such systems are also at high risk of experiencing so-called black swans (NUÑEZ and LOGARES 2012, TALEB 2007). A good example would be changes that occurs from a pandemic. With two sets of data (2019 and 2021), it was indicated that family ties while angling, lost strength, although it is worth noting that they were already not very strong before the pandemic. At that time recreational activities, even outdoors in fairly safe locations as angling (e.g. MIDWAY et al. 2021, KARPIŃSKI and SKRZYPCZAK 2022, SKRZYPCZAK et al. 2022), were prohibited or temporarily banned with the "closure" of some public spaces including recreation sites, e.g. parks forests or other locations by the water (e.g. FREEMAN and EYKELBOSH 2020, VOLENEC et al. 2021). In this period the preference to fish in rivers, which are less exposed, and on the other hand, more accessible internationally and in Poland than lakes (DILL 1993, MEYBECK 1995, SOBOLEWSKI et al. 2014), increased at the expense of artificial waterbodies. However, it is unclear whether these changes have had a temporal character or have already permanently entered people's consciousness.

The concept of final service is crucial because, between the start of angling and the final service drawn from angling, there are a number of activities of different nature. This character is mainly cultural in origin containing a whole series of services from the biotic and abiotic part of cultural ES (HAINES-YOUNG and POTSCHIN 2018). Identifying the cultural services of recreational fisheries in particular, rather than attempting to measure these services in monetary terms, is essential to better resource management (LIU et al. 2019, WINFIELD 2016) However, it should be remembered that anglers can look for a variety of ES benefits while fishing. These include, of course, *Provisioning* (JOBSVOGT et al. 2014, WINFIELD 2016). Although it should be clear that the nature of this ES is mainly the domain of commercial fishing – not recreational. Final ES is not well distinguished in policies, for example in the Guidance on the Application of CICES HAINES-YOUNG and POTSCHIN (2018) is not clear whether angling is more cultural or provisioning ES. The CICES spreadsheet (CICES 2023) points out, that angling is a cultural ES, for example "using the environment for sport and recreation; using nature to help stay fit", or "using nature to destress"– which is generally what angling is. But

from the other side in the above-mentioned Guidance (HAINES-YOUNG and POTSCHIN 2018) in the chapter "final services and concept" there is the following sentence: "If the focus is on the service of recreational fishing, the fish caught would be regarded as a final service" which is not entirely true and needs to be resolved, because in purely recreational fishing, fish are only the means to satisfy a set of needs, e.g.: excitement, self-fulfilment, social ties, and especially contact with nature (SKRZYPCZAK and KARPIŃSKI 2020).

Angling and its internal connections are not black and white. It is important to recognize this gradient shifting towards provisioning or cultural ES, for particular groups of people, especially for those in the non-identified *Mixed* group. As the results showed, *Mixed* takes on a different character forming, on the one hand, a group of unaffiliated solitary anglers angling least often in the presence of other people (3.6%) and affiliated anglers, who are similar in their preferences to anglers in the affiliated *Provisioning* group (making up a significant group of about 23.1% of the anglers surveyed). The matter of affiliation in an organization or angling club in the context of angler behavior has gathered some interest from researchers already (COPELAND et al. 2017, GUPTA et al. 2016, KOHL et al. 2002, SCHRAMM and GERARD 2004, SKRZYPCZAK and KARPIŃSKI 2020) and it is shown, that affiliated group could be crucial for the management of waters, especially, when they are cooperating with fishing site managers. They are more accepting of fish release and participate in water protection as well as tending to be more specialized, ergo, more precise and causing less harm to the environment. They are also potentially easier to educate. In contrast to them, there are unaffiliated anglers who are characterized in research as more consumptive and invasive (SCHRAMM and GERARD 2004, SKRZYPCZAK and KARPIŃSKI 2020). This article also shows that unaffiliated anglers are more difficult to characterize, less decisive, and less predictable in their choices and preferences. Unaffiliated anglers in the *Provisioning* group have the lowest preference for the "no-kill" but their willingness to take fish is no higher than the affiliated *Provisioning* group. Which may indicate less respect for wildlife. However, it should be noted that the possibilities and nature of affiliation also depend on the specifics of angling law in the particular country. *Cultural* anglers regardless of the association are rather related to the social nature of angling.

Choice of final angling ecosystem service versus ethical and legal status

The different approaches to handling caught fish and, consequently, the choice of the final ES also involve ethical issues of dealing with living organisms. The catch and release and catch and keep approaches are not right or wrong, and both approaches to angling have their pros and cons (ARLINGHAUS et al. 2007). At first glance, it appears that *Provisioning* angling is closer to subsistence fishing and is worse for the environment, because of the depletion of fish from waterbodies (BOOI et al. 2022). However, in the context of behavior toward a living organism, this behavior appears to be less controversial (ARLINGHAUS et al. 2007). If carried out in a proper, skillful manner, it seems more "merciful" than releasing a fish that is alive but not fully healthy. We should keep in mind that some of the fish due to various types of events (e.g.: lack of angling skills, conditions, swallowing the bait too deep) finally, do not survive and cannot be a part of the angling ES once again. Then it is not counted as a cultural part, nor provisioning. Studies indicate that mortality, depending on conditions, can be as high as 90%. However, such fish are not wasted, as in the vast majority of cases they provide biomass, but instead of for humans, for other predatory fish, or birds (BARTHOLOMEW and BOHNSACK 2005, LEWIN et al. 2018). The problem of human conduct with other living organisms and ethical issues in this regard has already been recognized by social geography researchers trying to explore the background and consequences of such behavior (among others: BRAITHWAITE and BOULCOTT 2007, KOTUS 2022, PANELLI 2010, ROSE et al. 2014, SNEDDON 2009). However, it seems that the golden mean on this issue will never be found due to the differences between the following opposite approaches: human, as the "crown of creation", and human, as merely a part of the world. This has also been turned into binding laws in some countries, such as Germany, Austria, and Switzerland (ARLINGHAUS et al. 2007, MICHEL and KAYASSEH 2011). The clause "prohibition of abuse of vertebrates" is indicated there. Angling falls under this clause and forces anglers to kill caught fish as quickly and "humanely" as possible (with few exceptions, such as protected fish caught by accident). The results of this work, as well as others (KARPIŃSKI and SKRZYPCZAK 2021, SKRZYPCZAK and KARPIŃSKI 2020), indicated that the tendency to release fish is more clear than the tendency to keep them. Among anglers opting to keep fish, unwillingness to release them is lower than in the corresponding opposite situation. There is either a bias of unwillingness to answer truthfully, but not really legally, or simply that the need to keep fish for anglers, in general, is lower than the need to

release them. It is also worth noting briefly here that in both non-cultural groups, the percentage of women reflected in the PDI index was significantly higher. This may be related to the characteristics of women in recreational fishing already indicated in the studies (SCHROEDER et al. 2006, SKRZYPCZAK and KARPIŃSKI 2020) as seeking more utilitarian, concrete benefits from angling.

Environmental and social aspects of the cultural and provisioning background of angling

The results show the greatest preference for fishing in lakes, regardless of any group of respondents. Lakes accumulate in the northern part of Europe and Poland's landscapes covering the main areas of the last glaciation where lakes are more common (MARKS 2012). Rivers are much more evenly geographically distributed throughout the country, therefore, are more accessible, while reservoirs and artificial waters were found throughout the whole of Poland, but they are predominantly located in the southern part (LAKES 2023). The nature of potential pressure magnitude on the respective water types seems to be more correlated with their quantity than with their accessibility (GUS 2022). The pressure on lakes is the highest, as 2 out of 3 anglers prefer them. The lowest is in artificial water bodies. However, it should be remembered that this is only a preference and may differ from the actual number of hours of angling on the types of waters and a single angler may prefer different types of waters at the same time and fish on them. The fact that for artificial reservoirs the most characteristic (21.9% preferring this type of water) is the most numerous group of *Cultural* anglers seems to be a positive trend. Although the preference is still rather negative. Due to the often anthropogenic nature of these reservoirs, they reduce somewhat the pressure on natural waters. *Cultural* anglers, however, seem to be more versatile in their preference for angling in different types of reservoirs. *Provisioning* anglers seem to be the least diverse in this regard, especially the most elusive in indicating pressure on reservoirs was the group of unaffiliated *Provisioning* anglers (4.8%), who are the most difficult to capture in any framework of choice or preference, whether water or socioeconomic, or involvement and they are the ones who could potentially represent the greatest source of uncertainty about behavior on the water. It is also interesting to note the lack of any preference was shown by 1 in 40 respondents. While their importance to the whole may seem marginal, one has to wonder whether this is due to indifference to angling on any water bodies, or whether there is another reason for it.

Aspects of angling in a presence of other people appear to be linked in some groups to a preference for the final ES used. The *Mixed* group is similar to the *Cultural* in cases of fishing alone or with family, but they prefer less angling with friends. On the other side are *Provisioning* anglers who are more likely to fish with family or alone, while the *Cultural* ones are most likely to fish in the company of friends. Perhaps the presence of people you do not directly influence makes anglers more hesitant to take the fish they catch. There is, some kind of pressure on *Provisioning* to behave "properly" (whatever anglers think it means) towards sustainable development but, in reality, another angler who might catch the fish in the future. This behavior is reflected in the theory of "Social Norms" in terms of both fellow anglers and bystanders, who can also influence anglers' decisions around the water (Berkowitz 2005, Grasmick and Green 1980). The theory indicates that individuals mistakenly perceive the attitudes and behaviors of other people as different from their own, when in fact they are not. It is called "pluralistic ignorance" and it often occurs with problematic or risky behaviors (which tend to be overestimated) and with healthy or protective behaviors (which tend to be underestimated).

As the study by Bova et al. (2017) showed, anglers can manifest this type of ignorance. Despite having a fairly high attachment to the rules, they tend to overestimate inappropriate behaviors in other anglers, especially as the social distance from those anglers increases. This may also be related to the aforementioned development of animal rights, which causes anglers to view others, usually non-anglers, as behaving inappropriately (Riepe and Arlingaus 2014).

One of the effects of pluralistic ignorance is that individuals may change their behavior to get closer to the misperceived norm, i.e. to behave on the fishing ground in the best manner and such behavior can occur among anglers and can be a positive factor as long as the angling community can show best practices and social disapproval for unethical or incorrect behavior. However, this situation is related not only to the perception of social norms, but also to other factors, not dependent on society, but on the internal desires of a particular angler or group of anglers, dependent on a number of socioeconomic factors or internal motivational needs. Among them, the aforementioned tendency of women to keep fish was identified. Women also show a greater desire to fulfill social ties while angling (Skrzypczak and Karpiński 2020) and so are more likely to fish in the company of family. These factors cannot be neglected and should be developed in further studies of the topic.

Conclusions

Given that *Cultural* anglers are the most numerous, and, in the largest proportion affiliated, there is a reasonable assumption that these are the anglers who will be most likely to submit to suggestions on environmental use from legislators as well as managers. Any form of education aimed at better use of the environment and reducing the negative impact on it should start in this community to have the best effect. Managers should seek to organize anglers, as this seems to be of environmental and social benefit to them even in small communities and on artificial waters. Affiliated *Cultural* anglers can serve as a driving force for introducing more sustainable angling solutions and, through their social control, influence other anglers as well. *Cultural* anglers are closer to the values of angling as an activity that is more about angling than catching a fish and their ES use is more broad and complicated to valuation than provisional anglers, which are easier to quantify in money.

With the introduction of the association factor, as the dividing line between anglers, respondents classified in the *Mixed* group for the most part adopt provisioning behavior, while the remaining part forms a separate group rather than being closer to the *Cultural* group. Affiliation is a clear reinforcement of the social component in the angling and cultural part of ES.

The result shows that anglers expecting measurable in biomass benefits (ES) from angling account for between 4.5% and 31.5% of anglers' population depending on the magnitude of desire to keep fish, but more rigorous research is needed to determine the specific behavioral characteristics of this group. The unaffiliated anglers are generally less likely to prefer artificial waters and, given their hard-to-define preferences, they may be the most unpredictable angling users of natural water bodies.

A comparison of behavioral preferences from the two surveys indicated that the pandemic had no substantial impact on the behavior shift of surveyed anglers.

Biases and future studies

The author is aware that this is a preliminary study that raises the issue of the heterogeneity of angler ecosystem services more than it solves it. In future research on this topic, researchers should focus on a more insightful attempt to divide anglers based on multiple differentiating criteria than the final ES criterion used in this article. For example, it is known what expectations and preferences for handling fish these groups have, but we do not know what social and environmental behaviors they exhibit during angling.

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Appendix 1

Table 1.1

The full survey questionnaire

Question	Options					MoE* [%]
I. Sociodemographic, economic, and engagement in angling						
Age	type in the correct value according to your best knowledge					1.1–2.7
Income (per month)						1.3–2.7
Place of residence (in thousands of inhabitants)						2.2–2.5
How long have you been angling for? (in years)						1.8–2.4
How often do you fish? (days in a year)						1.5–2.6
How much money do you spend on your hobby (equipment expenses, licenses, travel, etc.) per year (in Euro)?						1.1–2.4
What is the distance you travel to your most visited fishing spot? (in kilometers)?						2.3–2.7
Name of the municipality or district where you live						–
What is the name of your favorite fishing spot?						–
Gender	male		female	–		1.4
Education	primary		secondary	higher		2.0–2.7
What is your marital status?	married		not married	partnership		2.1–2.7
What is your employment status	employed		unemployed	–		2.3
Are you a member of an angling organization/association?	yes		no	–		2.1
II. Perceptions and behaviors towards angling**						
I release caught fish according to the 'no-kill' principle	1	2	3	4	5	1.3–2.7
The possibility of keeping each caught fish is important to me	1	2	3	4	5	1.5–2.7
I prefer to fish in lakes	1	2	3	4	5	1.3–2.7
I prefer to fish in rivers and streams	1	2	3	4	5	1.8–2.7
I prefer to fish in artificial water bodies (e.g., ponds, artificial lakes, etc.)	1	2	3	4	5	1.6–2.6
I fish alone	1	2	3	4	5	1.9–2.5
I fish with my family	1	2	3	4	5	1.9–2.5
I fish with my friends	1	2	3	4	5	1.8–2.5

* MoE – margin of sampling error [%] at 95% confidence interval calculated for the distinguished groups (see Table 3). MoE for Likert scale questions was calculated for every option from 1 to 5.

** If you do not agree with the statement, please circle "1" (Strongly disagree) or "2" (Disagree). If you agree with the statement, please circle "5" (Strongly agree) or "4" (Agree). If you do not have an opinion on a given topic or it is difficult to determine it, then please circle "3" (I have no opinion – neutral).

Appendix 2

The surveyed anglers – location of the communities of origin and favorite fishing grounds

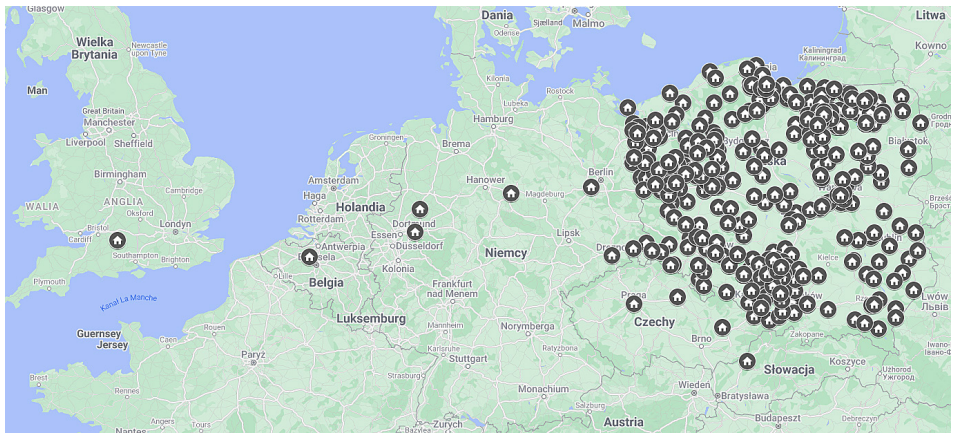

Fig. 2.1. Location of the communities of origin of the surveyed anglers (N = 685)
Source: own elaboration based on Google My Maps (Maps data: ©2023 GeoBasis-DE/BKG (©2009), Google, Inst. Geogr. National)

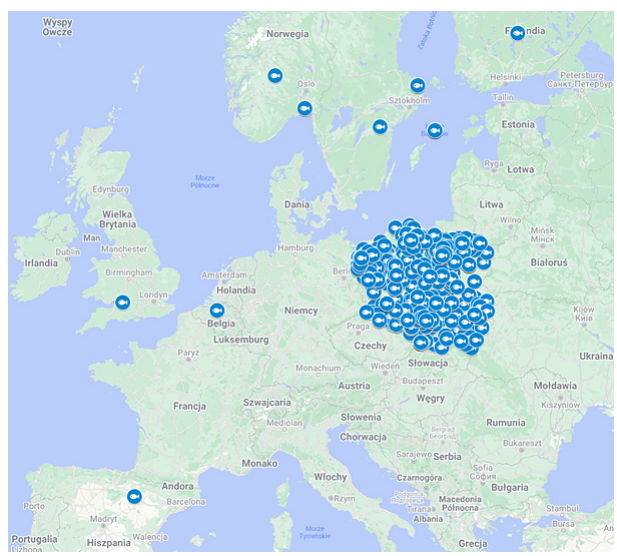

Fig. 2.2. Location of the favorite fishing grounds of the surveyed anglers (N = 680)
Source: own elaboration based on Google My Maps (Maps data: ©2023 GeoBasis-DE/BKG (©2009), Google, Inst. Geogr. National)

Appendix 3

Tree graph for different ES grups (1st node)

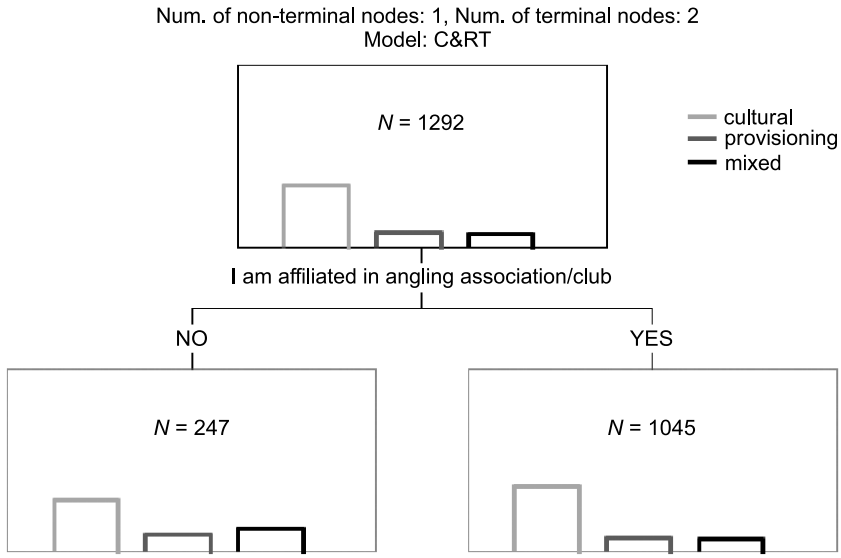

Fig. 3.1. Results of interaction tree pre-analysis for all distinguished ES preferred use groups (only 1st node)

Appendix 4

Supplementary Table for Figure 6

Table 4.1

Response of environmental and sociological preferences of anglers with different kinds of ecosystem services and various association statuses to NMDS 1 and NMDS 2

Anglers' characteristics	NMDS 1	NMDS 2
LAKE, preference for fishing in a lake	-0.1711	0.3335
RIVER, preference for fishing in rivers and streams	-0.9842	-0.1407
ARTIF_WB, preference for fishing in artificial ponds	-0.2487	-0.4752
ALONE_F, preference for fishing alone	-0.3169	0.5747
FAMILY_F, preference for fishing with family	-0.8473	0.2324
FRIEND_F, preference for fishing with a friend	-0.1294	-0.9796

Appendix 5

Supplementary Table for Figure 7

Table 5.1

Summary statistics for RDA of anglers' environmental and sociological preferences with different kinds of ecosystem services and various association status (response data) versus demographic-economic factors (explanatory variables, *VIF** < 10)

Axes	1	2	3	4	Total variance
Eigenvalues:	0.6340	0.1780	0.0226	0.0037	1.000
Pseudo-canonical correlation	0.9766	0.9990	0.8293	0.7257	–
Cumulative percentage variance					
of response data:	63.40	81.20	83.46	83.84	–
of fitted response data:	75.62	96.86	99.56	100.00	–
Sum of all eigenvalues	–				1.0000
Sum of all canonical eigenvalues	–				0.8384

* variance inflation factor

Appendix 6

Supplementary Table for Figure 8

Table 6.1

Summary statistics for redundancy analysis of environmental and sociological preferences among anglers with different kinds of ecosystem services and various association status (response data) versus engagement indices (explanatory variables, *VIF** < 10)

Axes	1	2	3	4	Total variance
Eigenvalues:	0.5668	0.1651	0.1237	0.0080	1.000
Pseudo-canonical correlation	0.9229	0.9560	0.9949	0.6877	–
Cumulative percentage variance					
of response data:	56.68	73.18	85.55	86.36	–
of fitted response data:	65.63	84.74	99.07	100.00	–
Sum of all eigenvalues	–				1.0000
Sum of all canonical eigenvalues	–				0.8636

* variance inflation factor

Native speaker correction by James Gypson

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