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EFFECT OF *FICUS ASPERIFOLIA* AQUEOUS EXTRACT ON SEMEN QUALITY, TESTICULAR HISTOLOGY AND REPRODUCTIVE PERFORMANCE OF NEW ZEALAND WHITE RABBITS RAISED UNDER TROPICAL CONDITION

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Key words: rabbits, *Ficus asperifolia* leaves extract, reproductive performance, semen characteristics.

Abstract

Thirty-six rabbits consisting of two groups of 18 bucks and 18 does were allotted into three treatment groups (T1, T2 and T3 : 0 ml, 10 ml and 20 ml of aqueous *Ficus asperifolia* leaves extract, respectively) on a weight equalization bases in a completely randomized design. Data collected on the semen characteristics and reproductive performance were subjected to One Way analysis of variance; significant means were separated using Duncan-Multiple Range Test while data collected on reproductive hormones were subjected to descriptive statistical representation. Results revealed that *Ficus asperifolia* leaves extract significantly ($p < 0.05$) influenced the testosterone and double head sperms which significantly ($p > 0.05$) reduced with increase in level of administration of aqueous *Ficus asperifolia* leaves extract. Rabbit does administered 10 ml and 20 ml aqueous *Ficus asperifolia* leaves extracts recorded similar and higher (83.33%) breeding efficiency; fertility index was significantly ($p < 0.05$) highest in rabbit does administered 20 ml (5.17) and lowest in control group (3.17). This study therefore concluded that *Ficus asperifolia* leaves extract improved reproductive performance in the does as does administered *Ficus asperifolia* leaves extract significantly ($p < 0.05$) exhibited higher breeding efficiency and fertility index.

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Introduction

Rabbit is an important livestock that can contribute to meat and protein production in developing countries due to its rapid growth rate, high reproduction potential and ability to utilize forage (BIOBAKU and DOSUMU 2003). This is because human population growth in developed countries is stabilizing while that of developing countries including Nigeria is still increasing rapidly. Hence, there is need to intensify the search for alternative sources of protein to meet up with this population challenge (MAILAFIA et al. 2010). However, there are myriad of problems confronting rabbit farming in these countries, which have resulted to a gross shortage of meat to meet up with the population challenge (NWORGU 2007). One of these problems is breeding problem.

Breeding challenges always increase in the hottest months of the year and these could be as a result of summer heat. Bucks can become sterile in extremely hot weather. The length of time sterility lasts is directly proportional to the length of exposure; this condition affects adult males more than maturing ones (MATOLLI 1982). When the temperature exceeds 29^{o}C for several consecutive days, male rabbits remain sexually active but may not be fertile for about 60 days (i.e mounting without conception). Other conditions that can inhibit conception include physical condition, nutritional deficiencies, decreasing daylight, inherited factors, molting, stress, age, diseases and abnormalities like the malformation or absence of any of the reproductive organs e.g. un-descended testicles (LEBAS 1983).

NWOKO and IBE (2005) opined that the reproductive performance of the male is an essential economic trait in the management of breeder stock and the evaluation of the ejaculate is an important aspect of the determination of the reproductive status of the male animals. Some medicinal plants and plant products have been used in handling primary medical difficulties due to their accessibility, availability and affordability in developing countries. In these countries, a variety of plants are claimed to have fertility regulating properties and a few have been tested for such effect (BAKER et al. 1999, TELEFO et al. 2002, GANGULY et al. 2007, CHERDSHEWASART et al. 2007).

Ficus asperifolia is one of these plants. It is a small or average size tree, terrestrial or epiphyte which can reach 20 m in height. It is found in Senegal, Uganda, Tanzania, Natal (South Africa), Madagascar and Cameroon. According to ADJANOHUN et al. (1996), *Ficus asperifolia* is abundant in the savannah regions, especially along river banks and marshy areas at an altitude of up to 1100 m. The leaves are enormous and displayed spirally, the limb is largely oval or has a form of ellipse and the roots are most

often fibrous. Traditional medicine of this same region indicates that the decoction of dry fruits of *Ficus asperifolia* is used to reverse some cases of sterility or infertility whereas the leaves are used as anthelmintic and purgative. Although there is no scientific evidence to support the ethnopharmacological reputation of *Ficus asperifolia* on female reproduction, tribes continue to popularly use it in the management of cases of sterility or infertility in women. Previous work done by OMONIWA and LUKA (2012) on the aqueous stem extract of *Ficus asperifolia* revealed that it possesses hypoglycemic and hypolipidemic properties on diabetic rats while NKAMIFIYA et al. (2010) also published that the leaves of *Ficus asperifolia* has a higher protein, crude fibre and mineral contents than some vegetables. This study therefore seeks to evaluate the effect of aqueous extracts of *Ficus asperifolia* leaves on the semen characteristics, testicular histology and reproductive performance of New Zealand white rabbits.

Materials and Methods

The research work was carried out at the Rabbitry Unit of the Directorate of University Farms (DUFARMS) of Federal University of Agriculture, Abeokuta (FUNAAB) Ogun State, Nigeria. The region lies between latitude 7º10'N and longitude 3º2'E and altitude 830 m above the sea level. The experimental site is located in the derived savannah vegetation zone of South-Western Nigeria with annual average rainfall of 1100 mm and peak rainfall temperature ranges from 28ºC in December to 36ºC in February with a yield average relative humidity of about 82% (GOOGLE EARTH 2019).

The study protocol was approved and conducted in line with the Animal Ethics Committee guidelines of Federal University of Agriculture, Abeokuta, Nigeria (FUNAAB 2013). Thirty six New Zealand White rabbits with average weight of 2.1 kg and 6 months old were purchased from reputable farms in Abeokuta. Before the arrival of the animals, the stable was thoroughly washed and disinfected in readiness for stocking. On the first day of arrival of the animals, they were given anti stress (Maxiyield) and duration of acclimatization was two weeks to enable the animals adapt to the environment.

Thirty six (36) New Zealand White (NZW) rabbit bucks and does were divided into two groups of 18 bucks and 18 does. Each group was randomly assigned to three experimental treatment groups on a weight equalization bases in a completely randomized design. Each treatment group was subdivided into six replicates with a rabbit per replicate. Treatment 1 was

orally administered 0 ml aqueous *Ficus asperifolia* leaves extract and served as control, Treatment 2 and Treatment 3 were administered the prepared aqueous *Ficus asperifolia* leaves extract orally with 10 ml and 20 ml daily respectively for 3 weeks consecutively. After three weeks of *Ficus asperifolia* leaf extracts administration, does were hand mated twice to ensure conception and palpated at the 14th day of the gestation for pregnancy test. Kindling boxes were introduced on the 28th day of gestation into the hutches to stimulate nest building, safe delivery and kits protection. The animals were housed under the same condition fed concentrate containing 16% crude protein, 7% crude fibre, 5% ether extract, 1.6% calcium, 0.5% phosphorus, 0.75% lysine, 0.36% methionine, 0.3% salt (NaCl), 10,250.8 MJ/kg Metabolizable Energy and supplied water ad-libitum. This was supplemented with Tridax procumbens twice a week to prevent bloating. The experiment lasted for 12 weeks.

The fresh leaves of *Ficus asperifolia* were harvested within the environment of Federal University of Agriculture, Abeokuta. The leaves were sorted to remove contaminants, dead matter, sand particles and were air dried for 10 days in the absence of sunlight to retain its nutrients. The air-dried leaves were finely powdered using electric blender. The powdered leaf meal obtained was stored until further use. 200 g of the leaf meal was measured into conical flasks and extracted with 1000 ml distilled water for 24 hours. The mixture was filtered into 500 ml conical flasks with Whatman paper no. 1. The solution was filtered, decanted and filtered three times using sieve to achieve aqueous leaves extract of *Ficus asperifolia*.

At the end of the 3rd week Ficus administration, 5 ml of blood sample was collected from 3 bucks and 3 does per treatment into heparinized sterile test tubes and immediately centrifuged (4000 g) for 15 minutes. Then, plasma was separated and stored at -20^{o}C until hormonal assay was carried out. Concentration of melatonin in blood plasma was determined using commercial kit according to PINTOR et al. (2001). Plasma concentration of FSH and plasma concentration of LH were determined in duplicate by RIA, using commercial kit according to UBILLA et al. (1992). Also, Testosterone was determined using commercial kit according to ASHBY et al. (1980).

Testes were harvested from three bucks that were randomly selected per treatment for morphometric analysis and histology. Testes were carefully separated and freed of tunica albuginea and all adhering connective tissues. The length of each testis was measured using a vernier caliper. The testes weight (Left and Right) were measured on electronic scale. In estimating the testicular histology, the harvested testes were fixed in 10% formalin, dehydrated in a graded series of ethanol saturated in benzene,

benzene-paraffin and embedded in paraffin wax (MASSANYI et al. 2000). Testes were sectioned on a microtome and serial 10 m thick sections were stained with haematoxylin and eosin. Lumen and germinal epithelium of the treatments were treatments were compared.

For the semen evaluation, the caudal epididymis was placed in beakers containing physiological saline (maintained at 37ºC) and several lacerations were made on it to enable the spermatozoa swim out. Sperm motility was immediately determined by placing a drop of the suspension on a clean glass slide under the cover slip and viewed on a binocular microscope. Sperm motility was assessed immediately by counting both motile and immotile spermatozoa per unit area at the magnification of ×40. Sperm viability was assessed using eosin-nigrosin test. The percentages of unstained (live) and stained (dead) spermatozoa were calculated by counting 100 spermatozoa per sample (ZEMJANIS 1977).

Data collected on the reproductive parameters include:

– gestation length: this was determined by the time interval between conception and kindlin;
– breeding efficiency: this was expressed in terms of percentage of does that kindled following mating;
– litter weight at birth: this was obtained by weighing all the kits kindled by a doe in a litter together;
– average birth weight: it was determined by dividing litter weight at birth by the total number of the kits;
– litter size at birth: it was determined by counting the total number of kits born per doe both still and live kits;
– litter size at weaning: this was determined by counting the remaining number of kits at the time of weaning;
– pre-weaning loss: this was determined by counting and recording number of kits that died before weaning;
– litter weight at weaning: this was obtained by weighing all the kits remaining in a litter together at the time of weaning;
– fertility index was determined by multiplying the total number of litter at birth by the breeding efficiency divided by 100;

$$\text{Fertility Index (FI)} = \frac{\text{breeding efficiency (BE)}}{\text{litter size at birth (LSAB)}} \cdot 100$$

– still birth: it was determined by counting the number of kits dead at birth in a litter;
– litter weight gain: this was obtained by subtracting litter weight at birth from litter weight at weaning;
– kit weight gain: it was determined by dividing litter weight gain by litter size at weaning;

– weaning rate: this was obtained by dividing litter size at weaning by litter size at birth multiplied by 100;
– doe weight at kindling: it was taken on the kindling day by digital weighing scale;

$$\text{Weaning rate (WR)} = \frac{\text{litter size weaning (LSAW)}}{\text{litter size at birth (LSAB)}} \cdot 100$$

– doe weight at weaning: it was taken through the weighing of the kit on weaning.

Data obtained were subjected to One Way analysis of variance in a complete randomized design (CRD). Significant differences ($p < 0.05$) among means were separated using Duncan-Multiple Range Test as contained in SAS (2010).

Results

The effects of oral administration of aqueous *Ficus asperifolia* leaves extract on the reproductive hormones of rabbits is presented in Table 1 and Figures 1–3. *Ficus asperifolia* leaves extract significantly ($p < 0.05$) influenced the testosterone where the lowest value (0.09 mg/ml) was observed in the rabbits orally administered 20ml of the extract and the highest value (0.69 mg/ml) for testosterone was observed in the control treatment orally administered 0ml of the extract. There were no significant ($p > 0.05$) difference in luteinizing hormones and follicles stimulating hormones of the rabbits orally administered the extract. The luteinizing hormones increased numerically with increasing levels of *Ficus asperifolia* leaves extract; the values range from 2.86 mg/ml to 5.85 mg/ml and follicles stimulating hormones values range from 5.31 mg/ml to 12.77 mg/ml.

Table 1

Reproductive hormones analysis of rabbits administered aqueous *Ficus asperifolia* leaves extract

Parameters	0 ml	10 ml	20 ml	SEM
Testosterone [mg/ml]	0.69^a	0.17^b	0.09^b	0.10
LH [mg/ml]	2.86	4.26	5.85	1.37
FSH [mg/ml]	10.21	5.31	12.77	2.23

Means having different alphabet across the column are significantly different ($p > 0.05$)
LH – luteinizing hormone, FSH – follicles stimulating hormones

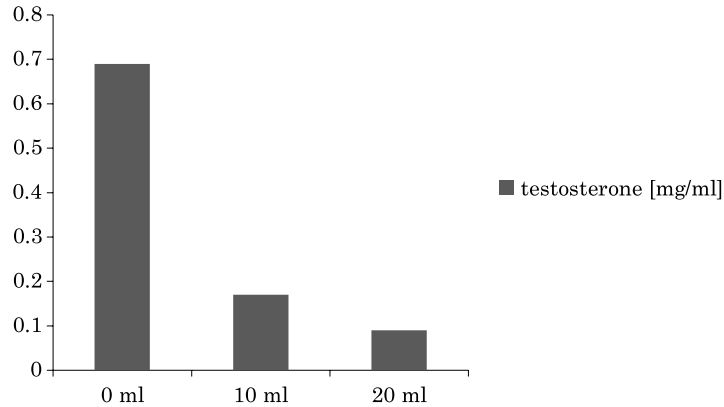

Fig. 1. Effect of *Ficus asperifolia* leaves extract on the testosterone of rabbits

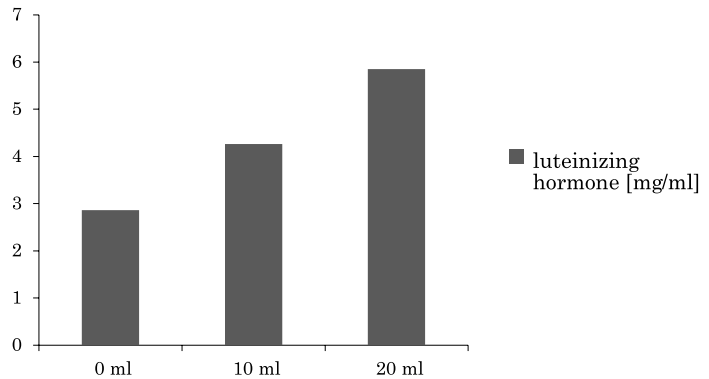

Fig. 2. Effect of *Ficus asperifolia* leaves extract on the luteinizing hormone of rabbits

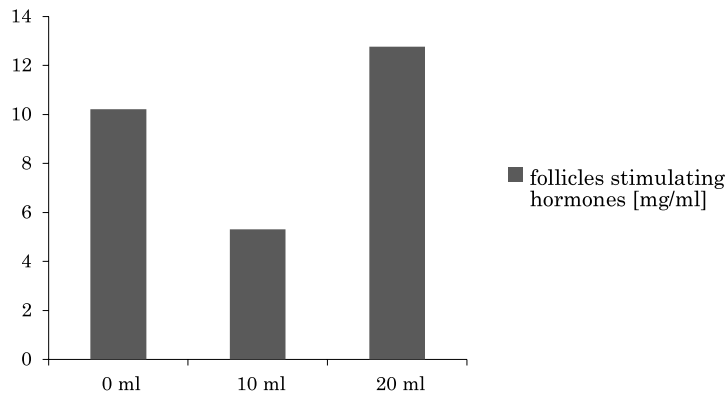

Fig. 3. Effect of *Ficus asperifolia* leaves extract on the follicles stimulating hormone of rabbits

The result obtained on the effect of oral administration of *Ficus asperifolia* on semen characteristics of New Zealand White rabbit is shown on Table 2. Oral administration of aqueous leaf extract of *Ficus asperifolia* at different levels (0 ml, 10 ml and 20 ml) had no significant effect ($P > 0.05$) on all the semen quality parameters measured in this study except on Double head sperms. Double head significantly ($p > 0.05$) reduced with increase in level of administration of aqueous *Ficus asperifolia* leaf extract. The highest value (6.00) of Double head was recorded in rabbit bucks administered 0ml of the extract while the least value (2.33) was observed in bucks given 20ml of the extract. Rabbit bucks administered 0 ml aqueous *Ficus asperifolia* leaf extract recorded the highest numerical values for liveability, live/death ratio and PH (96%, 0.38% and 7.7) respectively while individual motility was numerically highest in bucks administered 10 ml of the extract. Normal to abnormal sperm ratio and sperm concentration was observed in bucks administered 20 ml of the extract.

Table 2

Effect of oral administration of leaf extract of *Ficus asperifolia* on semen characteristics of New Zealand White rabbits

Parameters	Dosage			
	treatment 1 (0 ml)	treatment 2 (10 ml)	treatment 3 (20 ml)	SEM
Sperm motility [%]	94.67	94.67	96.33	10.70
Individual Motility	90.00	92.00	90.67	1.42
Liveability	93.33	87.67	94.67	2.05
Normal cell [%]	77.00	73.33	83.67	2.48
Abnormal cell	23.00	26.67	16.33	2.12
pH	7.70	7.11	7.35	0.13
Conc. [$\cdot 10^6$/ml]	344.50	348.33	363.00	13.71
Double head	6.00^a	4.33^{ab}	2.33^b	0.68
Free tail	0.88	0.58	0.33	0.44
Bent tail	2.67	3.67	4.00	0.63
Abnormal head	1.33	2.00	1.33	0.34

Table 3 shows the effect of *Ficus asperifolia* leaf extract on major testicular morphometry parameters administered at different levels. There was no significant ($P > 0.05$) difference between the animals for the parameters observed. There was no difference in testicular weight of the rabbit bucks administered 0 ml, 10 ml and 20 ml of aqueous *Ficus asperifolia* leaf

extract while the animals on 0ml of the extract has highest numeric testis length of 11.63 cm compare to 9.96 cm and 7.80 cm observed in animals with 10 ml and 20 ml of the extract respectively.

Table 3

Effect of oral administration of leaf extract of *Ficus asperifolia* on testicular morphometry of New Zealand White rabbits

Parameters	Dosage			
	treatment 1 (0 ml)	treatment 2 (10 ml)	treatment 3 (20 ml)	SEM
Testis weight [g]	3.33	3.33	3.33	0.28
Testis length [cm]	11.63	9.96	7.8	0.86

Effect of oral administration of leaf extract of *Ficus asperifolia* on testis histology of New Zealand White rabbits.

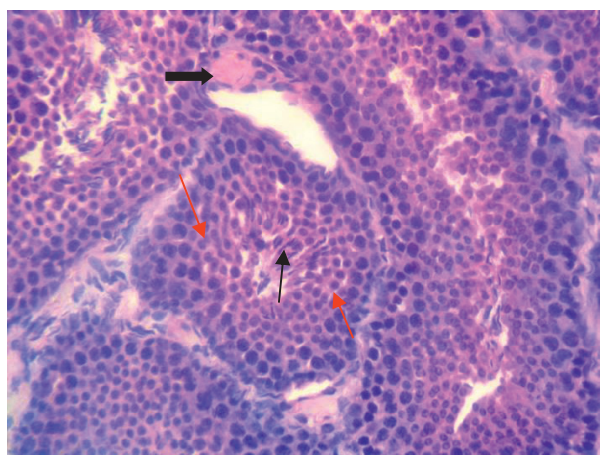

Fig. 4. Photomicrograph of testes tissue section of rabbit bucks administered 0 ml of *Ficus asperifolia*

There are numerous, closely packed, variably sized seminiferous tubule. The STs are packed full with abundant amounts of spermatogenic cells evidenced by the increased height of the germinal epithelium and reduced luminal space. Elongate spermatids (black arrow) and spermatocytes (red arrows) predominate. There is moderate congestion of the testicular blood vessels (thick arrow).

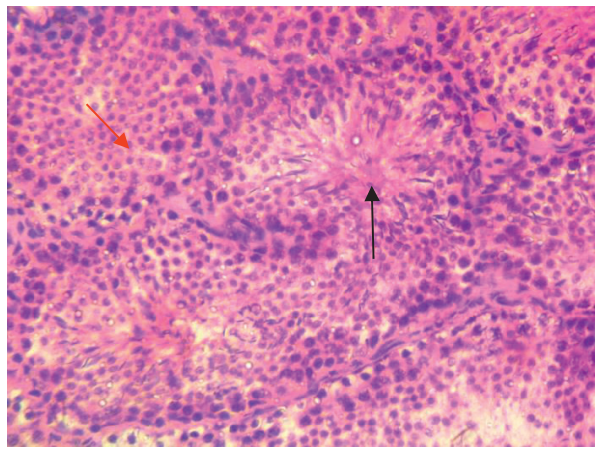

Fig. 5. Photomicrograph of testes tissue section of rabbit bucks administered 10 ml of *Ficus asperifolia*

There are numerous closely-packed large STs with regular outlines. These STs contain abundant amounts of spermatogenic cells. There is normal polarization and differentiation of the spermatogenic cells from the basal compartment to the luminal compartment. Elongate spermatids (black arrow) and spermatocytes (red arrows) predominate.

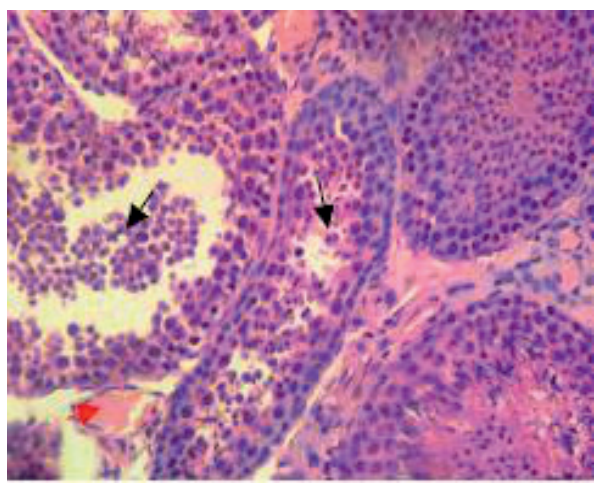

Fig. 6. Photomicrograph of testes tissue section of rabbit bucks administered 20 ml of *Ficus asperifolia*

There are numerous closely-packed, variably-sized STs with regular outlines. The STs show depletion and loss of spermatogenic cells from the basal aspects to the luminal aspects (black arrows). There is moderate congestion of the testicular blood vessels (red arrow).

Reproductive performance of rabbit does administered *Ficus asperifolia* leaves extract results presented in Table 4 show significant ($p < 0.05$) effect only for breeding efficiency, fertility index, doe weight at kindling and weaning. Doe administered *Ficus asperifolia* leaves extract significantly ($p < 0.05$) exhibited higher breeding efficiency and fertility index and reduced weight at kindling and weaning than the control. Rabbit does administered 10 ml and 20 ml aqueous *Ficus asperifolia* leaves extracts recorded similar and higher (83.33%) breeding efficiency compare to the 50% of rabbit does administered 0ml. Fertility index was significantly ($p < 0.05$) highest in rabbit does administered 20 ml (5.17) and lowest control group (3.17). Doe weight at kindling (2567.33g) and weaning (2600.00 g) was significantly ($p < 0.05$) higher in the control group than rabbit does administered *Ficus asperifolia* leaves extract.

Table 4

Reproductive performance of rabbit does administered *Ficus asperifolia* leaves extract

Parameters	Levels of *Ficus asperifolia* leaves extract			
	(0 ml)	(10 ml)	(20 ml)	SEM
Gestation length [d]	31.33	31.40	31.20	0.24
Breeding efficiency [%]	50.00^{b}	83.33^{a}	83.33^{a}	4.05
Litter weight at birth [g]	296.67	270.00	244.60	15.66
Average birth weight [g]	48.99	48.85	40.43	2.43
Litter size at birth	6.33	5.60	6.20	0.41
Litter size at weaning	4.00	5.20	4.00	0.54
Pre-weaning loss	2.33	0.40	2.00	0.60
Litter weight at weaning [g]	1588.33	2330.00	1650.00	248.29
Fertility index	3.17^{b}	4.67^{ab}	5.17^{a}	0.37
Still birth	0.00	0.00	0.20	0.08
Litter weight gain [g]	1286.67	2060.00	1405.40	254.50
Kit weight gain [g]	298.89	434.29	359.94	42.52
Weaning rate [%]	70.24	91.00	69.29	8.74
Doe weight at kindling [g]	2567.33^{a}	2222.00^{b}	2384.40^{ab}	58.82
Doe weight at weaning [g]	2600.00^{a}	2104.80^{b}	2350.00^{ab}	68.36

^{ab} Means on the same row having different superscripts are significantly different ($P < 0.05$).

Discussion

Testosterone hormone is produced by the interstitial cells of the testis and necessary for the completion of spermatogenesis. The testosterone values recorded was significant ($P < 0.05$) across the treatment and decreased with increasing level of *Ficus asperifolia* leaves extract. This result is an indication that *Ficus asperifolia* leaves extract can be used to reduce sexual drive in male animals; hence, it can be a biological method of castration for male animals, thereby reducing stress given to the animals in using other methods of castration and also to improve animal welfare. EL-HANOUN et al. (2014) reported that a good relationship exist between increased testosterone concentration and increased libido of male rabbits. The numerical increase observed in the value (12.77 mg/ml) of FSH of rabbits administered (20 ml) of the extract is in line with the report of OLUYEMI et al. (2007) that flavonoids increased follicle stimulating hormones (FSH) in rat models administered extract of *Garcinia kola.* Herbs balance the levels of hormones such as testosterone, luteinizing hormone and follicle stimulating hormones (KOUMANOV et al. 1982) and *Ficus asperifolia* is one of these herbs which its phytochemical screening have detected flavonoids, saponins, alkanoids, tannins, steroids and many others (OMONIWA et al. 2013).

The results of semen evaluation of New Zealand White rabbit administered aqueous leaf extract of *Ficus asperifolia* showed that the extract did not significantly influence semen characteristics parameters observed in the study except a significant increase in the number of coil tails. The significant increase observed in the number of coil tails in the animals administered aqueous *Ficus asperifolia* leaves extract and other indicators of abnormal sperm cells (bent tail and free tail) suggested that the extract can be used to reduce abnormal sperm cells thereby increasing chance of successful copulation. The values of sperm concentration observed in this research ranged from 344.50 to 363.00 $\cdot 10^6$/ml; it is conceivable that the increase in sperm concentration might lead to higher fertility which is supported by the findings of OYEYEMI et al. (2008). These values were higher than the recorded values of 136.00 to 184.00 $\cdot 10^6$/ml stated by AJAYI et al. (2009) on sperm motility of rabbits fed graded levels of blood-sunflower meal. OYEYEMI and OKEDIRAN (2007) reported that an increased concentration of spermatozoa is a signal to a possible high fertility rate by the reason of the number of spermatozoa available during service or insemination. Higher motility value obtained from animals administered the extract is an indication that the extract had supplied adequate nutrient to support sperm motility. OYEYEMI et al. (2002) reported that

adequate nutrition with high percentage of crude protein enhance motility and concentration of spermatozoa. Also, the results of this study are much higher than those reported by ABU and UCHENDU (2010), who studied the antispermatogenic effects of aqueous ethanolic extracts of *Hymenocardia acida* stem bark on sperm motility of laboratory rodents and obtained values of 23–28%. Sperm concentration in this study is higher than 126.00 to 154.00 · 10^6/ml and 123.30±1.76 to 138.30±1.20 reported by AHEMEN et al. (2013) on sperm quality and testicular morphometry of rabbits fed dietary levels of water spinach (*Ipomoea aquatic*) leaf meal and falls within the range of 50 to 350·10^6/mm^3 reported by BRACKETT (2004) and also similar to what was obtained by HAFEZ (1970) for rabbit bucks. The variation may be attributed to effect of the treatment and the breed or genetic line of the animal as indicated by ALVARINO (2000). High concentration of sperm recorded in this study is a sign of high possible fertility at the time of copulation.

The percentage normal sperm cells in this research ranged from 73.33 to 83.67% and were not significantly affected by *Ficus asperifolia* leaves extract. The percentage normal sperm cells value was higher in rabbit bucks administered 20 ml (83.67%) compared with 0 ml (77%) and 10 ml (73.33%). ARTHUR et al. (1989) discovered that high quality semen samples show an average of 25% dead sperms. The average value of percentage normal sperm (an indicator of sperm viability and fertilizing capacity) cells reported in this research was within the range of high quality samples. The percentage live sperm cells, which also indicate sperm viability and possibly higher fertilizing capacity, are those present for use during fertilization (AJALA et al. 2001). The percentage of abnormal sperm cells values in this research ranged from 16.33 to 26.67%. The percentage of abnormal sperm cells in bucks administered 20 ml extract were lower than the upper limit of 20% suggested as the least quantity recommendable for good reproductive potential and fertility in either normal mating or in artificial insemination (OYEYEMI and OKEDIRAN 2007). AJAYI et al. (2009) established the influence of quality feeding on sperm characteristics of rabbits. OYEYEMI et al. (1998) declared that quality nutrition with high percentage of protein will improve motility and concentration of spermatozoa.

The results of evaluation of testicular morphometry of New Zealand White rabbit fed aqueous leaf extract of *Ficus asperifolia* showed that the extract did not significantly influence testicular morphometry observed in this study. This result is in consonance with the submissions of BITTO and GEMADE (2001) who recorded a non-significant influence of pawpaw peel meal up to 30% on testicular morphometry of rabbit bucks and also agrees with the findings of OGUNLADE et al. (2006) who observed non-significant

differences in testis weight among rabbits fed fumonisin contaminated diets and AHEMEN et al. (2013) who fed water spinach leaf meal to male rabbits. The mean testicular weight values obtained in this study (3.33 g) is comparable to the range of 2.58±0.42 to 3.23±0.19 reported by AHEMEN et al (2013) in rabbits fed dietary levels of water spinach (*Ipomoea aquatica*) leaf meal and higher than the range (1.39–2.13 g) observed by ABU et al. *(*2016). The mean testicular weight (3.3g) observed in this study is lower than 3.1g reported by FRANCA et al. (2002) and higher than 6.7 g reported as the average testicular weight by HERBERT et al. (2005). Though not significant, the mean testicular length of rabbit bucks decrease with increase in the levels of administration of *Ficus asperifolia* leaves extract. Investigation on the morphometric parameters of reproductive tract have been observed to give invaluable information on adjudging the breeding and fertililizing ability of animals (OGBUEWU et al. 2009). GAGE and FRECKLETON (2003) reported that testes size, length and width of mammals are described as favourable pointer to the present and future spermatozoa production. Knowledge of the important morphometric qualities of the reproductive organ is important to enhance the opinion and forecast not only of sperm production ability, but likewise the storage potential and fertilizing capability of the breeder male. MOREIRA et al. (2001) verified in a study of Santa Ines sheep, that changes in testicular length and scrotal circumference is considered viable indicators of the effect of thermal stress on gonads. In accordance with EZEKWE (1998) and PERRY and PETTERSON (2001), testes size, length and width are high quality indicators of present and future sperm production. This enhances increased fertilizing potential in rabbits.

The effect of aqueous leaf extract of *Ficus asperifolia* on testis histology showed that, there was no observable difference in the seminiferous tubule of the observed animals from each of the treatments as the seminiferous tubule of the observed animals are numerous and closely packed. The seminiferous tubules are packed fully with abundant amount of spermatogenic cells evidence by increased height of germinal epithelium and reduced luminal space though the seminiferous tubules of animals on 20 ml of the extract show depletion and loss of spermatogenic cells from the basal aspect to the luminal aspect (Black arrows). This may be due to reduction in pH of the sperm as the extract is given at higher level. Animals on 0 ml and 10 ml of the extract had seminiferous tubule that is closely packed evident from the small size of the lumen. This non-differential observation reported in this study is in line with what was reported by CHRENEK et al. (2006) when comparing testicular histology of transgenic and non-transgenic line of rabbit and disagrees with report of EWUOLA and

EGBUNIKE (2002) on effects of dietary fumonisin B1 on the onset of puberty, semen quality, fertility rates and testicular morphology in male rabbits; they reported a degenerated seminiferous tubule on rabbits fed dietary fimonisin B1. It also contradict the report of IFEANYI et al. (2009) on Semen quality characteristics, reaction time, testis weight and seminiferous tubule diameter of buck rabbits fed neem (*Azadirachta indica A. Juss*) leaf meal based diets who also reported a decrease in size of seminiferous tubule of the animals fed test diet. The result from this study thus indicates that administering aqueous leave extract of *Ficus asperifolia* up to 10 ml did not have impairing effect on testicular histology though there is depletion and loss of spermatogenic cells at higher level (20 ml).

Significantly higher breeding efficiency exhibited by doe administered *Ficus asperifolia* leaves extract suggests that *Ficus asperifolia* leaves extract provided enough nutrients for maintenance and reproduction of the rabbits. This result is better and higher than the 16.67% breeding efficiency obtained from rabbits fed diet containing 7.5% Neem leaf meal reported by AYO-AJASA et al. (2018) and also disagrees with the 67 to 100% conception rate reported by ABDELLI-LARBI (2014) in New Zealand White breed of rabbits and the study of ODEYINKA et al. (2008) who fed Moringa leaf in place of *Centrosema pubescens*. Also the high fertility index reported in this study shows that *Ficus asperifolia* leaves extract prevented aspermic ejaculation from the bucks involved in the fertilization process and hence did not impair fertility (SZENDRO et al. 1984). This result could also be attributed to the high concentration of sperm recorded in this study which is a sign of high possible fertility at the time of copulation. Breeding efficiency and fertility index can be employed as traits to determine the future breeding programmes and lifetime productivity in rabbit production.

Conclusion

This study concluded that *Ficus asperifolia* leaves extract significantly ($p < 0.05$) reduced testosterone and double head sperm and improved reproductive performance in the does as does administered *Ficus asperifolia* leaves extract significantly ($p < 0.05$) exhibited higher breeding efficiency and fertility index and reduced weight at kindling and weaning than the control.

Recommendation

Aqueous *Ficus asperifolia* leaves extract can be used as biological method of castration for male animals and be used to boost breeding efficiency and fertility index which can be employed as traits to determine the future breeding programmes and lifetime productivity in rabbit production.

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References

Abdelli-Larbi O., Mazouzi-Hadid F., Berchiche M., Bolet G., Garreau H., Lebas F. 2014. *Pre-weaning growth performance of kits of a local Algerian rabbit population: Influence of dam coat color, parity and kindling season.* World Rabbit Science, pp. 231–240.

Abu A.H., Uchendu C.N. 2010. *Antispermatogenic effects of aqueous ethanolic extract of Hymenocardia acida stem bark in Wistar rats*. Journal of Medicinal Plants Research, 4(23): 24952502.

Adjanohoun E. 1996. *Traditional Medicine and Pharmacopoeia: Contribution to Ethnobotanical and Floristic Studies in Cameroon*. Scientific, Technical and Research Commission of the Organization of African Unity, Lagos, Nigeria, pp. 301–325.

Ahemen T., Abu A.H., Orakanya T.T. 2013. *Sperm quality and testicular morphometry of rabbits fed dietary levels of water spinach (Ipomoea aauatica) leaf meal*. Agriculture and Biology Journal of North America. Abjna, 2013.4.3.352.357.

Ajala O.O., Oyeyemi M.O., Akusu M.O., Eimunjeze H.E. 2001. *The effects of scrotal insulation on the testicles and spermatozoa characteristics of WAD goats.* Sokoto J. Vet. Sc., 3(1): 44–50.

Ajayi A.F., Raji Y., Togun V., Oyewopo A.O. 2009. *Caudal epididymal sperm characteristics and testicular morphometrics of rabbits fed graded levels of a blood-wild sunflower leaf meal (BWSLM) mixture diet*. Journal of Complementary and Integrative Medicine, 6(1): 1553–3840.

Alvarino J. 2000. *Reproductive performance of male rabbits*. In: Blasco, A. (Ed.), 7thWorld Rabbit Congress. World Rabbit Science 8(A): 13–35.

Ashby C.D., Danzer C.H., Swerdloff R.S. 1980. *Estrogen radioimmunoassay suitable for the monitoring of ovulation induction*. Clin. Chem., 26: 1143–1146.

Ayo-Ajasa O.Y., Egbeyale L.T., Abiona J.A., Njoku C.P., Amoo, A.B., Aseniyi O.E. 2018. *Gestational and reproductive performance of rabbit does fed diets containing neem (Azadirachta indica) leaf meal*. Nigerian Journal of Animal Production, 45(3): 79– 86. Published by Nigeria Society of Animal production, www.nsap.org.

Baker V.A., Hepburn P.A., Kennedy S.J. 1999. *Safety of phytosterolesters Part 1. Assessment of oestrogenicity using a combination of in vivo and in vitro assays*. Food Chem Toxicol., 37: 13–22.

Biobaku W.O., Dosumu E.O. 2003. *Effects of supplementing a diet based maize and rice bran with 3 different improved forages on feed intake, digestibility and growth in rabbits*. Nigerian Journal of Animal Production, 22: 179–184.

BITTO I.I., GEMADE M. 2001. *Preliminary investigations on the effect of Pawpaw peel meal on growth, visceral organ and endocrine gland weights, testicular morphometry and the haematology of male rabbits*. Global J.P. & Appl. Sci., 7(4): 611–625.

BRACKETT B.G. 2004. *Male Reproduction in Mammals*. In: *Duke's physiology of Domestic Animals* 12th ed. Edited by Reece, W.O. Cornel University Press, Ithaca, pp. 670–688.

CHERDSHEWASART W., KITSAMAI Y., MALAIVIJITNOND S. 2007. *Evaluation of the estrogenic activity of the Wild Pueraria mirifica* by vaginal cornification assay. J. Reprod. Dev., 53: 385–393.

CHRENEK P., TRANDZIK J., MASSANYI P., MAKAREVICH A., LUKAC N., DANA PESKOVICOVA D., PALEYANDA R. 2007. *Effect of transgenesis on reproductive traits of rabbit males*. Animal Reproduction Science, 99: 127–134.

EZEKWE A.G. 1998. *Gonadal and extragonadal sperm reserve and testicular histometry of post pubertal Muturu bulls*. Nig. J. Anim. Prod., 25: 106–110.

FRANCA L.R., RUSSELL L.D., CUMMINS J.M. 2002. *Is Human spermatogenesis uniquely poor?* ARBS Annual Review of Biomedical Science, 4: 19–40.

GAGE M.J.G., FRECKLETON R.P. 2003. *Relative testis size and sperm morphometry across mammals. No evidence for an association between sperm competition and sperm length*. Proceedings of Biological Science, 22: 625–632.

GANGULY M., BORTHAKUR M., DEVI N., MAHANTA R. 2007. *Antifertility activity of the methanolic leaf extract of Cissampelos pareira* in female albino mice. J. Ethnopharmacol., 2007(111): 688–691.

GOOGLE EARTH 2019. www.googleearth.com, access: 25.07.2021.

HAFEZ E.S. 1970. *Rabbits*. In: *Reproduction and breeding techniques for laboratories animals*. Philadelphia, pp. 273–298.

HERBERT U., OZOJE M.O. ADEJUMO D.O. 2005. *Effect of Leucaena and Gliricidia leaf meals on the seminal characteristics, testis weights and seminiferous tubule diameters of rabbits*. Anim. Res., 54: 173–178.

IFEANYI C.I.C., ISU R.N., AKPA A.C., IKENECHE N.F. 2009. *Enteric bacteria pathogens associated with Diarrhoea of children in the federal capital territory Abuja, Nigeria*. New York Science Journal, 2(7): 62–69.

KOUMANOV F., BOZADJIEVA E., ANDREEVA M., PLATONVA E., ANKOV V. 1982. *Clinical trial of Tribes tan*. Experimental Medicine, 1: 2–4.

LEBAS F. 1983. *Small-scale rabbit production*. World Animal Review, 46: 11–17.

MAILAFIA S., ONAKPA M. M., OWOLEKE O.E. 2010. *Problems and prospects of rabbit production in nigeria – a review*. Bayero Journal of Pure and Applied Sciences, 3(2): 20–25.

MASSANYI P., LUKAE N. HLUCHY S., SLAMEEKA J., JUREIK R., TOHMAN R., KOVAEIK J. 1999. *Seasonal variations in the metric analysis of the testis and epididymis in Fallow-Deer (Dama dama)*. Folia Veterinaria, 43(2): 67–70.

MATTIOLI C. 1982. *Effect of climate on reproduction*. Journal of Rabbits, 19(2): 38–39.

MOREIRA E.P., MOURA A.A.A., ARAUJO A.A. 2001. *Efeitos da insulação escrotal sobre a biometria testicular e parâmetros seminais em carneiros da raça Santa Inês criados no estado do Ceará*. Revista Brasileira de Zootecnia, 30(6): 1704– 1711.

NKAFAMIYA I.I., OSEMEAHON S.A., MODIBBO U.U., AMINU A. 2010. *Nutritional status of non-conventional leafy vegetables*, *Ficus asperifolia and Ficus sycomorus*. Afr. J. Food Sci., 4: 104–108.

NWOKO N., IBE S.N. 2005. *Effect of tread and season on semen quality and fertility of bucks in a humid topical environment*. Nigerian Journal of Animal Production, 32(2): 327–334.

NWORGU F.C. 2007. *Economic importance and growth rate of broiler chicken served fluted pumpkin (Telfaria occidentalis)*. African Journal of Biotechnology, 6(2): 167–174.

ODEYINKA S.M., OYEDELE O.J., ODEDIRE J.A. 2008. *Reproductive performance of rabbits fed Moringa oleifera as a replacement for Centrosema pubsescens*. 9th World Rabbit Congress. June, 10th–13th 2008, Verona-Italy.

OGBUEWU I.P., OKOLI I.C., ILOEJE M. 2009. *Semen quality characteristics, reaction time, testis weight and seminiferous tubule diameter of buck rabbits fed neem (Azadiractita indica A. juss) leaf meal based diet*. Iranian Journal of Reproductive Medicine, 7(1): 23–28.

OGUNLADE J.T., EWUOLA E.O., GBORE F.A. 2006.*Testicular and epididymal sperm reserves of rabbits fed fumonisms contaminated diets*. Int. Digital Organisation for Scientific Information, 1(1): 35–38.

OMONIWA B.P., LUKA C.D. 2012. *Antidiabetic and toxicity evaluation of aqueous stem extract of Ficus asperifolia* in normal and alloxan-induced diabetic albino rats. Asian J. Exp. Biol. Sci., 3: 726–732.

OMONIWA B.P., LUKA C.D., SOJI-OMONIWA O. 2013. *Effect of aqueous leaf extract of Ficus asperifolia on cardiac enzymes and lipid profile in male albino rats*. Journal of Medical Sciences, 13: 373–378, doi: 10.3923/jms.2013.373.378.

OYEYEMI M.O., OLUWATOYIN O., LEIGH O.O., PIDESIJI T., FISAYO A. 2008. *The spermiogram of male Wistar rats treated with aqueous leaf extract of Vernonia amygdalina*. Folia Veterinaria, 52(2): 98–103.

OYEYEMI M.O., OKEDIRAN B.S. 2007. *Testicular parameters and sperm morphology of chinchilla rabbits fed with different planes of soy meal*. Int. J. Morphol., 25(1): 139–144.

OYEYEMI M.O., OKE A., OLUSOLA C., AJALA O., OLUWATOYIN O., IDEHEN C.O. 2002. *Differences in testicular parameters and morphological characteristics of spermatozoa as related to age of West African Dwarf bucks*. Tropical Journal of Animal Science, 5(1): 99–107.

OYEYEMI M.O., AJALA O.O., AKUSU M.O., AREGBESOLA O. O.1998. *Effects of Starvation on semen characteristics of West African dwarf bucks*. Conference of Animal Science Association Nigeria 22–24, 1998 Lagos, Nigeria, pp. 128–130.

PERRY G., PETTERSON D. 2001. *Determining reproductive fertility in herd bulls*. University of Missouri Agriculture publication, 2011: 1–8.

PINTOR J., MARTON L., PELAEZ T., HOYLE C. H. V., PERAL A. 2001. *Involvement of melatonin MT3 receptors in the regulation of intraocular pressure in rabbits*. Eur. J. Pharmacol., 416: 251–254.

SAS 2007. *Statistical analysis systems package. SAS for Windows 9.1.3 portable version*. Statistical Systems Institute Inc., Cary, NY, USA.

SZENDRO S., TAGEL-DEM T.H., NEMECH B. 1984. *Effect of double mating on conception rate and litter size in rabbits*. Proc. World Rabbit Cong., 2: 124–128.

TELEFO P.B., MOUNDIPA P.F., TCHOUANGUEP F.M. 2002 *Oestrogenicity and effect on hepatic metabolism of the aqueous extract of the leaf mixture of Aloe buettneri, Dicliptera verticillata, Hibiscus macranthus and Justicia insularis*. Fitoterapia, 73: 472–478.

UBILLA E., ALVARINO J.M.R., ESQUIFINO A., AGRASAL C. 1992. *Effects of induction of parturition by administration of a prostaglandin F2a analogue in rabbits: Possible modification of prolactin, LH and FSH secretion patterns*. Anim. Reprod. Sci., 27: 13–20.

WATCHO P., NGADJUI E., ALANGO, N.P., BENOIT N.T., KAMANYI K. 2009. *Reproductive effects of Ficus asperifolia (Moraceae) in female rats*. African Health Science, 9(1): 49–53.

ZEMJANIS R. 1977. *Collection and evaluation of semen*. In: *Diagnostic and therapeutic techniques in animal reproduction*. The Williams and Wilkins Co. Baltimore.

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EMBRYO PRODUCTIVITY OF THE DONOR COWS INSEMINATED BY UNISEXUAL AND BISEXUAL SEMEN*

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Key words: embryo, cattle, blastocyst, transplantation, donor.

Abstract

The aim of the study was to determine the quantity and quality of embryos and effectivity of embryo transfer obtained from Simmental donor cows by artificial insemination with various semen types. The study indicated that cows fertilized with bisexual sperm had a higher fertilization efficiency than those fertilized with unisexual sperm. From one cow fertilized by bisexual sperm, an average of 10.0±2.25 transplantable and 2.8±1.65 was non-transplantable embryos were obtained. When used the unisexual semen, 6.2±4.30 and 4.0±2.02 embryos respectively were obtained. Thus, using bisexual semen resulted in an average of 2.6 more embryos.

The superovulation of two donors fertilized by unisexual sperm was unsuccessful. The above suggests that conditions close to natural ones created greater survival possibilities for a higher number of transplanted embryos which means more successful reproduction.

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Introduction

Embryo transplantation as a biotechnological method for accelerated reproduction opens up enormous opportunities for realizing the reproductive and biological potential of animals, with the use of an individually targeted genetic reserve with economically useful features, given phenotypic and genotypic characteristics, as well as their subsequent maximum replication in herds of recipients with the least valuable indicators. Despite the successes achieved in the technology of embryo transplantation, the issues of finding new methods for the selection of donors and recipients, induction of superovulation with a higher yield of embryos, reducing the complexity of the process, and animal stress – are still valid (HANSEL et al. 1986, GORDON 2003, MAPLETOFT et al. 2016).

In order to increase the production of milk and meat products, it is necessary to use biotechnological methods to improve animal husbandry, as well as to conduct correct selection and slaughter of animals based on their genetic potential (THORNE 2013).

The main stages of embryo transplantation using the in vivo technique is superovulation, egg fertilization, zygote formation, and then its fragmentation and blastomerization. Knowledge of the oocyte morphology makes it possible to control the development of the donor cow embryos, starting from the process of obtaining embryos by washing, and thus allows the assessment of the embryo and determining its further development (BAYMISHEV 1999, BABINTSEVA et al. 2012, POLYANTSEV et al. 2012).

A breeding cow gives birth to about 6–8 calves during its lifetime. Embryo transplantation is the only selection method to obtain hundreds of calves with good breed characteristics. Studies have shown that this method produces 50–60 transplant embryos obtained from one sample per year. This means that a high-yielding cow can gave a minimum of 25–30 calves embryos (AIATHANULY and SANJJAVYN 2012). Due to the transplantation of cattle embryos, it is possible to improve the breeding stock reproductive activity and to secure high productivity as soon as possible.

Following the experts, the X chromosome semen is demanded by the dairy livestock sector, and the Y chromosome semen by the beef cattle breeding sector. Fertilization of ova with sexually separated sperm, as opposed to normal sperm, allows obtaining individuals of the desired sex with a probability of 93%. When transplanting embryos not divided by sex, the birth rate of heifers is up to 55%, which is of no small importance in dairy cattle breeding, where there is a high demand for heifers with high milk production (DMITRY 2017).

Transplantation of unisexual embryos results in a major economic impact, for example:

– growth of heifers up to 95 pieces per 100 pieces. It is used to form a herd of high-yielding breeding cows without buying expensive animals from abroad;
– high productivity of cows born through the transplantation: increase average annual gross milk yield which can reach 16–20 thousand kg (compared with 4–8 thousand kg of the usual ones);
– the cost of livestock management is reduced several times due to reducing the duration of the service period (this is facilitated by the high fertility of the sexed semen and high survival ability of the transplanted embryos). Due to the reduction in the number of difficult calving and birth of dead calves, the economic losses are reduced;
– the average daily gain in live weight of male cattle generated by this technology significantly exceed the average weight gain of those of the "local" herds and increased the profitability of livestock meat farms.

The development or non-development of embryos in the uterus of recipients directly depends on their quality. During transplantation to recipients, based on their morphological characteristics, the embryos assigned to the highest category developed in 70%, while those assigned to the satisfactory group in 44% (ANZOROV et al. 2005, BAYTLESOV et al. 2007, GAVRIKOV 2012).

Proper evaluation of the embryos directly influences the results of transplant biotechnology. The use of modern scientific achievements and the latest technologies is a priority for the development of a competitive livestock industry. Therefore, in the North-Eastern region of Kazakhstan, research began determined of the embryo productivity of Simmental through fertilization by unisexual and bisexual semen.

The aim of the study was to determine the quantity and quality of embryos and effectivity of embryo transfer obtained from Simmental donor cows by artificial insemination with various semen types.

Materials and Methods

The research were carried out within the period from 2017 to 2019 in the "Galitskoye" LLP and "Pobeda" LLP in the Pavlodar region. The cows were kept in the barn all year round and fed in the TMR system. In the ration, roughage preserved in a mixed form was used, i.e. maize silage, haylage, concentrated mix, molasses, and mineral premix. Additionally, selenium licks were used. Cows were divided into 3 feeding groups depend-

ing on their yield, i.e. with the highest yield (more than 40 kg of milk), with average yield (20–30 kg of milk) and the lowest yield (less than 20 kg of milk). The fourth group consisted of dry cows that did not receive concentrated feed.

Healthy dairy cows without gynecological diseases were selected for the study, with milk production of 6000–8000 kg per lactation, an average live weight of 500–650 kg, and from 2 to 5 lactations. Thirteen Simmental animals with higher milk yields were selected. Selected donor cows were subjected to gynecological examination to determine their basic health, mainly the function of the sexual organs, and to determine the moment of egg collection. In addition, donor cows were selected based on zootechnical characteristics.

Pluset hormone (follicle-stimulating hormone; Calier, Spain) was used to activate superovulation of donor cows. The donors achieved superovulation within 11 days after hormone administration. For this purpose, the Pluset hormone was intramuscularly injected twice (morning and evening) for 4 days (in lowering doses). The hormone was injected as follows: on the first two days 1.5 ml in the morning and evening; on the third and fourth day – 10.0 ml twice per 1.0 ml. After all, cows were inoculated, 4.0 ml of prostaglandin (magestrofan) were injected additionally in the morning and the evening in order to rapidly ovulate and collect the embryos. After the hormonal treatment, signs of ovulation appeared in each of the test cows were defined based on external characteristics and behavioral changes. Then, double artificial insemination was performed with bull semen assigned to each donor (2 doses in the morning and 2 doses in the evening). In an aim to determine the embryo quantity and quality of the donor cows, two groups were identified. The first group was artificially inseminated by the unisexual sperm (X sperm), the second one with bisexual one (X, Y sperm). On the seventh day after artificial insemination of donors, the embryos were washed with Dulbecco saline from the uterine horns. The rinsed saline from the uterine horns along with the outflowing contents were collected into a container and the volume of the injected and withdrawn saline was examined. The container with the saline was kept in a laboratory with a temperature not lower than +20 degrees, settled for 15–20 minutes, and, after the embryos had been settled on the bottom of the bottle, the upper layer of the saline. The saline solution which had collected at the bottom of the bottle, up to 5 cm in volume, was carefully shaken and poured into the petri dish. The embryos were detected by microscopy (Nikon SMZ-745, magnification 0.67–5). After detection, each embryo was subjected to stereomicroscopic examination at 50–60 magnification. The quality of the embryos was assessed with the use of morphological indicators.

The obtained data on the quantity and quality of embryos obtained from donor cows were statistically analyzed. The arithmetic means (X) and standard deviations (Sd) were calculated.

The results

Selected donors which had superovulation were artificially inseminated with bisexual and unisexual semen. 8 donors were artificially inseminated with bisexual semen and 5 donors with same-sex semen. 4 portions of semen were used per animal. The embryo quantity of donors artificially fertilized with different sperm is shown in Table 1 and the diagram.

Table 1

Influence of bisexual and unisexual spermatozoids on donor embryo quantity and quality

#	Semen type	Number of donors (*n*)	Quantity of embryos obtained [%]		Quantity of transplantable embryos [%]		Quantity of non-transplantable embryos [%]	
1	bisexual sperm (X,Y)	8	102	100	80	78.4	22	21.6
2	unisexual sperm (X)	5	51	100	31	60.8	20	39.2
Total		13	153	100	111	78.4	42	21.6

As can be perceived from the above Table 1, 102 embryos were washed out of 8 donors artificially inseminated with bisexual sperm. 78.4% of the obtained embryos were transplantable, the remaining 21.6% were non-transplantable. Of all 5 donors artificially fertilized with same-sex sperm, 51 embryos were washed away. 60.8% of the embryos were suitable for transplantation and 39.2% were not suitable for transfer. If we compare the transplantability of the embryos washed out from donors, the donors impregnated with bisexual sperm have 17.6% more embryos, and the non-transplantable embryos fertilized with unisexual sperm have 2 times more embryos. 153 embryos were obtained from all cows. Of these, 111 were transplantable and 42 were without transplants.

The quantity and quality of embryos obtained through superovulation are presented in Table 2.

Table 2

Quantity and quality of embryos obtained from donor cows

#	Donor number	Embryos quantity		Embryos quality			
		total		transplantable		non-transplantable	
		n	%	*n*	%	*n*	%
Inseminated by bisexual sperm							
1	KZS178874122	20	100.0	20	100.0	–	–
2	KZS178685616	14	100.0	12	85.7	2	14.3
3	KZS178865888	1	100.0	1	100.0	–	–
4	KZS178863784	19	100.0	16	84.2	3	15.8
5	KZS178873964	7	100.0	7	100.0	–	–
6	KZS178863784	10	100.0	9	90.0	1	10.0
7	KZS178779002	14	100.0	12	85.7	2	14.3
8	KZS178777715	17	100.0	3	17.6	14	82.4
Total		102	100.0	80	78.4	22	21.6
Inseminated by unisexual sperm							
1	KZS178924313	–	–	–	–	–	–
2	KZS178865458	28	100.0	23	82.2	5	17.8
3	KZS178865471	14	100.0	3	21.4	11	78.6
4	KZS178780636	–	–	–	–	–	–
5	KZS178780424	9	100.0	5	55.6	4	44.4
Total		51	100.0	31	60.8	20	39.2

Through artificial insemination with bisexual sperm from 8 donor cows, a total of 102 embryos were obtained. It is reported that on average 12.8 embryos can be obtained from one cow. The number of embryos received from each cow was different. For example, the largest number of embryos (20) was received from the KZS178874122 cow, while the smallest embryo (1) from the KZS178865888 cow. 78.4% of the washed out embryos were allocated transplantable, 21.6% non-transplantable. The developmental stages of embryos obtained from each cow are found in different proportions. In general, the proportion of transplantable embryos varied from 17.6 to 100.0%, while the proportion of non-transplantable embryos – from 10.0 to 82.4%.

In the results of the superovulation of donors inseminated with same-sex sperm induced by the gonadotropin hormone Pluset, in the order mentioned above. We confirmed that out of 5 experimental cows, 51 embryonated eggs were obtained and an average of 10.2 embryos from one cow. 60.8% of all embryos washed from the uterus were transplant embryos of

normal structure and development. The percentage of embryos unfit for transplant, whose development was late or morphologically changed, was 39.2%.

A variety of indicators of superovulation proves that individual characteristics and physiological differences in donor cows artificially inseminated by unisexual sperm were also formed at a fairly high level. The number of embryos, cells obtained from each cow, and the proportion of transplantable and non-transplantable embryos were different. For example, the number of embryos washed out from each cow was calculated in the range of 9–28, the number of transplantable embryos was 3–23, and the number of the non-transplantable ones was 4–11.

One we can conclude that superovulation was unproductive in 2 donors subjected to the treatment. It means that the effect of the hormone on donors is different. Table 3 presents the results of a comparison of the indicators of superovulation of the Simmental donor cows artificially inseminated by unisexual and bisexual sperm. As indicated in the Table, an average of 12.8 embryos were received from a single donor fertilized with bisexual sperm, including 10 transplantable and 2.8 non-transplantable embryos.

Table 3

Average quantity and quality of embryos received from donor cows (superovulation was provoked)

Semen type	Number of donors (*n*)	Number of embryos received, total	Average number of embryos per cow	Transplantable embryos	Non-transplantable embryos
		n	X±Sd	X±Sd	X±Sd
Bisexual (X, Y)	8	102	12.8±2.28	10.0±2.25	2.8±1.65
Unisexual (X)	5	51	10.2±5.68	6.2±4.30	4.0±2.02

As a result of superovulation, on average 10.2 embryos per specimen were received from the Simmental donor cows inseminated by unisexual sperm, of which 6.2 are transplantable, 4.0 non-transplantable.

Now let us compare the results of the superovulation, fertilized by bisexual sperms in this experiment. On average 12.8 embryos were received from the donor cow fertilized by bisexual sperm, of which 10.0 were transplantable, 2.8 non-transplantable. It was reported that superovulation of the Simmental breed cows, fertilized by unisexual sperm, resulted in high rates of 10.2; 6.2; 4.0. All this highlights that the number of the embryos received from a Simmental cow fertilized by bisexual sperm

is 2.6 more, the number of transplantable embryos is 3.8 more, while in unisexual embryos the number of non-transplantable embryos is conversely 1.2 lower.

Statistical processing of the obtained data indicates that the difference between the number of transplantable, non-transplantable embryos and those obtained from bisexual and unisexual semen undoubtedly varies. The mentioned data indicates that insemination with various sperm types significantly affects the result of cattle fertilization. This demonstrates that cattle fertilization directly depends on various types of spermatozoids. The number of spermatozoids contained in unisexual sperm is 10 times lower than in bisexual sperm. This indicates that the donors artificially inseminated by unisexual sperm have a large number of non-transplantable embryos.

The development of transplanted embryos in the uterus of recipient cows directly depends on their quality. It was confirmed by many experiments that according to the morphological parameters, the highest-category embryos develop at 70%, whereas in satisfactory, middle groups the indicators do not exceed 44%. Therefore, the correct assessment, as the embryos develop, has a great influence on the results of embryo transplant biotechnology.

After the artificial insemination of superovulated cows, we washed out the 7-day-old embryos that descended from the oviduct into the uterus horn cavity using a Foley catheter. Only those embryos are transplanted to the recipient whose development corresponded to the natural development of the embryo at this stage. Only in this case, the fetus would continue to develop after transplantation.

Table 4

Stages of development of embryos obtained from donor cows inseminated by bisexual sperm

Donor number	All embryos		Embryos developmental stage							
			early morula		compact morula		early blastocyst		non-transplantable	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
KZS178874122	20	100	–	–	2	10.0	18	90.0	–	–
KZS178685616	14	100	–	–	3	21.4	9	64.3	2	14.3
KZS178865888	1	100	–	–	–	–	1	100	–	–
KZS178863784	19	100	1	5.3	2	10.5	14	73.7	2	10.5
KZS178873964	7	100	–	–	3	42.8	4	57.2	–	–
KZS178863784	10	100	–	–	4	40.0	5	50.0	1	10.0
KZS178779002	14	100	1	7.1	2	14.3	10	71.5	1	7.1
KZS178777715	17	100	7	41.2	3	17.6	–	–	7	41.2
Total	102	100	9	8.8	19	18.6	61	59.8	13	12.8

As shown in Table 4, 8.8% of the embryos obtained from the donor cows were early morula, 18.6% – compact morula, 59.8% – early blastocyst, and 12.8% – unfertilized eggs. Consequently, the morula embryos made up 27.4%, and the blastocyst embryos – 59.8%. In contrast, the morula embryos showed that the level of development from the blastocyst stage was lower.

This means that the obtained embryos developed at different stages. The degree of the embryo's development did not depend on the number of embryos obtained but on the individual characteristics of the development of animals. Most embryos were early blastocysts. It can be noted that at the stage of the development of embryos obtained from each of the experimental animals, there are some deviations. For example, early morula embryos deviate by 5.3–41.2%, compact morula – by 10.0–42.8%, early blastocyst – by 50.0–100.0%, unfertilized egg – by 7.1–41.2%. In comparison with the processes running in natural conditions, we assume that this is due to a large amount of foaming in heterogeneous uteruses, as well as their slow maturation and prolonged course of ovulation. Endocrinological regulation of processes in the gonads and the function of the fallopian tube after the hormonal treatment show what promotes the development of eggs. Table 5 shows the results of the classification of embryos by developmental stages obtained from donor cows fertilized by unisexual sperm.

Table 5

Developmental stages of embryos obtained from donor cows fertilized by unisexual sperm

Donor number	All embryos		Embryos developmental stages							
			early morula		compact morula		early blastocyst		unfertilized eggs	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
KZS178924313	–	–	–	–	–	–	–	–	–	–
KZS178865458	28	100	2	7.1	8	28.6	15	53.6	3	10.7
KZS178865471	14	100	2	14.3	–	–	3	21.4	9	64.3
KZS178780636	–	–	–	–	–	–	–	–	–	–
KZS178780424	9	100	1	11.1	2	22.2	3	33.3	3	33.4
Total	51	100	5	9.8	10	19.6	21	41.2	15	29.4

In this case 9.8% of the embryos obtained from donor cows were early morula, 19.6% compact morula, 41.2% early blastocyst, and 29.4% – unfertilized eggs. In both donors fertilized by unisexual sperm, superovulation was unproductive. The number of embryos at different stages of development and their proportion for experimental cows were different. For example, the minimum and maximum percentage difference of the early morula

varies from 7.1 to 14.3, the compact morula from 19.6 to 28.6, the early blastocyst from 21.4 to 53.6, and the unfertilized egg from 10.7 to 64.3. Donors receiving a relatively large number of embryos, and cows receiving a smaller number of embryos, demonstrated the priority development of early blastocyst. In addition, it should be noted that in the Simmental cows a large follicle formation inhibited the development of the embryo. Inversely, a small follicle formation accelerates its formation. It can be assumed that the delay and acceleration of the development of the embryo is a phenomenon arising from the simultaneous rupture of the bubbles formed in the eggs, and the uneven release of the eggs.

Table 6

Comparison of developmental stages of embryos obtained by fertilization with bisexual and unisexual semen

Spermatozoids types	Number of specimens	Quantity of embryos obtained, total		Early morula		Compact morula		Early blastocyst		Unfertilized eggs	
		n	%	*n*	%	*n*	%	*n*	%	*n*	%
Bisexual sperm	8	102	100	9	8.8	19	18.6	61	59.8	13	12.8
Unisexual sperm	5	51	100	5	9.8	10	19.6	21	41.2	15	29.4

A donor fertilized with bisexual sperm has an average of 8.8% early morula, 18.6% compact morula, 59.8% early blastocyst, and 12.8% unfertilized egg (Table 6). As for the Simmental cow, fertilized by unisexual sperm, the development of the early morula embryos is shown by 9.8%, compact morula 19.6%, early blastocyst 41.2%, unfertilized egg 29.4%.

If we compare the embryos fertilized by bisexual semen, each embryo has the early blastocyst developed in maximum quantity (bisexual – 59.8%, unisexual – 41.2%). The next stage of the development of embryos is a compact morula, which shares 18.6% in the bisexual scenario and 19.6% in the unisexual one. The most poorly formed embryo species are as follows: early morula in two breeds makes up 8.8% in the bisexual scenario, and 9.8% in the unisexual one. As for the unfertilized egg, the percentage of embryos fertilized by unisexual sperm was 2.3 times higher (in the bisexual scenario 12.8%, the unisexual one 29.4%). In this narrow tube of 0.25 ml, one dose of sperm contains at least 15–25 million germ cells. And a single dose of unisexual sperm of 0.25 ml contains only 2 million germ cells.

The actual difference lies in the fact that in the cows inseminated by bisexual sperm, in contrast to donors fertilized by unisexual sperm, the proportion of unfertilized eggs is around 16.6% more (29.4% and 12.8%). Early blastocyst embryos in bisexual embryos were more than 18.6% greater (in unisexual embryos 41.2, in bisexual 59.8). Besides, it was observed that the early morula and compact morula embryos did not have many differences, i.e. their share is only 1% (early morula 8.8 and 9.8%, compact morula 18.6 and 19.6%).

Pregnancy rates after embryo transplantation rarely exceed 50%, and in most cases are even lower. According to the reports, the onset of pregnancy at the transplantation of embryos into the lower and middle third of the uterine horn is 25–37.5%, and when they are transplanted into the upper third, it reaches 40—50% or more. According to BRIGIDA (2017), such difference in the embryo engraftment is unlikely to be related to the technique of transplantation or the quality of embryos, since in all cases these indicators were the same; at the same time, the optimal location of the seven-day embryo has a direct effect on the hormone-mediated signaling system by the feedback mechanism.

In this regard, it can be assumed that the upper third of the uterine horn is an optimal site for implantation of a seven-day embryo (OVCHINNIKOV and SMYSLOVA 1985, SREENAN 1976). The results of the study are presented in Table 7.

Table 7

Results of the embryos implantation

Farm name	Embryos transplanted in the upper part of the uterus horn		Implanted embryos		Non-implanted embryos	
	n	%	*n*	%	*n*	%
unisexual embryos						
"Galitskoye" LLP	7	100.0	3	42.8	4	57.2
"Pobeda" LLP	5	100.0	4	80.0	1	20.0
Total	12	100.0	7	58.3	5	41.7
bisexual embryos						
"Galitskoye" LLP	27	100.0	15	55.6	12	44.4
"Pobeda" LLP	15	100.0	10	66.7	5	33.3
Total	42	100.0	25	59.5	17	40.5

Table 7 above shows that out of 12 unisexual embryos 7, or 58.3%, continued to develop in the uterus of the recipient heifers, the remaining 5 embryos, or 41.7%, stopped their development. However, the results of

the embryo transplantation in different farms were different, amounting to 42.8–80.0%. Bisexual embryo transplantation studies show that 25, or 59.5%, of the 42 embryos continued to develop, while the remaining 17 embryos, or 40.5%, stopped developing. The results of the study on embryo transplantation on different farms show a difference of about 11%. Based on these data, we conclude that the development of unisexual and bisexual embryos is approximately the same. The remaining transplantable 38 bisexual and 19 unisexual embryos were kept deep-frozen in liquid nitrogen at –196ºC.

From the obtained data it was found that the results of transplantation at the localization of implanted embryos in the middle third of the uterine horn do not have significant differences.

Discussion

The current study resulted in new, theoretically substantiated, and tested in practice data on the morphology of ovaries and embryos obtained from donor cows at the induction of polyovulation, extraction, collection, and transplantation of embryos. All studies proved that most of the embryos washed out from the donor uterus formed compact morula and early blastocyst. However, it does not exclude that there are early morulae and expanded and released blastocysts. Comparing with natural conditions, such rapid and late development of embryos in the eggs of females is associated with a variety of bubbles formed in excess, and prolonged ovulation. Many bubbles formed in the egg cannot develop evenly. Some of them develop normally, the others earlier, the next ones – late. Because of this, the maturation of the bubbles varies. Due to various maturated bubbles, the eggs gradually decrease and the ovulation process lasts 4–12 hours (HASLER et al. 2003).

The combination of spermatozoids with eggs prolongs the process of fertilization for a while. Besides, the body increases the estrogen hormone released from a large number of bubbles. Due to this, the embryo moves much faster along the oviduct to the cavity of the uterine horns than it does in a natural. As a result of the imbalance of hormones in the organism, normal conditions inside the uterus also change. For these reasons, we can conclude that the development of embryos obtained from superovulated cows is also changed. In addition, we can say that the influence of such factors as the hormone type, cattle breed, time and method of producing embryos, reproductive cycle, repetition of superovulation, is significant.

As a result of research by ERNST and SERGEYEV (1989) and SERGEYEV and AMARBAYEV (1987), 1512 embryos were received from donors within 6 and 7 days after fertilization. The morphological assessment was performed. According to the authors, 27.4% of all embryos taken on the 6th day were early morula, 69.3% morula, 3.3% early blastocyst. On the 7th day, the number of morulae decreased, while the proportion of blastocyst species increased. In particular, the early morula changed for 9%, morula for 10.2%, early blastocyst for 79.5%, expanded blastocyst for 1.3%. It is described that 38.2% of all examined embryos were morphologically normal and at appropriate developmental stages. The degenerated embryos accounted for 24.1%, unfertilized eggs – 37.7%. The number of embryos transplanted from adult cows was higher compared to heifers (33.6% and 46.8%, respectively). In heifers, the incidence of unfertilized eggs is higher (42.8% and 28.4%).

In Bavaria (Germany), AYATKHANULY et al. (2010) received 791 embryos from 47 donors of the Simmental race and determined the stages of their development. 63.8% of the embryos obtained as part of the study were suitable for transfer, 15.1% were unfit for transplantation, and 21.1% of the eggs were unfertilized. The embryos obtained on the 7th day after fertilization were classified according to the stages of their development. The results were as follows: 22.8% – early morula, 49.62% – morula, 16.26% – early blastocyst, 11.32% – expanded blastocyst.

Another study proved that 69.9% of embryos obtained from 202 donor cows with the use of follitropin were suitable for transplantation. Of these, 76.6% were morules, 23.4% were blastocysts (WILLETT et al. 1951).

Scientists at the Kazakh Research Institute of Animal Breeding and Feed Production conducted research to generate the calf of the required breed through the in vivo and in vitro methods. As a result of the in vivo study, 38 same-sex embryos were obtained. Of these, 26 were transplanted fresh, the remaining 12 were cryopreserved (KARYMSAKOV et al. 2017). Scientists of the Pavlodar State University conducted comparative research to determine the quantity and quality of embryos received from 6–10 year old adult Simmental breed donor cows and 18–24 month old heifers. From 7 adult cows acquired 58 embryos, 29 embryos from heifers were received within the study. Of the embryos received from adult cows, 49 were transplantable, 9 non-transplantable. Of the embryos received from heifers, 16 were transplantable, 3 non-transplantable. Embryos from adult donor cows were in 6.9% at the early morula stage, 39.7% – compact morula, 27.6% – early blastocyst, 17.2% – blastocyst, 8.6% – expanded blastocyst. Embryos from heifers were in 10.3% at the early morula stage, 51.7% – compact morula, 34.5% – early blastocyst, 3.5% – blastocyst (AYAT-

KHAN et al. 2015). According to research by Brigida (2017), 1218 embryos came from 138 milk donors. That is on average 8.8 embryos were collected from one donor. Of these cases, 685 (56.2%) were transplanted, 98 (8.2%) were partially degenerated, 171 (14.0%) were degenerated and 264 (21.6%) were unfertilized eggs. In 2015, at the animal breeding association in Neustadt Aisch (Germany), a group of scientists headed by NOHNER received 3999 transplantable embryos from 386 donor cows, on average 10.4 embryos per donor (NOHNER 2016).

Conclusion

Based on the results of the research carried out in North-East Kazakhstan, it was found that: It was determined that the Simental breed of cattle significantly affects the result of superovulation. The cows fertilized by bisexual sperm form more embryos than those fertilized by unisexual sperm (12.8±2.28; 10.2±5.68). An average of 10.0±2.25 of a cow's embryos are transplantable, 2.8 ± 1.65 are non-transplantable; in the unisexual embryos are 6.2±4.30, 4.0± 2.02 washed out. Consequently, on average more than 2.6 embryos were obtained from bisexuals. 8.8% of all embryos obtained by fertilization with bisexual sperm developed to the early morula stage, 18.6% compact morula, 59.8% early blastocyst, 12.8% unfertilized egg. It can be noted that at the stage of development of embryos obtained from each of the experimental animals, there are significant deviations. For example, embryos at the early stage of morula deviate from 5.3 to 41.2%, compact morula from 10.0 to 42.8%, early blastocyst from 50.0 to 100.0%, unfertilized egg from 7.1 to 41.2%. As for the unisexual semen, 9.8% of all developed embryos were early morula, 19.6% compact morula, 41.2% early blastocyst, 29.4% unfertilized egg. In both donors fertilized by unisexual sperm, superovulation was unproductive. The survival rate in transplanted unisexual embryos was 58.3% but in bisexual 59.5%. However, the engraftment of unisexual embryos to the uterus of the recipient heifers in various farms ranged from 42.8–80.0%. In bisexual embryos, this indicator was 55.6–66.7%.

It is noted that for successful transplantation, embryos must reach a certain stage of development. Creating conditions similar to the natural ones improves opportunities to successfully breed and increases the number of transplanted calves.

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References

AIATHANULY M., SANJJAVYN G. 2012. *Janýarlardyń uryǵyn kóshirip otyrǵyzý*. Pavlodar Ýlanbaatar, 40.

ANZOROV V.A. 2005. *Sravnitelnaya charakteristika i otsenka zhiznesposobnosti embrionov, poluchennykh ot zdorovykh i problemnykh korov-donorov s raznym urovnem molochnoy produktivnosti*. Agricultural Biology, 6: 63–68.

AYATKHAN M., ATEIKHAN B., SEITEUOV T.K. 2015. *Rezultaty izucheniya morphologii razvitiya embrionov ot superovulirovannykh korov i telok simmentalnoy porody*. Vestnik of State University Named After Shakarim. Semei, 72: 211–213.

AYATKHANULY M., LEIDING K., NOONER K.H.P. 2010. *Quantitative and qualitative study of embryos obtained from the German Simmental breed donor cows. International scientific practical conference*. Barnaul ASU, 30–33.

BABINTSEVA T.V., SUTYGINA A.N., NOVYKH N.N., BAUMAN N.E. 2012. *Morphological and functional characteristics of the ovaries of cows*. Scientific notes of the Kazan State Academy of Veterinary Medicine, 209: 55–59.

BAYMISHEV Kh.V. 1999. *Morphologiya yaichnikov I reproduktivnye kachestva telok v zavisimosti ot vozrasta i dvigatelnoy aktivnosti*. Veterinary, 11: 33–35.

BAYTLESOV E.U., TITOVA V.A., Tyapugin Y.A. 2007. *Aspekty embrionalnoy smerti v skotovodstve*. Veterinary pathology, 2: 128–131.

BRIGIDA A.V. 2017. *Morphologicheskoye obosnovaniye usovershenstvovannoi tekhnologii transplantatsii embrionov krupnogo rogatogo skota* (the dissertation for the degree of Candidate of Biological Sciences), Orenburg, 81.

ERNST L.K., SERGEYEV N.I. 1989. *Transplantation of embryos of farm animals*. Moscow VO "Agropromizdat", 8: 302.

GAVRIKOV A.M. 2012. *Biotechnology for the reproduction of beef cattle by embryo transplantation*. Bykovo village, 125.

GORDON, I. 2003. *Laboratory Production of Cattle Embryos*. CABI Publishing, 577.

HANSEL W. 1986. *New concepts of the control of corpus luteum function*. J. Reprod. Fert., 2: 755–768.

HASLER J.F., CAULEY A.D., LATHROP W.F., FOOTE R.H. 2003. *Effect of onor-embryo-resipient interactions on pregnancy rate in a large-scale bovine embryotransfer program*. Theriogenology, 27(1): 139–168.

KARYMSAKOV T.N., BEKENOV D.M., SPANOV A.A. 2017. *Sravnitelnye resultaty prozhivlyayemosti seksirovannykh embrionov. poluchennykh metodami in vivo I in vitro*. Scientific and Production Journal Dairy and Beef Cattle Breeding, 6: 9–11.

MAPLETOFT J., GARCIA GUERRA A., DIAS F.C.F., SINGH J., ADAMS G.P. 2015. *In vitro and in vivo embryo production in cattle superstimulated with FSH for 7 days*. Anim. Reprod., 12: 151–388.

NOHNER H.P. 2016. J*ahrestagung der Arbeitsgemeinschaft Embryotransfer deutschsprachiger*. Lander (AET-d) am 16 und 17 Juni 2016 in Neustadt a. d. Aisch. Zeitschrift zuchtwahl und besamung, p.176.

OVCHINNIKOV A.V., SMYSLOVA N.I. 1985. *Transplantation of embryos in dairy and sheep cattle*. VIZH. Dubrovitsy, pp. 16–18.

POLYANTSEV N.I., AFANASYEV A.I. 2012. *Obstetrics, gynecology and biotechnology of animal reproduction*. St. Petersburg, M., Krasnodar, Lan, 399.

SERGEYEV N.I., AMARBAYEV A.M. 1987. *Embryo transfer in cattle*. Alma-Ata "Kainar", p. 160.

SREENAN J.M. 1976. *Egg transfer in the effect of site of transfer*. Jg. Grassland Anim. Prod. Assoc., 11: 110–115.

THORNE W.J. 2013. *Fertility of beef recipients following a fixed-time embryo transfer protocol that includes follicle stimulating hormone diluted in hyaluronan*. Texas A&M University.

WILLETT E.L., BLACK W.G., CASIDA L.E., STONE W.H., BUKNER P.J. 1951. *Successful transplantation of a fertilized bovine ovum*. Science, 113: 247–248.

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ALLELOPATHIC POTENTIAL OF COTTONWOOD (*POPULUS DELTOIDES* BARTR. EX MARSH.) LEAF EXTRACTS ON SOME RICE (*ORYZA SATIVA* L.) CULTIVARS

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Key words: agroforestry, allelochemicals, allelopathy, plant interactions, seed germination.

Abstract

An experiment was conducted to declare the allelopathic potential of *Populus deltoides* leaf extracts on four local *Oryza sativa* L. cultivars. The Laboratory study consisted of seed germination, radicle, and plumule growth phases. The experiment was performed under five leaves extract concentrations (0%, 25%, 50%, 75%, and 100%). The results revealed that seed germination indices were significantly reduced and inhibited by increasing leaf extract concentration. The development of the plumule and radicle showed a significant reduction in concentration changes. The current study demonstrated that there was a strong allelopathic effect of cottonwood on rice cultivars. It is important to consider that the *P. deltoides* was not recommended as a suitable tree for agroforestry with rice until further research has been conducted on field experiments under the shelter of cottonwood, to identify the allelochemicals in other parts of the cottonwood tree and the soil litter content in the stands.

Introduction

Allelopathy is defined as the natural inhibitory interaction between plants and the environment by producing and releasing some specific chemical compounds into the surroundings. The allelopathic compounds are produced through secondary metabolism in plants that can influence other plant species in agricultural systems, natural systems, or both (agroforestry systems). This phenomenon is a biological force that reduces plant performance by harmful effects such as resource competition, affecting the

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germination and growth disorder of neighboring plant species through chemical interference. Allelopathic compounds that are called allelochemicals can generate different inhibition on the growth of the other plants and induce chemical interactions between organisms or plant species to affect their development, health, behavior, or even population biology (WANG et al. 2020). Therefore, a quantitative analysis is needed to understand the roles of allelopathy as a biological phenomenon in agroforestry systems, study the allelopathic interactions of trees species on agricultural plants, help the past decades' knowledge gaps and guide future research (ZHANG et al. 2021, BANUELAS et al. 2019).

Agroforestry systems involve the combination of cultivated woody tree species with annual crops in the same spatial and temporal arrangements. This combination leads to economic profits, a sustainable environmental land-use system, and multi-layer cultivation. Nevertheless, interactions are inevitable. One of them is allelopathic interactions between the release of allelochemicals from dead and falling leaves of trees and inhibition of seed germination and growth. Allelochemicals could be found in the various parts of a plant, released from the litter, and affect the development of neighbor crop plants in agroforestry systems. Therefore, It must be determined that the allelopathic compatibility of crops with trees before incorporating them into an agroforestry system because released allelochemicals by some trees could affect the establishment and development of crops and reduce economic and environmental efficiency. Otherwise, allelopathic interference effects will recognize as the primary reasons for low productivity in agroforestry systems (JOHN et al. 2006, AMOO et al. 2008).

Tree species (especially multi-purpose ones) as an integral part of agroforestry programs could conserve the soil and increase agricultural soil productivity and quality by helping to add the organic matter into it, improving the water holding capacity of it, biodiversity of microbes, nutrients concentrations, declining pests population and conservation of the soil. However, several tree species have negative effects on the performance of crops through mono-culture plantation and allelopathy. The roots, leaves, and bark of these species release allelochemicals into the soil and caused negative interaction depending on the concentration of allelochemicals. These interactions inhibited the growth of neighbor plants (AMOO et al. 2008, GARIMA and DEVI 2017).

Furthermore, There have been many studies that reported about the suppressive effects of the tree species' leaves extracts on the germination of other agricultural species, such as allelopathic effects of *Senna siamea*, *Albizia lebbeck*, *Azadirachta indica*, *Cedrela odorata*, *Leucaena leucocephala*, *Gliricidia sepium*, *Eucalyptus grandis*, *Terminalia superba* and *Tec-*

tona grandis trees species on seeds of *Zea mays* (Maize), *Vigna unguiculata* (Cowpea), *Lycopersicon esculentum* (Tomatoes), and *Hibiscus esculentus* (Okra) (ABUGRE et al. 2011). *Azardiracta indica*, *Vitellaria paradoxa*, and *Parkia biglobosa* trees on germination and growth of *Vigna unguiculata* (cowpea) (ALEEM et al. 2014). *Pinus halepensis* and *Quercus coccifera* as two agroforestry trees on the germination of *Triticum aestivum* (wheat), *Hordeum vulgare* (barley), *Lens culinaris* (lentil), *Cicer arietinum* (chickpea), and *Vicia faba* (faba bean) (ALRABABAH et al. 2009). Also Allelopathic potential of *Tetrapleura tetraptera* leaf extracts on *Lycopersicon esculentum*, *Abelmoschus esculentum*, *Amaranthus spinosus*, *Capsicum annum*, and *Solanum melongena* (AMOO et al. 2008). The aqueous leaf extracts of *Pinus sylvestris*, *Broussonetia papyrifera*, and *Pinus tabulaeformis* could promote *Amygdalus pedunculata* seed germination and seedling growth (WANG et al. 2021).

On the other hand, there are represents that showed the allelopathy in ecosystem-level of forests (BLANCO 2007). Many kinds of the literature indicated the trees' foliage leachates had the allelopathic potential on the plants of understory that leaf extracts could influence seedling growth in the natural condition (TEIXEIRA DA SILVA et al. 2015).

The allelopathic capacity of forest trees has been reported previously such as allelopathic effects of *Albizia julibrissin* (ABEDI and ABDI 2021), *Melia azedarach* (MAJEED et al. 2017), *Zanthoxylum armatum*, *Ougeinia oojeinensis* and *Boehmeria rugulosa* (VASISHTH et al. 2020).

Since agroforestry is a leading alternative for food security and forest conservation, it seems that identifying the local trees and crops with the minimum allelopathic interaction is necessary for the best agroforestry system management. Scientific studies could be used to identify the best crops cultivars and the best accompanying tree species to improve the agroforestry system's productivity and move from monoculture to multicultural plantation. A successful agroforestry system management depends on identifying local tree crops with a minimum accumulation of toxins in the soil (THAPALIYAL et al. 2008, ALRABABA et al. 2009).

In recent years, the studies focused on the chemical aspects of agricultural plants and forest trees plantation relationship (LU et al. 2017). Evaluation of allelopathic effects of leaf and bark extracts of *Dalbergia sissoo* on wheat germination under the different concentrations by SIYAR et al. (2018) observed that the mean germination time significantly increased. The final results suggested the negative effects of allelopathy of this tree species on wheat. In addition, GARIMA et al. (2017) introduced the *P. deltoids* as a multipurpose tree species but had unfavorable effects on crops through allelopathy. They explored the allelopathy effects of *P. del-*

toides leaf extracts on wheat (*Triticum Aestivum*) germination and radicle and plumule length in laboratory conditions and determined that increase in the concentration of leaf extract had more inhibition effects. LU et al. (2017) introduced the poplar as an allelopathic species and reported the impact of monoculture plantation of poplar on the rhizosphere microbial community. SHER et al. (2011) noted the allelopathic potential of *Populus euphratica* against *Sorghum vulgare*, *Setaria italica,* and *Triticum aestivum*. The poplar leaves have toxic and reduce the germination, plumule, and radical growth, fresh and dry weight of all studied crops in laboratory experiments. AMOO et al. (2008) studied the allelopathic potentials of *Tetrapleura tetraptera* leaves extract as a multi-purpose tree species on *Lycopersicon esculentum*, *Abelmoschus esculentum*, *Amaranthus spinosus*, *Capsicum annum,* and *Solanum melongena* as five agriculture crops and reported the significant preventive effects on crops seedling growth. SINGH et al. (2001) studied the allelopathic interface of *P. deltoides* in seven winter seasons crops including *Triticum aestivum* (wheat), *Lens culinaris* (lentil), *Phaseolus mungo* (black gram), *Avena sativa* (oat), *Trifolium alexandrinum* (clover), *Brassica juncea* (Indian mustard) and *Helianthus annuus* (sunflower). Their conclusion showed a significant reduction (10–30%) in germination, height, and biomass of crops. SHARMA et al. (2000) investigated the allelopathic effects of *Populus deltoides* on wheat in the laboratory condition and showed that germination and growth of wheat were suppressed by extraction added to the soil.

In the north of Iran, *P. deltoides* is an important fast-growing and frequently cultivated deciduous tree species for timber, fuelwood, and fodder. That is planted within the rice field borders.

Rice (*Oryza sativa* L.) is a common crop in Guilan province and is ranked the second most widely used crop in the world after maize, which has a significant impact on global food in all of the world (RAHAMAN et al. 2021). So, its high-yield planting is crucial. Nevertheless, there was no study on the allelopathy interaction of these two species. There is little knowledge available in the field of the trees' farming effects on crops in the agro-ecosystem in Iran. Therefore, this study was performed to assess the allelopathy potential effects of cottonwood (*Populus deltuides*) under different leaf extracts concentration on four local variants of rice that originated from Guilan province in the north of Iran. No study has determined these effects before. We examined the possibility of the allelopathic effects of poplar leaves extracts on rice germination, plumule, and radicle growth. The present laboratory investigation was to take the first step for revealing the species inhibitory effects of *Populus deltoides* on the germination of rice cultivars in north Iran and the most promising species to set up field exper-

iments and finally establish agroforestry systems. The present laboratory investigation was to take the first step toward revealing the species inhibitory effects of *Populus deltoides* on the germination of rice cultivars in the north of Iran. *P. deltoides* plantations are the most beneficial species to set up field experiments, and eventually establish agroforestry systems.

Materials and Methods

Plant materials

All experimental steps were performed in the laboratory at Ahar Faculty of Agriculture and Natural Resources at the University of Tabriz. Laboratory study consists of two phases, seed germination and radicle, and plumule growth phases by the following instructions.The experimental seeds were four local rice Varieties including Shiroodi, Gharib, Alikazemi, and Hashemi. All seeds were collected from the fields of Guilan province, North of Iran. The region belonged to a humid climate zone.

Preparation of aqueous leaves extracts

Fresh leaves were collected directly from the fully mature cottonwood trees at the different parts of the trees canopy (from lower, middle, and top parts) and air-dried. The leaves were powdered and screened with a one-millimeter sieve to remove visible extra residues. The 1% leaf aqueous extracts were prepared by soaking air-dried leaves powder in 100 ml of distilled water and were shaken on the shaker for one hour. The leaves' aqueous extracts were stored in a refrigerator at 4ºC for 24 hours, and these processes were repeated for two days. The Leaf aqueous extracts were filtered through a Whatman filter of 50 uniform sizes and were prepared in 100% (100 ml of aqueous leaf extract without distilled water), 75% (75 ml. Leaf extract with 25 ml; distilled water), 50% (50 ml Leaf extract with 50 ml distilled water) and 25% (25 ml; leaf extract with 75 ml distilled water).

Seed germination phase

A hundred healthy local seeds in each rice cultivar as experimental treatments including T1 (Shiroodi), T2 (Gharib), T3 (Alikazemi), and T4 (Hashemi) were counted and placed in sterilized Petri dishes (9 cm diameter) on double layers of filter paper. Each treatment was replicated four times for experimental replication. The germination experiment was performed at 25º C temperature. Samples were irrigated by leaf extractions

daily (100%, 75%. 50%, 25%, and 0% (as control, consisting of distilled water) (BOKHARY 1978). The germination was complete within six days (When the seed's germination completely stopped). This experiment was laid out in a completely randomized design with four treatments in four replications (GARIMA et al. 2017, NANDAL and DHILLON 2007).

Germination percentage was determined by counting the number of germinated seeds for six days on a daily observation basis.

Radicle and plumule growth phase

Ten seeds from the previous phase were transferred to the large culture vessels. They were kept at room temperature for 10 days and irrigation continued using each extract. Then, radicle and plumules lengths were measured and recorded. Seedlings of each replication were dried oven-dried at 70ºC until weight loss accrued and weighted to determine the dry biomass weight (CHATURVEDI and JHA 1992). The experimental design at this phase also included a completely randomized design with four treatments in four replications.

The germination index was determined by counting the number of germinated seeds (GARIMA et al. 2017).

Statistical analysis and indices calculations

Statistical analysis was performed by Tukey test among growth indices (including the germination rate, germination percentage, germination inhibition, plumule length, radicle length, total length, fresh weight, dry weight, tissue moisture content, weight vigor index, length vigor index, seed vigor index, plumule vigor index) and concentrations (S0 (Control), S1 (25%), S2 (50%), S3 (75%) and S4 (100%) in Rcommander (Rcmdr) package (FOX and BOUCHET-VALAT 2020) of R software at $P \leq 0.05$ (SIYAR et al. 2018):

$$\text{GR (germination rate)} = \Sigma \text{ (seeds germinating per day)}$$
$$\text{GP (germination percentage)} = (\text{total germinated seeds} / \text{total seeds}) \cdot 100$$
$$\text{GI (germination inhibition)} = [(\text{treatment GP} - \text{control GP})/\text{control GP}] \cdot 100$$
$$\text{WVI (weight vigor index)} = (\text{GP}/100) \cdot \text{TW (total weight)}$$
$$\text{LVI (length vigor index)} = (\text{GP}/100) \cdot \text{TL}$$
$$\text{SVI (seed vigor index)} = (\text{RL} + \text{PL}) \cdot \text{GP}$$

where:
PL – plumule length
RL – radicle length
TL – total length
FW – fresh weight – shoot and root fresh weights
DW – dry weight – shoot and root dry weights
TMC – tissue moisture content
PVI – plumule vigor index.

Result

Seed germination rate (GR) and percentage (GP) reduced significantly in increasing leaf extract concentration compared with control concentration (S0) in all treatments (Figure 1, *a*, *b* and *c*; Table Anx 1). In addition, this increase was significant between treatments (Table 1). Therefore, the seed germination was inhibited significantly in the highest concentrations. The most inhibition was in the highest leaf extract concentration (S4 = 100%).

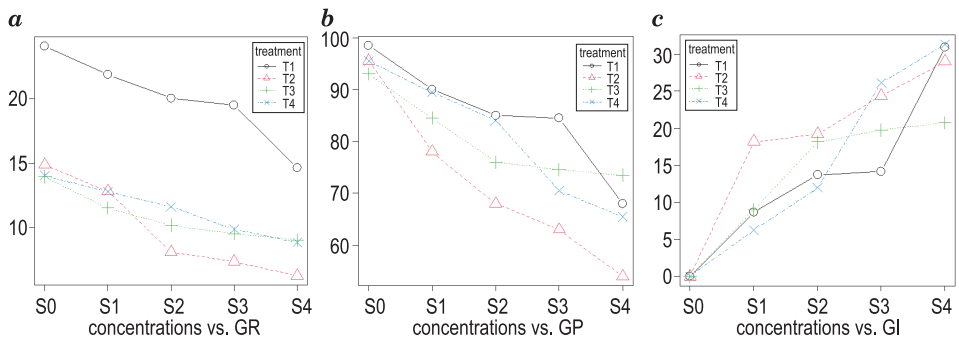

Fig. 1. Seed germination rate (GR, *a*), germination percentage (GP, *b*), and germination inhibition (GI, *c*) in different leaf extract concentrations

Table 1

ANOVA-Tukey test between treatments

	Specification	Sum of squares	df	Mean square	F	Sig.
GR	between groups	1327.427	3	442.476	56.854	.000*
	within groups	591.484	76	7.783	–	–
	total	1918.911	79	–	–	–
GP	between groups	1924.200	3	641.400	4.689	0.005*
	within groups	10395.600	76	136.784	–	–
	total	12319.800	79	–	–	–
GI	between groups	291.022	3	97.007	0.704	0.553ns
	within groups	10473.609	76	137.811	–	–
	total	10764.631	79	–	–	–
PL	between groups	12.497	3	4.166	6.094	0.001*
	within groups	51.952	76	0.684	–	–
	total	64.449	79	–	–	–
RL	between groups	31.805	3	10.602	4.590	.005*
	within groups	175.559	76	2.310	–	–
	total	207.364	79	–	–	–
TL	between groups	33.415	3	11.138	2.963	0.037*
	within groups	285.651	76	3.759	–	–
	total	319.066	79	–	–	–

Cont. Table 1

FW	between groups	0.011	3	0.004	3.134	0.030*
	within groups	0.085	76	0.001	–	–
	total	0.096	79	–	–	–
DW	between groups	0.017	3	0.006	48.517	.000*
	within groups	0.009	76	0.000	–	–
	total	0.026	79	–	–	–
SVI	between groups	124593.065	3	41531.022	5.266	0.002*
	within groups	599420.991	76	7887.118	–	–
	total	724014.055	79	–	–	–
LVI	between groups	46.564	3	15.521	3.382	0.022*
	within groups	348.827	76	4.590	–	–
	total	395.391	79	–	–	–
WVI	between groups	0.013	3	0.004	1.451	0.235ns
	within groups	0.233	76	0.003	–	–
	total	0.246	79	–	–	–
PVI	between groups	4.656E7	3	1.552E7	3.382	0.022*
	within groups	3.488E8	76	4589826.250	–	–
	total	3.954E8	79	–	–	–
TMC	between groups	1618.877	3	539.626	17.362	.000*
	within groups	2362.182	76	31.081	–	–
	total	3981.058	79	–	–	–

* Significant difference in $p \leq 0.05$; ns no significant difference

Note: GR – germination rate; GP – germination percentage; GI – germination inhibition; PL – plumule length; RL – radicle length; TL – total length; FW – fresh weight; DW – dry weight; TMC – tissue moisture content; WVI – weight vigor index; LVI – length vigor index; SVI – seed vigor index; PVI – plumule vigor index

Development of plumule, radicle, and total lengths showed a significant decrease with concentration changes irregularly. However, increasing in concentration of leaf extracts generally reduced the length of growth of plumule and radicle compared to the control level (S0 = Distilled water). The relevant effects appeared on the seedlings' survival by delay in radical growth (Figure 2; *a*, *b*, and *c*).

Therefore, the seedlings' growth decreased significantly with an increase in the concentration of extracts in all rice cultivars (Table 1, Table 2).

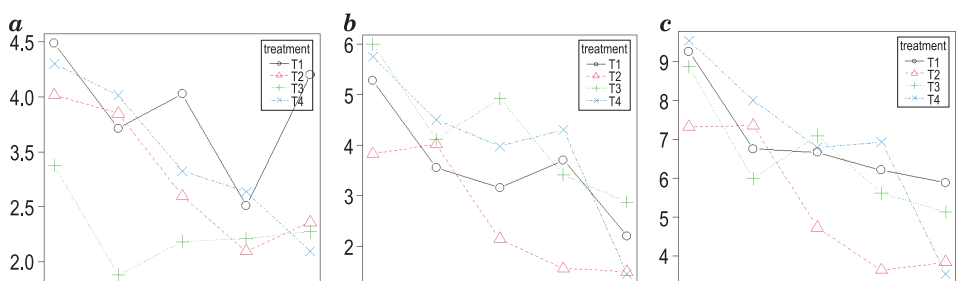

Fig. 2. Plumule length (PL, *a*), radicle length (RL, *b*), and total length (TL, *c*) in the different leaf extract concentrations

Table 2

Pearson correlations test

Specification		GR	FW	DW	PL	RL	TL	GP	GI	SVI	LVI	WVI	PVI	TMC
GR	correlation	1	0.127 ^{ns}	-0.530*	0.487*	0.348*	0.499*	0.733*	-0.523*	0.645*	0.608*	0.541*	0.608*	0.551*
	Sig	–	0.260	.000	.000	.002	.000	.000	.000	.000	.000	.000	.000	.000
FW	correlation	–	1	0.224*	0.512*	0.288*	0.463*	0.406*	-0.433*	0.584*	0.514*	0.805*	0.514*	0.510*
	Sig	–	–	0.045	.000	.010	.000	.000	.000	.000	.000	.000	.000	.000
DW	correlation	–	–	1	-0.162 ^{ns}	-0.262*	-0.283*	-0.303*	0.137 ^{ns}	-0.224*	-0.289*	-0.081 ^{ns}	-0.289*	-0.714*
	Sig	–	–	–	0.152	0.019	0.011	0.006	0.224	0.046	0.009	0.477	0.009	.000
PL	correlation	–	–	–	1	0.204 ^{ns}	0.614*	0.451*	-0.314*	0.937*	0.616*	0.576*	0.616*	0.514*
	Sig	–	–	–	–	0.069	.000	.000	0.005	.000	.000	.000	.000	.000
RL	correlation	–	–	–	–	1	0.898*	0.622*	-0.502*	0.394*	0.853*	0.557*	0.853*	0.409*
	Sig	–	–	–	–	–	.000	.000	.000	.000	.000	.000	.000	.000
TL	correlation	–	–	–	–	–	1	0.704*	-0.546*	0.738*	0.964*	0.707*	0.964*	0.560*
	Sig	–	–	–	–	–	–	.000	.000	.000	.000	.000	.000	.000
GP	correlation	–	–	–	–	–	–	1	-0.841*	0.721*	0.848*	0.866*	0.848*	0.547*
	Sig	–	–	–	–	–	–	–	.000	.000	.000	.000	.000	.000
GI	correlation	–	–	–	–	–	–	–	1	-0.573*	-0.703*	-0.779*	-0.703*	-0.415*
	Sig	–	–	–	–	–	–	–	–	.000	.000	.000	.000	.000
SVI	correlation	–	–	–	–	–	–	–	–	1	0.800*	0.789*	0.800*	0.614*
	Sig	–	–	–	–	–	–	–	–	–	.000	.000	.000	.000
LVI	correlation	–	–	–	–	–	–	–	–	–	1	0.829*	1.000*	0.601*
	Sig	–	–	–	–	–	–	–	–	–	–	.000	.000	.000
WVI	correlation	–	–	–	–	–	–	–	–	–	–	1	0.829*	0.632*
	Sig	–	–	–	–	–	–	–	–	–	–	–	.000	.000
PVI	correlation	–	–	–	–	–	–	–	–	–	–	–	1	0.601*
	Sig	–	–	–	–	–	–	–	–	–	–	–	–	.000

* Correlation is significant at the 0.05 level

Fresh weight (FW) decreased in T1, T3, and T4 cultivars by increasing extract concentrations but slightly improved in S2 and S4. In contrast, dry weight (DW) showed an insignificant increasing trend with increasing concentrations of extracts (Figure 3; *b*; Table 1). These results were consistent with the tissue moisture content (TMC) results diagram (Figure 3; *c*).

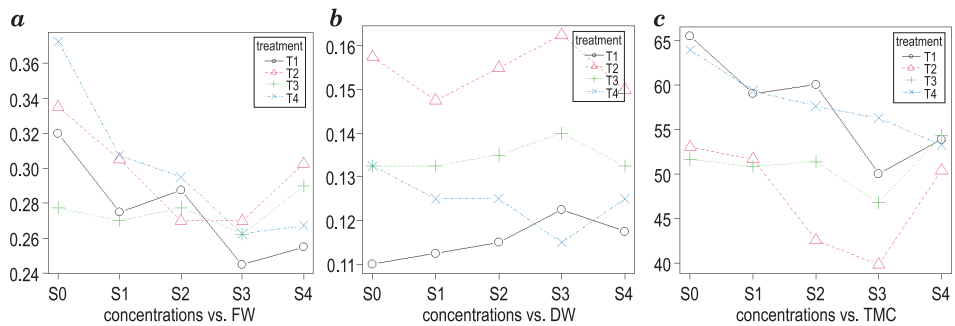

Fig. 3. Fresh weight (FW, *a*), Dry weight (DW, *b*), and Tissue moisture content (TMC, *c*) in different leaf extract concentrations

All leaf extracts significantly suppressed the growth of plumule and radicle of rice culti vars. In addition, the weight and length vigor indices (WVI and LVI, respectively) and plumule vigor index (PVI) reduced with increasing leaf extract concentration regularly (Figure 4; *a*, *b* & *c*; Table Anx 1).

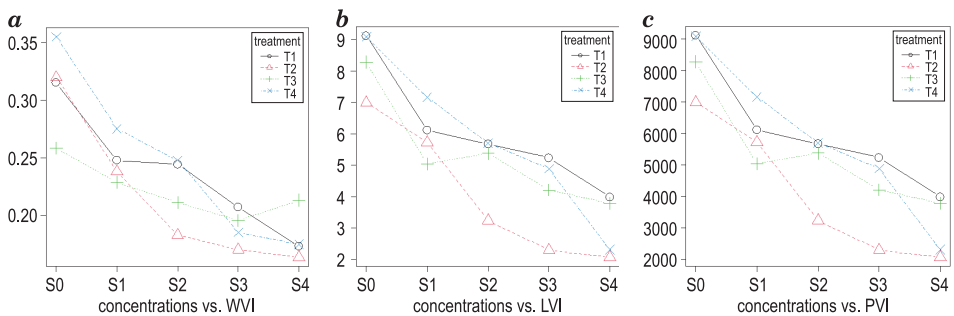

Fig. 4. Weight vigor index (WVI, *a*), length vigor index (LVI, *b*), and plumule vigor index (PVI, *c*) in different leaf extract concentrations

Note:
Leaf extract consecration levels (X-axis): in control (S0), 25% (S1), 50% (S2), 75% (S3) and 100% (S4) consecrations
Indices (Y-axis): GR – germination rate; GP – germination percentage; GI – germination inhibition; PL – plumule length; RL – radicle length; TL – total length; FW – fresh weight; DW – dry weight; TMC – tissue moisture content; WVI – weight vigor index; LVI – length vigor index; SVI – seed vigor index; PVI – plumule vigor index; T1, T2, T3, and T4 – treatments of the local rice cultivars

Pearson correlation test to assess the linear correlation among variables showed that there was a non-significant correlation among PL and RL (0.204), DW and PL (0.162), DW and GI (0.137), GR and FW (0.127), DW and WVI (0.081).

In contrast, There was a strong significant positive correlation ($r \geq 0.7$) between SVI and WVI (0.789), TL and SVI (0.738), GR and GP (0.733), GP and SVI (0.721), TL and GP (0.704), TL and WVI (0.707), RL and TL (0.898), WVI and GP (0.866), RL and LVI (0.853), RL and PVI (0.853), GP and LVI (0.848), GP and PVI (0.848), LVI and WVI (0.829), WVI and PVI (0.829), FW and WVI (0.805), SVI and LVI (0.800), SVI and PVI (0.800), PL and SVI (0.937), TH and LVI (0.964) and TL and PVI (0.964). In addition, There was a strong significant negative correlation ($r \geq -0.7$) between GP and GI (-0.841), GI and WVI (-0.779), DW and TMC (-0.714), GI and LVI (-0.703), and GI and PVI (-0.703) – Table 2.

Discussions

The present study revealed a decrease in rice seedling growth with an increase in the cottonwood leaves extract concentration. Furthermore, this inhibited behavior consisted of seed germination indices and plumule and radicle growth. These results were consistent with KHALID et al. (2021). They concluded that the fresh and dry leaves extract of *Populus nigra* negatively affected the germination and seedling growth of *Brassica campestris* and indicates some allelochemicals might be present in this tree. Also, a similar result was reported by ZUBAY et al. (2021). The allelopathic treatment of *Populus tremula* L. leaves extracts on some medicinal and aromatic plants showed that Poppy and Angelica proved to be the most sensitive species to the treatments. The allelochemicals existing in the higher concentrations of leaf extracts reduced the photosynthetic activity of seedlings and caused a decrease in the dry weight of the target plants (LI et al. 2021).

This study showed that germination and seedling growth significantly reduced in all tested crops in response to *P. deltoides* leaves leachate. The researchers found that the soil of poplar stands was rich in phytotoxic phenolic (SINGH et al. 2001, ZUBAY et al. 2021). Therefore, it is recommended to examine soil content in poplar stands or rice fields in terms of the allelochemicals present in the different soil horizons.

In our study, the allelopathic effects were severe at higher concentrations. This result does not support the MAJEED et al. (2017) that showed the higher extracts concentration of *P. deltoides* leaves had no effects on

the seedling growth parameters. The germination percentage (GP) of wheat was not influenced by the higher concentration of poplar leaves extracts and on the contrary, it was promoted by lower extract concentrations. In contrast, our results were supported by the WANG et al. (2021) that has reported a significant reduction in seed germination and seedling growth of *Amygdalus pedunculata* under concentration increase of some *Pinus* species leaves aquatic extracts. CATALAN et al. (2013) pointed out that *Populus alba* had an intense allelopathic effect and they also emphasized on the extracts' concentration level impacts on prohibition process. Moreover, inhibitory effects of *P. deltiodes* leaf extracts on crops in laboratory or field experiments were shown by SHARMA et al (2000) and SINGH et al. (2001). They also demonstrated its stimulant effects at lower concentrations. Conversely, experiments on the extracts of *Melia azedarach* leaves at all concentration levels revealed inhibitory effects on the seedling growth of wheat (MAJEED et al. 2017). As a result, the allelopathy process is strongly dependent on the type of adjacent or target plants. Consequently, allelopathic effects correlated with the target plant, the type of allelochemicals, and extract concentrations.

Based on the conclusion of the LIU et al. (2003), more inhibition of seedling growth might lead to a theory that more extractions levels contain the higher contents of allelochemicals. In other words, the inhibitory effect of allelopathic plants on other plants was strongly correlated with the concentration percentage and type of allelochemicals (MAJEED et al. 2017).

In addition, the tree-crop-soil interaction demonstrated that the reduction in the growth of the crops associated with *P. deltoides* phenolic presence was released from tree leaves in the soil in this interaction process (SINGH et al. 2001).

Since it is not unexpected that cottonwood is a deciduous tree species and the fallen leaves in autumn and winter on the field floor may be released and accumulate the allelochemicals into the soil. Therefore, soil study is strongly recommended (WANG et al. 2021) because, in field experiments, this process may cause the reduce yield of crops by falling litter under the shelter of cottonwood (KHAKET et al. 2014).

The growth reduction of crops was due to allelopathic interference of phytotoxin phenolics content of cottonwood leaves. Therefore, it is suggested that the content of *P. deltoides* leaf extract should be examined because it was shown the presence of bioactive compounds (that have a role in the allelopathic effects), alkaloids, flavonoids, steroids, terpenoids, saponins, tannins, phlorotannins, glycoside, triterpenes, and phytosteroid in the aqueous leaf extract of *Populus nigra* (INAYAT et al. 2020). In the

same experiment, *Populus tremula* L. trees synthesize compounds typically derived from the shikimate-phenylpropanoid pathway (phenol glycosides, hydroxy-cinnamates, flavonoids, and condensed tannins) (ZUBAY et al. 2021). However, terpenoids and fatty acids are also present in considerable concentrations. The most likely responsible compounds are the phenolic acids. They decompose from decaying leaves to soil.

According to the results of the present study, *Oryza sativa* L. is a sensitive crop species and has poor growth yield under the influence of cottonwood leaf extraction. Similar conclusions were obtained from the KOUL et al. (1991).

They reported a laboratory experiment to investigate the allelopathic activity of leaf leachates from eight commonly grown farm tree species (*Acacia nilotica*, *Dalbergia sissoo*, *Bauhinia variegata*, *Ficus bengalensis*, *Morus alba*, *Populus deltoides*, *Salix babylonica*, and *Leucaena leucocephala*) on the seed germination and the early growth parameters of rice. All leaf leachates inhibited seed germination and seedling growth (with a maximum reduction by *L. Leucocephala*, and *A. nilotica*). Therefore, it was recommended to use more parameters to measure allelopathic effects for suggesting some feasible crops species for a multicultural agroforestry system.

Overall, the type of extractions (leaves, roots, bark, flowers, or fruits), type of target plant, type of allelochemicals, and amount of concentration is the most efficacious factors in an allelopathy interaction. A change that accrued in any aspect could affect the allelopathic potential and reduce the germination of crops (MAJEED et al. 2017). A lower extraction concentration level of *P. deltoides* increases the total length growth of wheat (MAJEED et al. 2017), but all concentrations had high effects on reducing the growth of all rice cultivars in our study. Therefore, the target plant selection is a fundamental phase.

Declining enzyme activity, impairing mineral ions uptake, decreasing cells division and energy limitation, inhibiting photosynthesis, damage to cells, reducing the plant's ability to remove active oxygen, destroying the structure of plant cell membranes, thus weakening the protective effect has introduced as the main factors that resulting from allelochemicals activity in plants. Consequently, they could limit the seeds' germination and seedlings' growth (ABEDI and ABDI 2021, ZHANG et al. 2021, LI et al. 2021).

Conclusions

The current study demonstrated that the allelopathic effects of cottonwood should be attended seriously on tested rice cultivars because it is planting increasingly inside or around the rice fields in recent years in the north of Iran. While according to the results of this study, the fall of cottonwood leaves on rice fields is affected adversely and has inhibitory effects on the growth characteristics of rice seedlings and products. Therefore, it is necessary to consider that the cottonwood is not recommended as a suitable species for agroforestry with rice, but further research is needed to conduct mixed cultivation with other agricultural species and crops. Also, an additional study under natural field conditions is essential for evaluating the effect of local natural conditions on plant growth and an additional study on natural conditions seems to be necessary to assess the impacts of local natural conditions on plant growth, crop yield, and the physical and chemical properties of soil under field cultivation. Further research is necessary to identify the allelochemicals in other parts of the cottonwood tree (such as roots by secreting allelochemicals into the soil) that prohibit or promote the growth of rice or other crops. The results of this experiment may provide a consequential basis for further field experimental studies.

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References

Abedi R., Abdi S. 2021. *Allelopathic effects of persian silk tree (albizia julibrissin) on seedling biomass attributes of tree of heaven (Ailanthus altissima).* Iranian J. For. Range Protection Res., 18(2): 150–164.

Abugre S., Apetorgbor A.K., Antwiwaa A., Apetorgbor M.M. 2011. *Allelopathic effects of ten tree species on germination and growth of four traditional food crops in Ghana*. J. Agri. Tech., 7(3): 825–834.

Aleem M.O., Alamu L.O., Olabode O.S. 2014. *Allelopathic effects of some selected tree species on the germination and growth of cowpea (Vigna unguiculata* L. *Walp.).* Open J. For., 4: 310–315.

Alrababah M.A., Tadros M.J., Samarah N.H., Ghosheh H. 2009. *Allelopathic effects of Pinus halepensis and Quercus coccifera on the germination of Mediterranean crop seeds*. New For., 38: 261–272.

Amoo S.O., Ojo A.U., Van Staden J. 2008. *Allelopathic potential of Tetrapleura tetraptera leaf extracts on early seedling growth of five agricultural crops*. South African J. Bot., 74: 149–152.

Banuelas D.C., Questad E.J., Bobich E.G. 2019. *Interactions between the invasive Schinus molle (Peruvian pepper tree) with six plant species commonly found in Southern California nature reserves.* Urban For. and Urban Greening, 43(6): 126348.

Blanco J.A. 2007. *The representation of allelopathy in ecosystem-level forest models.* Ecological Modelling, 209(2–4): 65–77.

Bokhary U.G. 1978. *Allelopathy among prairie grass and its possible ecological significant*. Annals Bot., 42: 127–136.

Catalan P., Vázquez De Aldana B.R., De Las Heras P., Fernández-Seral A., Pérez-Corona M.E. 2013. *Comparing the allelopathic potential of exotic and native plant species on understory plants: are exotic plants better armed?* Anales de Biologia, 35: 65–74.

Chaturvedi O.P., Jha A.N. 1992. *Studies on allelopathic potential of an important agroforestry species*. For. Ecol. Manag., 53: 91–98.

Fox J., Bouchet-Valat M. 2020. *Rcmdr: R Commander*. R package version 2.7–1, https://socialsciences.mcmaster.ca/jfox/Misc/Rcmdr/.

Garima, Devi M. 2017. *Allelopathy in agroforestry. A review*. J. Pharmacogn. Photochem., 6(3): 686–688.

Garima, Pant K.S., Devi M. 2017. *Effect of allelochemicals present in Populus deltoides leaf extract on wheat (Triticum aestivum) in poplar-based agroforestry system*. Intl. J. Chem. Stud., 5(3): 785–787.

Inayat N., Zahir M., Rehmanullah A.M. 2020. *Phytochemical screening and allelopathic evaluation of aqueous and methanolic leaf extracts of Populus nigra* L. Pure App. Biol., 9(1): 956–962.

John J., Patil R.H., Joy M., Nair A.M. 2006. *Methodology of allelopathic research: 1. Agroforestry systems*. Allelopathy, 18(2): 173–214.

Khaket T.P., Kumar V., Singh J., Dhanda S. 2014. *Biochemical and physiological studies on the effects of senescence leaves of Populus deltoides on Triticum vulgare*. Sci. World J., 126051: 1–7.

Khalid S., Amreen, Bibi S, Taskeen S.A., Shumail H., Ul Haq S.I. 2021. *Allelopathic effect of Morus alba, Populus nigra, and Euphorbia helioscopia on Brassica campestris*. Pure Appl. Biol., 10(2): 532–538.

Koul V.K., Raina A., Khanna Y.P., Tickoo M.L., Singh H. 1991. *Evaluation of the allelopathic influence of certain farm-grown tree species on rice (Oryza sativa* L. *C.V. PC 19)*. Indian J. For., 14(1): 54–57.

Li C., Yang X., Tian Y., Yu M., Shi S., Qiao B., Zhao C., Mao L. 2021. *The effects of fig tree (Ficus carica* L.*) leaf aqueous extract on seed germination and seedling growth of three medicinal plants*. Agronomy, 11: 2564.

Liu D.L., An M., Johnson I.R., Lovett J.V., 2003. *Mathematical modeling of allelopathy. III. A Model for curve-fitting allelochemical dose responses*. Nonlinearity Biol. Toxicol. Med., 1(1): 37–50.

Lu Q., Zhang J., Chen L. 2017. *Impact of monoculture of poplar on the rhizosphere microbial communities over time*. Pedosphere, 30(4): 487–495.

Majeed A., Muhammad Z., Ahmad H. 2017. *Allelopathic effects of leaf extracts of three agroforestry trees on germination and early seedling growth of wheat (Triticum aestivum* L.*)*. Azarian J. Agri., 4(3): 69–73

Nandal D.P.S., Dhillon A. 2007. *Allelopathic effects of poplar (Populus deltoides Bartr. ex Marsh.): an assessment on the response of wheat varieties under laboratory and field conditions*. Indian J Agrofor., 9(2): 125–127.

Rahaman F., Juraimi A.S., Rafii M.Y., Uddin M.K., Hassan L. 2021. *Allelopathic Effect of Selected Rice (Oryza sativa) Varieties against Barnyard Grass (Echinochloa cruss-Gulli)*. Plants, 10: 1–20.

Sharma N.K., Samra J.S., Singh H.P. 2000. *Effect of aqueous extracts of Populus deltoides M" on germination and seedling growth. 1. Wheat*. Allelopathy J., 7(1): 56–68.

Sher Z., Hussain F., Ahmad B., Wahab M. 2011. *Allelopathic potential of Populus euphratica Olivier*. Pak. J. Bot., 43(4): 1899–1903.

Singh H.P., Kohli R.K., Batish D.R. 2001. *Allelopathic interference of Populus deltoides with some winter season crops*. Agronomie, 21: 139–146

Siyar S., Sami S., Hussain F., Hussain Z. 2018. *Allelopathic effects of Sheesham extracts on germination and seedling growth of common wheat*. Cercetari Agronomice in Moldova, 4(176): 17–26.

Teixeira Da Silva J.A., Karimi J., Mohsenzadeh S., Dobránszki J. 2015. *Allelopathic potential of select gymnospermous trees*. J. For. Environ. Sci., 31(2): 109–118.

THAPALIYAL S., SINGH BALI R., SINGH B., TODARIA N.P. 2008. *Allelopathic effects of tree of economic importance on germination and growth of food crops*. J. Herbs Spices Med. Plants, 13(4): 11–23.

WANG X., ZHANG R., WANG J., DI L., WANG H., SIKDAR A. 2021. *The Effects of leaf extracts of four tree species on amygdalus pedunculata seedlings growth*. Front. Plant Sci., 11: 587579.

WANG Y., ZHAO M., LIU T. 2020. *Extraction of allelochemicals from poplar alkaline peroxide mechanical pulping effluents and their allelopathic effects on Microcystis aeruginosa*. J. Bio Resour. Bio Prod., 5: 276–282.

VASISHTH A., KOKLIYAL N., JOSHI R., DHANAI C.S. 2020. *Allelopathic effects of tree species on germination and seedling growth of traditional crops of Garhwal Himalayas*. Indian J. Agrofor., 22 (2): 56–61.

ZHANG Z., LIU Y., YUAN L., WEBER E., VAN KLEUNEN M. 2021. *Effect of allelopathy on plant performance*. Ecol. Letters, 24: 348–362.

ZUBAY P., KUNZELMANN J., ITTZES A. ZAMBORINE E.N. SZABO K. 2021. *Allelopathic effects of leachates of Juglans regia* L.*, Populus tremula* L.*, and juglone on germination of temperate zone cultivated medicinal and aromatic plants*. Agroforest Syst, 95: 431–442.

Annex

Table Anx 1

The results of multiple comparisons of Tukey test

Dependent variable	Concentration (I)	Concentration (J)	Mean difference (I–J)	Std. error	Sig.
GR	1	2	1.99369	1.54619	.698
		3	4.25613	1.54619	.056
		4	5.18431*	1.54619	.011*
		5	7.04150*	1.54619	.000*
	2	1	-1.99369	1.54619	.698
		3	2.26244	1.54619	.589
		4	3.19063	1.54619	.247
		5	5.04781*	1.54619	.014*
	3	1	-4.25613	1.54619	.056
		2	-2.26244	1.54619	.589
		4	.92819	1.54619	.975
		5	2.78538	1.54619	.380
	4	1	-5.18431*	1.54619	.011*
		2	-3.19063	1.54619	.247
		3	-.92819	1.54619	.975
		5	1.85719	1.54619	.751
	5	1	-7.04150*	1.54619	.000*
		2	-5.04781*	1.54619	.014*
		3	-2.78538	1.54619	.380
		4	-1.85719	1.54619	.751
FW	1	2	.03687*	.00984	.003*
		3	.04375*	.00984	.000*
		4	.06625*	.00984	.000*
		5	.04750*	.00984	.000*
	2	1	-.03687*	.00984	.003*
		3	.00688	.00984	.956
		4	.02938*	.00984	.031*
		5	.01063	.00984	.817
	3	1	-.04375*	.00984	.000*
		2	-.00688	.00984	.956
		4	.02250	.00984	.161
		5	.00375	.00984	.995

Cont. Table Anx 1

FW	4	1	-.06625*	.00984	.000*
		2	-.02938*	.00984	.031*
		3	-.02250	.00984	.161
		5	-.01875	.00984	.324
	5	1	-.04750*	.00984	.000*
		2	-.01063	.00984	.817
		3	-.00375	.00984	.995
		4	.01875	.00984	.324
DW	1	2	.00375	.00650	.978
		3	.00062	.00650	1.000
		4	-.00188	.00650	.998
		5	.00187	.00650	.998
	2	1	-.00375	.00650	.978
		3	-.00312	.00650	.989
		4	-.00562	.00650	.908
		5	-.00187	.00650	.998
	3	1	-.00062	.00650	1.000
		2	.00312	.00650	.989
		4	-.00250	.00650	.995
		5	.00125	.00650	1.000
	4	1	.00188	.00650	.998
		2	.00562	.00650	.908
		3	.00250	.00650	.995
		5	.00375	.00650	.978
	5	1	-.00187	.00650	.998
		2	.00187	.00650	.998
		3	-.00125	.00650	1.000
		4	-.00375	.00650	.978
PL	1	2	.55312	.29351	.335
		3	.75937	.29351	.083
		4	1.17812*	.29351	.001*
		5	.93438*	.29351	.018*
	2	1	-.55312	.29351	.335
		3	.20625	.29351	.955
		4	.62500	.29351	.219
		5	.38125	.29351	.693

Cont. Table Anx 1

PL	3	1	-.75937	.29351	.083
		2	-.20625	.29351	.955
		4	.41875	.29351	.613
		5	.17500	.29351	.975
	4	1	-1.17812*	.29351	.001*
		2	-.62500	.29351	.219
		3	-.41875	.29351	.613
		5	-.24375	.29351	.920
	5	1	-.93438*	.29351	.018*
		2	-.38125	.29351	.693
		3	-.17500	.29351	.975
		4	.24375	.29351	.920
RL	1	2	1.16875	.44637	.077
		3	1.66562*	.44637	.003*
		4	1.97187*	.44637	.000*
		5	3.21250*	.44637	.000*
	2	1	-1.16875	.44637	.077
		3	.49687	.44637	.799
		4	.80313	.44637	.382
		5	2.04375*	.44637	.000*
	3	1	-1.66562*	.44637	.003*
		2	-.49687	.44637	.799
		4	.30625	.44637	.959
		5	1.54688*	.44637	.008*
	4	1	-1.97187*	.44637	.000*
		2	-.80313	.44637	.382
		3	-.30625	.44637	.959
		5	1.24062	.44637	.052
	5	1	-3.21250*	.44637	.000*
		2	-2.04375*	.44637	.000*
		3	-1.54688*	.44637	.008*
		4	-1.24062	.44637	.052
TL	1	2	1.72187*	.52074	.012*
		3	2.42500*	.52074	.000*
		4	3.15000*	.52074	.000*
		5	4.14687*	.52074	.000*

Cont. Table Anx 1

TL	2	1	-1.72187*	.52074	.012*
		3	.70312	.52074	.661
		4	1.42812	.52074	.057
		5	2.42500*	.52074	.000*
	3	1	-2.42500*	.52074	.000*
		2	-.70312	.52074	.661
		4	.72500	.52074	.634
		5	1.72187*	.52074	.012*
	4	1	-3.15000*	.52074	.000¬*
		2	-1.42812	.52074	.057
		3	-.72500	.52074	.634
		5	.99688	.52074	.319
	5	1	-4.14687*	.52074	.000*
		2	-2.42500*	.52074	.000*
		3	-1.72187*	.52074	.012*
		4	-.99688	.52074	.319
GP	1	2	10.12500*	2.46965	.001*
		3	17.37500*	2.46965	.000*
		4	22.50000*	2.46965	.000*
		5	30.37500*	2.46965	.000*
	2	1	-10.12500*	2.46965	.001*
		3	7.25000*	2.46965	.035*
		4	12.37500*	2.46965	.000*
		5	20.25000*	2.46965	.000*
	3	1	-17.37500*	2.46965	.000*
		2	-7.25000*	2.46965	.035*
		4	5.12500	2.46965	.242
		5	13.00000*	2.46965	.000*
	4	1	-22.50000*	2.46965	.000*
		2	-12.37500*	2.46965	.000*
		3	-5.12500	2.46965	.242
		5	7.87500*	2.46965	.017*
	5	1	-30.37500*	2.46965	.000*
		2	-20.25000*	2.46965	.000*
		3	-13.00000*	2.46965	.000*
		4	-7.87500*	2.46965	.017*

Cont. Table Anx 1

GI	1	2	-10.50816*	2.41756	.000*
		3	-15.76414*	2.41756	.000*
		4	-21.11811*	2.41756	.000*
		5	-28.05624*	2.41756	.000*
	2	1	10.50816*	2.41756	.000*
		3	-5.25598	2.41756	.201
		4	-10.60994*	2.41756	.000*
		5	-17.54808*	2.41756	.000*
	3	1	15.76414*	2.41756	.000*
		2	5.25598	2.41756	.201
		4	-5.35396	2.41756	.186
		5	-12.29210*	2.41756	.000*
	4	1	21.11811*	2.41756	.000*
		2	10.60994*	2.41756	.000*
		3	5.35396	2.41756	.186
		5	-6.93813*	2.41756	.041*
	5	1	28.05624*	2.41756	.000*
		2	17.54808*	2.41756	.000*
		3	12.29210*	2.41756	.000*
		4	6.93813*	2.41756	.041*
PVI	1	2	2358.62500*	4.70730E2	.000*
		3	3371.18750*	4.70730E2	.000*
		4	4210.43750*	4.70730E2	.000*
		5	5324.25000*	4.70730E2	.000*
	2	1	-2358.62500*	4.70730E2	.000*
		3	1012.56250	4.70730E2	.210
		4	1851.81250*	4.70730E2	.002*
		5	2965.62500*	4.70730E2	.000*
	3	1	-3371.18750*	4.70730E2	.000*
		2	-1012.56250	4.70730E2	.210
		4	839.25000	4.70730E2	.391
		5	1953.06250*	4.70730E2	.001*
	4	1	-4210.43750*	4.70730E2	.000*
		2	-1851.81250*	4.70730E2	.002*
		3	-839.25000	4.70730E2	.391
		5	1113.81250	4.70730E2	.136

Cont. Table Anx 1

PVI	5	1	-5324.25000*	4.70730E2	.000*
		2	-2965.62500*	4.70730E2	.000*
		3	-1953.06250*	4.70730E2	.001*
		4	-1113.81250	4.70730E2	.136
LVI	1	2	2.35862*	.47073	.000*
		3	3.37119*	.47073	.000*
		4	4.21044*	.47073	.000*
		5	5.32425*	.47073	.000*
	2	1	-2.35862*	.47073	.000*
		3	1.01256	.47073	.210
		4	1.85181*	.47073	.002*
		5	2.96562*	.47073	.000*
	3	1	-3.37119*	.47073	.000*
		2	-1.01256	.47073	.210
		4	.83925	.47073	.391
		5	1.95306*	.47073	.001*
	4	1	-4.21044*	.47073	.000*
		2	-1.85181*	.47073	.002*
		3	-.83925	.47073	.391
		5	1.11381	.47073	.136
	6	1	-5.32425*	.47073	.000*
		2	-2.96562*	.47073	.000*
		3	-1.95306*	.47073	.001*
		4	-1.11381	.47073	.136
WVI	1	2	.06456*	.01070	.000*
		3	.09050*	.01070	.000*
		4	.12250*	.01070	.000*
		5	.13075*	.01070	.000*
	2	1	-.06456*	.01070	.000*
		3	.02594	.01070	.120
		4	.05794*	.01070	.000*
		5	.06619*	.01070	.000*
	3	1	-.09050*	.01070	.000*
		2	-.02594	.01070	.120
		4	.03200*	.01070	.030*
		5	.04025*	.01070	.003*

Cont. Table Anx 1

WVI	4	1	-.12250*	.01070	.000*
		2	-.05794*	.01070	.000*
		3	-.03200*	.01070	.030*
		5	.00825	.01070	.938
	5	1	-.13075*	.01070	.000*
		2	-.06619*	.01070	.000*
		3	-.04025*	.01070	.003*
		4	-.00825	.01070	.938
TMC	1	2	3.30825	2.26444	.591
		3	5.64381	2.26444	.103
		4	10.28544*	2.26444	.000*
		5	5.56706	2.26444	.111
	2	1	-3.30825	2.26444	.591
		3	2.33556	2.26444	.840
		4	6.97719*	2.26444	.023*
		5	2.25881	2.26444	.856
	3	1	-5.64381	2.26444	.103
		2	-2.33556	2.26444	.840
		4	4.64163	2.26444	.253
		5	-.07675	2.26444	1.000
	4	1	-10.28544*	2.26444	.000*
		2	-6.97719*	2.26444	.023*
		3	-4.64163	2.26444	.253
		5	-4.71837	2.26444	.238
	5	1	-5.56706	2.26444	.111
		2	-2.25881	2.26444	.856
		3	.07675	2.26444	1.000
		4	4.71837	2.26444	.238
SVI	1	2	82.56250*	26.23192	.019*
		3	120.11875*	26.23192	.000*
		4	165.93125*	26.23192	.000*
		5	168.28750*	26.23192	.000*
	2	1	-82.56250*	26.23192	.019*
		3	37.55625	26.23192	.609
		4	83.36875*	26.23192	.018*
		5	85.72500*	26.23192	.014*

Cont. Table Anx 1

SVI	3	1	-120.11875*	26.23192	.000*
		2	-37.55625	26.23192	.609
		4	45.81250	26.23192	.412
		5	48.16875	26.23192	.361
	4	1	-165.93125*	26.23192	.000*
		2	-83.36875*	26.23192	.018*
		3	-45.81250	26.23192	.412
		5	2.35625	26.23192	1.000
	5	1	-168.28750*	26.23192	.000*
		2	-85.72500*	26.23192	.014*
		3	-48.16875	26.23192	.361
		4	-2.35625	26.23192	1.000

* The mean difference is significant at the 0.05 level

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TOTAL PHENOLIC, FLAVONOID CONTENTS AND ANTIOXIDANT ACTIVITY OF *CEDRUS ATLANTICA* EXTRACTS

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Key words: *Cedrus atlantica*, sawdust, antioxidant activity, total phenolic, flavonoid.

Abstract

This study is conducted to determine the total polyphenol, flavonoid contents and the antioxidant activity by DPPH (2,2-diphenyl-1-picryl hydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging effect of hydroalcoholic extracts of *Cedrus atlantica* (Pinaceae). Two synthetic antioxidants were used as standards: ascorbic acid and trolox. The wood extracts by soxhlet, maceration and ultrasound of *C. atlantica* had a high antioxidant capacity. The results of the antioxidant activity by DPPH and ABTS radical scavenging have indicated better activities for the soxhlet extract (IC_{50} = 163.08 and 134.385 µg/ml for DPPH and ABTS, respectively) than for the maceration (IC_{50} = 348.88 and 473.166 µg/ml, for DPPH and ABTS, respectively) and the ultrasound extracts (IC_{50} = 501.47 and 923.902 µg/ml, for DPPH and ABTS, respectively) compared to the standards: ascorbic acid and trolox (IC_{50} = 10,278 and 30.154 µg/ml for DPPH and ABTS, respectively). The total polyphenol and flavonoid content of the extracts by soxhlet, maceration, and ultrasound methods revealed that the ultrasound extract (187.835 mg GAE/g and 37.2 mg RE/g, respectively) had higher polyphenol and flavonoid contenst than the soxhlet (157.731 mg GAE/g and 6.4 mg RE/g, respectively) and maceration (123.298 mg GAE/g and 29.2 mg RE/g, respectively) extracts.

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Introduction

Natural products, such as plant extracts, provide endless opportunities for new drug discovery due to the unequaled abundance of chemical variation (Cos et al. 2006). Traditional medicinal plants include a diverse spectrum of chemicals that can be utilized to treat both chronic and infectious disorders (Duraipandiyan et al. 2006). These plants have been the foundation of all medicine discoveries around the world for decades, with secondary metabolites identified from these plants having substantial biological activities required for health (Brooks and Brooks 2014). In the context of our work, we were interested in the species C. atlantica Manetti, an important forest tree species found in northern Africa and one of the most commercially and ecologically important species in Morocco's Mediterranean mountains belonging to the Pinaceae family *(*Moukrim et al. 2020*).* C. atlantica commonly known as Atlas cedar which has been studied for a variety of of bio-functions, including anti-inflammatory (Shinde et al. 1999*),* insecticidal (Ainane et al. 2019) activities, as well as analgesic effect (Emer et al. 2018), anticancer (Huang et al. 2020, Chang et al. 2021), antimicrobial (Zrira and Ghanmi 2016) and antioxidant (Belkacem et al. 2021) effects. Its natural range is very disjointed, divided into seven biogeographical blocks in the mountains of North Africa (Boudy 1950, Panestos et al. 1992, M'hirit 1999). Morocco has the largest area, estimated at about 140,000 ha, and represents the main source of timber in the country (Hceflcd 2015).). Atlas cedar has two kinds of twigs (long and short) and foliage in the form of isolated needles on young twigs and on the year's shoots, rigid with an acute apex and measuring 1 to 2 cm (Arbez et al.1978). The cone, 5 to 8 cm long, is cylindrical at the top and flattened or depressed at the base, green before maturity then brown and matures in two years (Boudy 1952, Toth 1971, Riou-Nivert 2007). From an ecological point of view, these species are essentially mountainous and well adapted to cold climate (Boudy 1950). They are mesophilic species occupying the bioclimatic stages ranging from the upper semi-arid to subhimude with cool to very cold variants (M'hirit 1982). their development requires average annual temperature of 8 to 12°C (Gaussen 1955) and a rainfall of 440 to 1403 mm (Toth 1978, Mediouni and Yahi 1994). Thus, in Morocco, three types of cedar forests can be distinguished (Pujos 1966): low (below 1800 m), medium (between 1800 and 2100 m) and high (above 2100 m). The highest ones can reach 2500 m in the Eastern High Atlas (M'hirit 1982, Abourouh 1994). The Moroccan cedar forest, produces nearly 100,000 m^3/year of logs (*Bilan des reboisements*… 1998). The waste from the sawing and machining workshops of this wood is estimated at about 8% in

the form of sawdust and nearly 30% in the form of slabs and sawing waste (EL AMMARI 1996), i.e. an annual production of about 18,000 tons. Therefore, this raw material is an important source that must be valorized.

Oxidative stress is a growing public health concern. If it is not properly managed, it can cause premature aging and significantly increases the risk of developing diseases such as diabetes, inflammation and cancer (POPRAC 2017, FULOP et al. 2020). It also affects the shelf life of foods (fresh and processed) (SALAMA et al. 2020). As a result, the search for a new source of antioxidants is essential. In this context, natural antioxidants, such as isolated extracts of medicinal plants, are a promising source (ABDIN et al. 2020, OWON et al. 2021).

This study aims to convert wood waste into renewable resources by investigating the influence of different extraction methods on total phenolic content, flavonoids and antioxidant activity in order to find new ways to valorize *C. atlantica* wood waste.

Materials and Methods

Sample preparation and extraction

The sawdust of Moroccan cedrus used was collected from a sawmill in Azrou (Middle Atlas region). Grinding of the samples was carried out until obtaining a fine and homogeneous powder.

Hydroalcoholic extraction which was carried out by two polar solvents: SHULTZ and FLORY (1953) have shown that binary solvent mixtures can affect solubility, resulting in improved solubility when compared to individual pure solvents. Moreover, the low toxicity of water-ethanol mixtures makes them particularly appealing for a variety of medical applications (HOOGENBOOM et al. 2008). The experiment was carried out 3 trials to express the yield values concerning the dry matter. In each trial, 100 g of the raw material was mixed with 700 ml of the solvents (ethanol – 80%, water – 20%). The extraction methods used were soxhlet, maceration and ultrasound.

The mixture collected was subjected to vacuum pressure using a rotary evaporator, to obtain the extracts.

The yield was calculated by using the equation:

$$\text{Yield [\%]} = (M_E / M_{S)} \cdot 100$$

where:
M_E – the mass of the extract [g]
M_S – the mass of sawdust [g].

Antioxidant properties

Total phenolic

The Folin-Ciocalteu method, as described by LISTER and WILSON (2001), was used to determine the total phenolic content. In brief, 0.5 mL of extract solution was mixed with 2.5 mL of Folin-Ciocalteu reagent diluted with 1:10 distilled water, then 4 mL Na_2CO_3 (7.5%, w/v) was added. The mixture was then incubated in a water bath at 45°C for 30 min and the absorbance was measured at 765 nm using a UV-Vis spectrophotometer against a blank sample. The gallic acid standard curve was obtained under the same conditions as described above, with a concentration range of (5–250 ug/l). The total phenolic content was measured in gallic acid equivalents (mg GAE/g extract).

Flavonoids

The flavonoid content was determined using a method developed by DEWANTO et al. (2002), 1 mL of sample solution (30 mg/mL) was added to 6.4 mL of distilled water in a test tube. Sodium nitrite solution (5%, 0.3 mL) was added to the mixture and held for 5 min. after that, 0.3 mL of 10% aluminum chloride was added. After 6 min, 2 mL of 1 M sodium hydroxide was finally added. The absorbance of the mixture at 510 nm was measured immediately against a standard curve prepared by rutin. Flavonoid contents were expressed as mg rutin equivalent (mg RE/g extract).

DPPH radical scavenging activity

The DPPH radical scavenging activity of cedar wood extracts was measured to assess their anti-free radical activity. The purple DPPH is reduced to the yellow 2,2-diphenyl-1-picryl hydrazine in the presence of free radical scavengers (ATHAMENA et al. 2010). In methanol, a DPPH solution (0.2 mM) was prepared, and 0.5 ml of this solution was added to 1.5 ml of extract solution at various concentrations (15% to 100%). After incubation for 30 min and at room temperature, the absorbance was measured at 517 nm. Ascorbic acid was used as standard and at different concentrations.

ABTS radical scavenging assay

To make an ABTS radical solution, 2 mM ABTS was mixed with a 70 mM potassium persulfate solution. The mixture is allowed to stir for 24 hours in the dark at room temperature before use. After that, the solution is diluted with methanol to give an absorbance of 0.700±0.02

at 734 nm. Then, 2 mL of this solution, 200 µL of extract or positive control is added, after 30 min. The absorbance obtained at 734 nm is noted (MÜLLER et al. 2011).

Results and Discussion

Yield of extraction

The yields of the extracts sawdust of C. atlantica are ranged from 3.43% for the maceration to 9.13% for the ultrasound. This results of the yields confirm that, the extraction method as well as the duration significantly influence the yield of the extracts (WILKINSON et al. 2003, ABERCHANE et al. 2004).

The studies conducted by DERWICH et al. (2010) showed that, the yields obtained by the needles (1.8%), seeds (2.6%) (RHAFOURI et al. 2014) and wood (3.41%) (FIDAH et al. 2016) of C. atlantica were lower than ours (Table 1).

Table 1

The extraction yield of the extraction methods

Extracts	Extraction times	Yield [%]
Soxhlet	10 h	9.13±0.28
Maceration	24 h	3.43±0.07
Ultrasound	10 min	7.26±0.64

Dosage of polyphenols and flavonoids

The results of the total polyphenols and flavonoids of hydroalcoholic extracts of *C. atlantica* sawdust showed the presence of polyphenols and flavonoids in variable amounts. The total phenolic compound of soxhlet, maceration and ultrasound extracts are ranged from 123.298 to 187.835 mg GAE/g. Flavonoids amount varied from 6.4 to 37.2 mg RE/g. The Ultrasound extract has the highest phenolics and flavonoids content (187.835 mg GAE/g and 29.2 mg RE/g, respectively).

Table 2

Total polyphenols and flavonoids of the extracts of *C. atlantica*

Specification	Total polyphenols [mg GAE /g of extract]	Flavonoids [mg RE/g of extract]
Soxhlet	157.731±4.537	6.4±0.466
Maceration	123.298±2.148	29.2±1.178
Ultrasound	187.835±4.464	37.2±1.528

The studies led by HOFMANN et al. (2020) allowed us to confirm that our cedarwood extracts contain more polyphenols than the extracts of Atlas cedar cones, which were extracted by the same solvents (ethanol 80%), and did not exceed 30.94 mg EQ/g. In addition, JAIN et al. (2015) indicated that the extracts of *C. deodora* contained a low amount of polyphenol, which ranges from 0.017 to 0.023 mg EQ/g, for the alcoholic (ethanol 70%) extract and aqueous extract, respectively. The results obtained by FADEL et al. (2016) from Algerian *Cedrus*, showed that the total of flavonoid content was 16.8 mg EQ/g. This showed that the Moroccan *Cedrus* oils extracted by maceration and ultrasound contains more Flavonoids than Algerian *Cedrus*.

Antioxidant activity

The antioxidants activity is related to the constituents, which can protect the organism system against the potential harmful effect of oxidative stress (FERNÁNDEZ-AGULLÓ et al. 2013).

DPPH scavenging activity

The antioxidant capacity of cedarwood hydroalcoholic extracts was tested using DPPH radical scavenging, which is a method for measuring the free radical scavenging property of various samples (LI et al. 2020). The essay is considered when the purple-colored free radical DPPH is reduced to a stable yellow-colored diamagnetic molecule as a result of a reaction with the hydrogen-donating scavenger (ARIKA et al. 2019). The extracts were evaluated in terms of their IC_{50} value, with each extract being compared to the IC_{50} = 10.278 µg/ml of ascorbic acid, which was used as a reference substance. Figure 1 summarized the DPPH radical scavenging activities of various extracts. The scavenging capacity of cedarwood extracts for DPPH free radical showed remarkable scavenging activity. The IC_{50} values of the extracts were 163.08, 348.88 and 501.47 µg/ml for soxhlet, maceration and ultrasound extracts, respectively. It was observed that cedarwood extract by soxhlet possesses the most potent DPPH radical-scavenging activity followed by the extract by maceration and ultrasound.

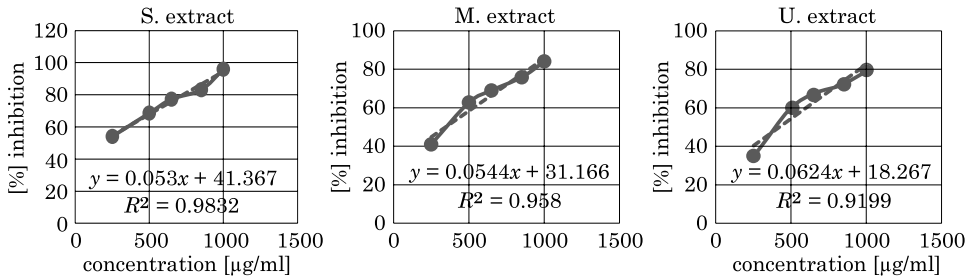

Fig. 1. DPPH radical-scavenging activity of hydroalcoholic *C. atlantica* extracts (S: Soxhlet, M: Maceration and U: Ultrasound)

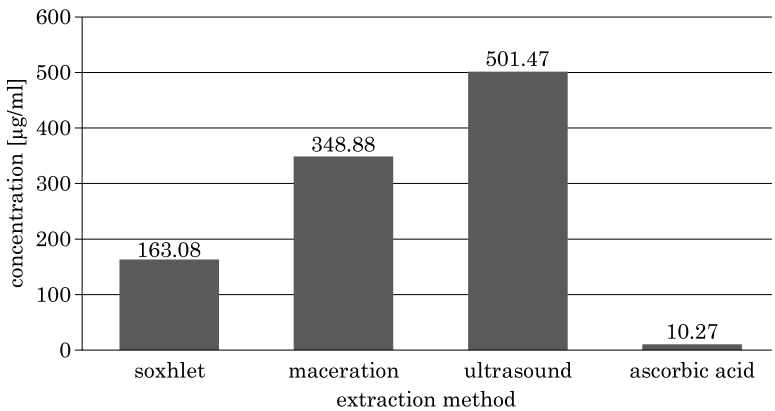

Fig. 2. IC_{50} values [µg/ml] of hydroalcoholic *C. atlantica* extracts and standard

The IC_{50} value for *C. atlantica* seeds extract was found to be 400 µg/ml in studies led by NAIMI et al. (2015). As a result of our findings, the *C. atlantica* extract obtained by soxhlet and maceration was consistent with the literature. A recent study of *C. atlantica* wood essential oil extracted by hydrodistillation by JAOUADI et al. (2021) revealed that the IC_{50} values were significantly higher (IC_{50} = 16264 µg/mL and 15559 µg/mL) than the IC_{50} values observed in our study.

ABTS assay

Hydroalcoholic extracts of *C. atlantica* wood were evaluated for their ABTS radical cation scavenging activity. Trolox was used as standard. The extracts extracted by soxhlet, maceration and ultrasound showed good ABTS radical cation scavenging activity with IC_{50} values of 134.385, 473.166 and 923.902 µg/ml, respectively, compared to Trolox (IC_{50} = 30.154 µg/ml). It was also found that the soxhlet extract had the most potent ABTS radical scavenging activity.

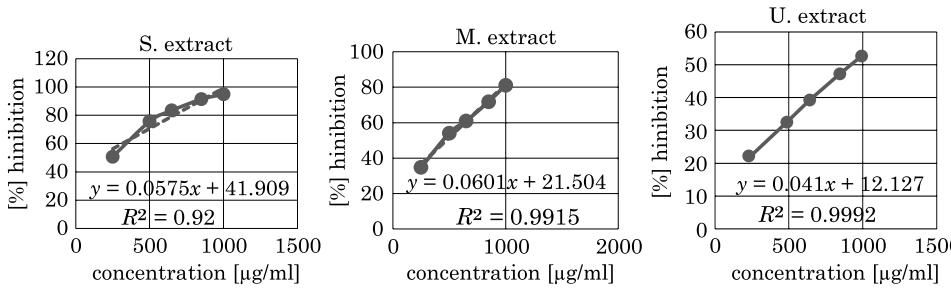

Fig. 3. Free radical scavenging activity of the extracts of *C. atlantica* by ABTS method (S: Soxhlet, M: Maceration and U: Ultrasound)

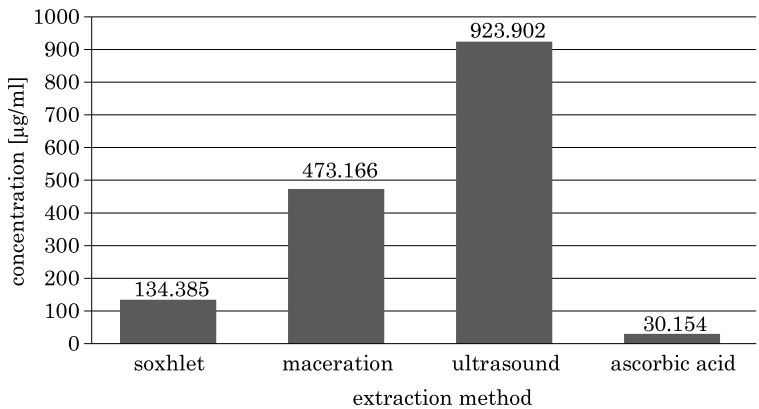

Fig. 4. Comparison between IC_{50} values [µg/ml] of hydroalcoholic *C. atlantica* extracts and standard

BELKACEM et al. (2021) found that *C. atlantica* acetone, methanol and ethanol extracts had low IC_{50} values reached about 147.46 µg/mL, 158.4 µg/mL, and 160.9 µg/mL, respectively According to JAIN et al. (2015) the IC_{50} values of *C. deodora* were 122.42 and 115.29 µg/mL for aqueous and ethanol (70%) extracts, respectively. Accordingly, the literature is in agreement with the ABTS result for *C. atlantica* extract obtained by soxhlet.

The results found in this study showed that the extraction method affects the antioxidant activity (DJOUAHRI et al. 2013), which could be mainly attributed to phenolic compounds (REZGUI et al. 2020): The high content of phenolic compounds in the extracts of soxhlet and maceration gives it a powerful antioxidant activity. However, it was observed that, even though, the ultrasound extract is the richest in polyphenols and flavonoids, it is the least reducing. This is explained by several studies that have found that antioxidant activity is not only related to the amount of phenolic compounds present, but is also strongly related to their chemical

structures (BELHAOUES et al. 2020) and, therefore the effect of other components which may act as antioxidants and provide effective protection against free radicals.

Conclusion

In the present work, we converted these wastes into renewable energy sources. The comparative study of antioxidant activity of cedar sawdust extracts was presented using DPPH and ABTS radical scavenging methods, as well as an evaluation of the total polyphenol and flavonoid content of *C. atlantica* wood extracts. Our results demonstrated a strong radical scavenging effect of *C. atlantica* soxhlet extract (IC_{50} = 163.018 and 134.385 µg/ml for DPPH and ABTS, respectively) compared to ascorbic acid and Trolox standards (IC50 = 10,278 and 30, 154 µg/ml respectively). According to the results obtained in this study, it is concluded that the soxhlet method allows us to obtain the most powerful extract of *C. atlantica* wood against DPPH and ABTS radicals and it can be considered as a good source of antioxidants.

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References

ABDIN M., SALAMA M.A., RIAZ A., AKHTAR H.M.S., ELSANAT S.Y. 2020. *Enhanced the entrapment and controlled release of Syzygiumcumini seeds polyphenols by modifying the surface and internal organization of Alginate based microcapsules*. J. Food Process. Preserv., 45(1): e15100.

ABERCHANE M., FECHTAL M. CHAOUCH A. 2004. *Analysis of Moroccan atlas cedarwood oil (Cedrus atlantica Manetti)*. Journal of Essential Oil Research, 16(6), 542–547.

ABOUROUH M. (1994). *Les ectomycorhizes du Cèdre de l'Atlas: état des connaissances et perspectives*. Le Cèdre de l'Atlas, Cedrus atlantica, pp. 337–348.

AINANE A., KHAMMOUR F., CHARAF S., ELABBOUBI M., ELKOUALI M., TALBI M., BENHIMA R., CHERROUD S., AINANE T. 2019. *Chemical composition and insecticidal activity of five essential oils: Cedrus atlantica, Citrus limonum, Rosmarinus officinalis, Syzygium aromaticum and Eucalyptus globules*. Materials Today: Proceedings, 13: 474–485.

ARBEZ M., FERRANDES P., UYAR N. (1978). *Contribution à l'étude de la variabilité géographique des cèdres*. Annales des Sciences Forestières, (ol. 35(4): 265–284). EDP Sciences.

ARIKA W., KIBITI C.M., NJAGI J.M., NGUGI M.P. 2019. *In vitro antioxidant properties of dichloromethanolic leaf extract of Gnidia glauca (Fresen) as a promising antiobesity drug*. Journal of Evidence-based Integrative Medicine, 24, 2515690X19883258.

ATHAMENA S., CHALGHEM I., KASSAH-LAOUAR A., LAROUI S., KHEBRI S. 2010. *Activité antioxydante et antimicrobienne d'extraits de Cuminum cyminum* L. Lebanese Science Journal, 11(1), 69–81.

BELHAOUES S., AMRI S., BENSOUILAH M. 2020. *Major phenolic compounds, antioxidant and antibacterial activities of Anthemis praecox Link aerial parts*. South African Journal of Botany, 131:200–205.

Belkacem N., Khettal B., Hudaib M., Bustanji Y., Abu-Irmaileh B., Amrine C. S.M. (2021). *Antioxidant, antibacterial, and cytotoxic activities of Cedrus atlantica organic extracts and essential oil*. European Journal of Integrative Medicine, 42: 101292.

Boudy P. (1950). *Economie forestière Nord-africaine*-Tome 2: *monographies et traitements des essences forestières,* E. larose, Paris.

Bilan des reboisements de Ia campagne 96–97. 1998. Ministere Charge des Eaux et Forets et de Ia Conservation du Maroc, Rabat.

Boudy P. 1952. *Guide du forestier en Afrique du Nord*. La Maison rustique. Paris.

Brooks B.D., Brooks A.E. 2014. *Therapeutic strategies to combat antibiotic resistance. Advanced drug delivery reviews*, 78: 14–27.

Chang K.F., Chang J.T., Huang X.F., Huang Y.C., Li C.Y., Weng J., Hsiao C., Hsu H.J., Tsai N.M. 2021. *Cedrus Atlantica extract suppress glioblastoma growth through promotion of genotoxicity and apoptosis: in vitro and in vivo studies*. International Journal of Medical Sciences, 18(11): 2417.

Cos P., Vlietinck A.J., Berghe D.V., Maes L. 2006. *Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'*. Journal of Ethnopharmacology, 106(3): 290–302.

Derwich E., Benziane Z., Boukir A. 2010. *Chemical composition and in vitro antibacterial activity of the essential oil of Cedrus atlantica*. Int. J. Agric. Biol., 12(3), 381–385.

Dewanto V., Wu X., Adom K.K., Liu R.H. 2002. *Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity*. Journal of Agricultural and Food Chemistry, 50(10): 3010–3014.

Djouahri A., Boudarene L., Meklati B.Y. 2013. *Effect of extraction method on chemical composition, antioxidant and anti-inflammatory activities of essential oil from the leaves of Algerian Tetraclinis articulata (Vahl) Masters*. Industrial Crops and Products, 44: 32–36.

Duraipandiyan V., Ayyanar M., Ignacimuthu S. 2006. *Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India*. BMC Complementary and Alternative Medicine, 6(1) : 1–7.

El Ammari A. 1996. *Etude de rendement en sciage du cèdre du Moyen Atlas central. Mémoire de 3eme cycle*. ENFI, Rabat, Morocco.

Emer A.A., Donatello N.N., Batisti A.P., Belmonte L.A.O., Santos A.R., Martins D.F. 2018. *The role of the endocannabinoid system in the antihyperalgesic effect of Cedrus atlantica essential oil inhalation in a mouse model of postoperative pain*. Journal of Ethnopharmacology, 210: 477–484.

Fadel H., Benayache F., Benayache S. (2016). *Antioxidant properties of four Algerian medicinal and aromatic plants Juniperus oxycedrus* L.*, Juniperus phoenicea* L.*, Marrubium vulgare* L. *and Cedrus atlantica (Manetti ex Endl).* Der Pharmacia Lettre, 8(3): 72.

Fernández-Agulló A., Pereira E., Freire M.S., Valentao P., Andrade P.B., González-Álvarez J., Pereira J.A. (2013). *Influence of solvent on the antioxidant and antimicrobial properties of walnut (Juglans regia* L.*) green husk extracts*. Industrial Crops and Products, 42: 126–132.

Fidah A. 2016. *Etude de la durabilité naturelle des bois de Cedrus atlantica (Manetti) et de Tetraclinis articulata (Vahl) et évaluation de la bioactivité de leurs huiles essentielles sur les champignons basidiomycètes lignivores.* Le Catalogue Collectif des Thèses en Afrique. Maroc. Université Mohammed V, Agdal, Faculté des Sciences, Rabat.

Fulop T., Pawelec G., Dupuis G., Kotb R., Friguet B., Witkowski J. M., Larbi A. (2020). *Immunosenescence, oxidative stress, and cancers.* In: *Cancer Immunology*, pp. 513–531. Springer, Cham.

HCEFLCD. 2015. *Synthèse de l'Inventaire Forestier National Marocain*. Direction de Développement Forestier, Rabat.

Hofmann T., Visi-Rajczi E., Albert L. 2020. *Antioxidant properties assessment of the cones of conifers through the combined evaluation of multiple antioxidant assays.* Industrial Crops and Products, 145: 111935.

HOOGENBOOM R., THIJS H.M., WOUTERS D., HOEPPENER S., SCHUBERT U.S. 2008. *Tuning solution polymer properties by binary water-ethanol solvent mixtures*. Soft Matter, 4(1): 103–107.

HUANG X.F., CHANG K.F., LEE S.C., SHEU G.T., LI C.Y., WENG J.C., HSIAO C.Y., TSAI N.M. 2020. *Extract derived from cedrus atlantica acts as an antitumor agent on hepatocellular carcinoma growth in vitro and in vivo*. Molecules, 25(20): 4608.

JAIN S., JAIN A., JAIN S., MALVIYA N., JAIN V., KUMAR D. (2015). *Estimation of total phenolic, tannins, and flavonoid contents and antioxidant activity of Cedrus deodara heart wood extracts*. Egyptian Pharmaceutical Journal, 14(1): 10.

JAOUADI I., CHERRAD S., BOUYAHYA A., KOURSAOUI L., SATRANI B., GHANMI M., CHAOUCH A. 2021. *Chemical variability and antioxidant activity of Cedrus atlantica Manetti essential oils isolated from wood tar and sawdust*. Arabian Journal of Chemistry, 14(12): 103441.

LI Y.X., ZHANG C., PAN S., CHEN L., LIU M., YANG K., ZENG X., TIAN J. 2020. *Analysis of chemical components and biological activities of essential oils from black and white pepper (Piper nigrum* L.*) in five provinces of southern China*. Lwt, 117: 108644.

LISTER E., WILSON P. (2001). *Measurement of total phenolics and ABTS assay for antioxidant activity (personal communication)*. Crop Research Institute, Lincoln, New Zealand, 7: 235–239.

MEDIOUNI K., YAHI N. 1994. *Phytodynamique et autoécologie du Cedrus atlantica dans le Djurdjura*. Ann. Rech. For. Maroc, 27: 278–105.

M'HIRIT O. 1982. *Etude ecologique et forestiere des cedraies du Rif marocain* (essai sur une approche multidimensionnelle de la phytoecologie et de la productivite du cedre) (*Cedrus atlantica* Manetti).

M'HIRIT O. 1999. *Le cèdre à travers le réseau Silva Méditerranea «Cèdre» Bilan et perspectives*. Forêt Méditerranéenne, XX(3): 99–100.

MOUKRIM S., LAHSSINI S., RIFAI N., MENZOU K., MHARZI-ALAOUI H., LABBACI A., RHAZI M., WAHBY I., EL MADIHI M., RHAZI L. 2020. *Modélisation de la distribution potentielle de Cedrus atlantica Manetti au Maroc et impacts du changement climatique*. Bois & Forets des Tropiques, 344: 3–16.

MÜLLER L., FRÖHLICH K. BÖHM V. 2011. *Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (aTEAC), DPPH assay and peroxyl radical scavenging assay*. Food Chemistry, 129: 139–148, https://doi.org/10.1016/j.foodchem.2011.04.045.

NAIMI F., BOUSTA D., BALOUIRI M., MESKAOUI A.E. 2015. *Antioxidant and free radical-scavenging properties of seeds flavonoids extract of Cedrus atlantica Manetti, Linum usitatissimum* L. *and Ocimum basilicum* L. *species*. Journal of Applied Pharmaceutical Science, 5(8): 95–99.

OWON M., OSMAN M., IBRAHIM A., SALAMA M.A., MATTHÄUS B. (2021). *Characterisation of different parts from Moringa oleifera regarding protein, lipid composition and extractable phenolic compounds*. OCL: Oilseeds Fats, Crops Lipids, 28: 12.

PANETSOS K.P., CHRISTOU A., SCALTSOYIANNES A. 1992. *First analysis on allozyme variation in cedar species (Cedrus sp.)*. Silvae Genetica, 41(6): 339–342.

POPRAC P., JOMOVA K., SIMUNKOVA M., KOLLAR V., RHODES C.J., VALKO M. (2017). *Targeting free radicals in oxidative stress-related human diseases*. Trends in Pharmacological Sciences, 38(7): 592–607.

PUJOS A. 1966). *Les milieux de la cédraie marocaine. Etude d'une classification des cédraiesdu Moyen Atlas et du Rif en fonction du sol et du climat de la régénération actuelle dans les peuplements*. Ann. Rech. For. Maroc. 8 (Rapport), pp. 1–283.

RHAFOURI R., STRANI B., ZAIR T., GHANMI M., AAFI A., EL OMARI M., BENTAYEB A. 2014. *Chemical composition, antibacterial and antifungal activities of the Cedrus atlantica (Endl.) Manettiex Carrière seeds essential oil*. Mediterranean Journal of Chemistry, 3(5) : 1034–1043.

RIOU-NIVERT P. 2007. *Fiche extraite de la Flore forestière française*, t. (III) région méditerranéenne. Forêt-Entreprise, 174: 14–16 Gaussen, 1955.

SALAMA M., EL HARKAOUI S., NOUNAH I., SAKR H., ABDIN M., OWON M., OSMAN M., IBRAHIM A., CHARROUF Z., MATTHÄUS B. (2020). *Oxidative stability of Opuntiaficus-indica seeds oil blending with Moringaoleifera seeds oil*. OCL: Oilseeds Fats, Crops Lipids., 27: 53.

SHINDE U.A., KULKARNI K.R., PHADKE A.S., NAIR A.M., MUNGANTIWAR A.A., DIKSHIT V.J., SARAF M.N. 1999. *Mast cell stabilizing and lipoxygenase inhibitory activity of Cedrus deodara (Roxb.)* Loud. wood oil. Indian J. Exp. Biol., 37(3): 258–261.

SHULTZ A.R., FLORY P.J. 1953. *Phase Equilibria in Polymer – Solvent Systems. III. Three-component Systems 1*. Journal of the American Chemical Society, 75(22): 5681–5685.

TOTH J. (1971). *Le cèdre (Cèdre de l'Atlas-Cedrus atlantica) en France*. Bulletin de la Vulgarisation Gorestière, 4: 1–9.

TOTH J. 1978. *Contribution à l'étude de la fructification et de la régénération du cèdre de l'Atlas (Cedrus atlantica M.) dans le sud de la France* (thèse. Doc. Ing. Fac. St.). Jérôme. Marseille, France.

WILKINSON J.M., HIPWELL M., RYAN T., CAVANAGH H.M. 2003. *Bioactivity of Backhousia citriodora. Antibacterial and antifungal activity*. Journal of Agricultural and Food Chemistry, 51(1): 76–81.

WOJTUNIK-KULESZA K.A., KASPRZAK K., ONISZCZUK T., ONISZCZUK A. 2019. *Natural monoterpenes. Much more than only a scent*. Chemistry & Biodiversity, 16(12): e1900434.

ZRIRA S., GHANMI M. 2016. *Chemical composition and antibacterial activity of the essential of Cedrus atlantica (Cedarwood oil)*. Journal of Essential Oil Bearing Plants, 19(5): 1267–1272.

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REQUIREMENTS FOR VEGETATIVE GROWTH OF *HOHENBUEHELIA MYXOTRICHA* AND ITS ANTIMYCOTIC ACTIVITY

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Key words: xylotrophic basidiomycetes, *Hohenbuehelia*, macromorphological characters, growth conditions, antimycotic activity.

Abstract

Biometric characteristics of the hyphae and micromorphological features of *Hohenbuehelia myxotricha* mycelia, such as clamps, hyphal loops, crystals were observed by light and electron microscopy. The basic requirements for *H. myxotricha* growth and acquisition of antimycotic activity have been investigated. The highest mycelial growth (18.2 mm/day) of *H. myxotricha* was observed on beer wort agar medium. Suitable conditions for maximal *H. myxotricha* mycelia production were found after 14 days of liquid static cultivation at 25°C, pH 4.5, 30 g/L of glucose, and 2 g/L of yeast extract. The inhibitory effect of *H. myxotricha* was evaluated against *Aspergillus niger*, *Issatchenkia orientalis*, and *Candida albicans* strains. The optimal period for *A. niger* growth inhibition was 14 days while 21 days of *H. myxotricha* cultivation was more appropriated for tested *Saccharomycetales* growth inhibitions. The most suitable for promotion of antimycotic metabolites against all tested pathogenic fungi were glucose and yeast extract.

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Introduction

The cosmopolitan in distribution genus *Hohenbuehelia* Schulzer belonging to Basidiomycota phylum is a relatively small genus with about 40 species known (KIRK et al. 2008). Basic attention has been paid to taxonomically and phylogenetically analysis of *Hohenbuehelia* species based on morphological and molecular data (THORN et al. 2000, KOZIAK et al. 2007, MENTRIDA 2016, CONSIGLIO et al. 2018, LUBIAN et al. 2018, BIJEESH et al. 2019). In contrast, only some cultural characteristics and growth of *H. petaloides* mycelium were investigated *in vitro* (YOO et al. 2001, ZHU et al. 2007). Moreover, *Hohenbuehelia* species have been documented to possess different therapeutical activities. Water and ethanol extracts of *Hohenbuehelia* sp. inhibited the growth of the bacteria *Acinetobacter baumannii*, *Bacillus cereus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and fungi *Geotrichum candidum*, *Saccharomyces cerevisiae* (BALA et al. 2012). Compound produced of *Nematoctonus robustus* as known anamorph of *Hohenbuehelia* sp. were active against different pathogenic microorganisms: *A. calcoaceticus*, *Bacillus brevis*, *B. subtilis*, *Candida albicans*, *Micrococcus luteus*, *Mucor miehei* (Current Name: *Rhizomucor miehei* (Cooney & R. Emers.) Schipper), *Nematospora coryli*, *Phodotula glutinis*, *Paecilomyces variotii*, *Penicillium notatum* (Current Name: *Penicillium chrysogenum* Thom), *Saccharomyces cerevisiae* (STADLER et al. 1994). Metabolites from crude extracts of submerged cultures of *H.* grisea exhibited antimicrobial activity against *B. subtilis*, *C. albicans*, *C. tenuis* (Current name: *Yamadazyma tenuis* (Diddens & Lodder) M.A.B. Haase), *M. luteus*, *Mucor plumbeus*, *M. hiemalis*, *Pichia anomala* (Current Name: *Wickerhamomyces anomalus* (E.C. Hansen) Kurtzman, Robnett & Bas.-Powers, *R. glutinis*, *Staphylococcus aureus* (SANDARGO et al. 2018a). Polysaccharides isolated from *Hohenbuehelia serotina* possessed antioxidant (LI et al. 2012, 2017), anti-proliferation (LI et al. 2012, 2017, ZHANG et al. 2014), and can be considered as a radioprotective agent in view of the ability of neutral polysaccharides to significantly improving the activity of glutathione peroxidase (GSH-Px) and increasing the contents of glutathione (GSH) as well as ceruloplasmin in plasma after treated with 6 Gy-radiation (LI et al. 2015). Isolated from *Hohenbuehelia grisea* culture liquids bioactive compounds like 4-hydroxypleurogrisein and pleurotin derivatives (pleurotin, leucopleurotin, dihydropleurotinic acid) displayed antiviral and antimicrobial activities (SANDARGO et al. 2018b). Bioethanol production using *Hohenbuehelia* sp. (strain ZW-16) was also reported (LIANG et al. 2013). Of particular interest is now *Hohenbuehelia* species like *H. grisea* (REALE 2018), *H. portegna*, *H. paraguayensis*,

H. mastrucata (LUBIAN et al. 2018, 2021) due to their potential to be used as biological control of nematodes. One of the rare *Hohenbuehelia* species is *H. myxotricha* which morphology and microanatomy have been studied (ANGELI and SCANDURRA 2012) on the basis of material from Palermo (Sicily). According to taxonomic concept of CONSIGIO et al. (2018) *H. myxotricha* is considered as a synonym of *H. grisea*. Studies of this fungus *in vitro* are very limited. To date, only a few studies were focused on extracellular enzymatic activity (KRUPODOROVA et al. 2014), alternative substrates for its mycelia cultivation (KRUPODOROVA and BARSHTEYN 2015), antimicrobial activity of mycelia and culture liquid (KRUPODOROVA et al. 2016, KRUPODOROVA et al. 2022), and antioxidant potential (KRUPODOROVA et al. 2022). In view of the intensive taxonomic and therapeutic investigations that have been made of *Hohenbuehelia* spp., there is the lack of knowledge in terms of *Hohenbuehelia* spp. morphology, growth and therapeutical activity of mycelium. The aim of current study was to study *H. myxotricha* morphology, growth requirements and antimycotic activity.

Material and Methods

Fungal strains, culture maintenance

Hohenbuehelia myxotricha 1599 was kindly supplied by the Mushroom Culture Collection (IBK) of the M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (BISKO et al. 2021). Stock cultures were maintained on beer-wort agar-agar slants at 4ºC.

The *Issatchenkia orientalis* Kudryavtsev 301, *Candida albicans* (C.P. Robin) Berkhout 17/138 and clinical strains of *C. albicans* 311, 315, 319 were obtained from the Culture Collection of Microorganisms of the Institute of Epidemiology and Infectious Diseases of the National Academy of Medical Sciences of Ukraine. *Aspergillus niger* Tiegh. IFBG 134 was kindly obtained from the Collection of strains of microorganisms and plant lines of the Institute of Food Biotechnology and Genomics of the National Academy of Sciences of Ukraine. Stock cultures were maintained on potato-dextrose-agar (PDA) slants at 4ºC.

Micro-morphological description

Vegetative mycelium of *H. myxotricha* growing on PDA (the 7th day of growth) was recorded using Zeiss light microscope (MBI-15) and scanning electron microscopy (Jeon JSM – 6060 LA, Japan). A small piece is sepa-

rated from the colony and placing on glass slides in reagent (a drop of sterilized water or Congo red). Preparations for scanning electron microscopy (SEM) were prepared according to QUATTELBAUM and CARNER (1980).

Effect of media on mycelial growth

The effects of different media beer wort agar (WA: liquid beer wort, diluted with distilled water to a density of 8° on the Balling scale, 20.0 g agar), Czapek Dox Agar (CZA, Sigma Aldrich, USA), Malt Extract Agar (MEA, Difco, USA), Potato Dextrose Agar (PDA, Difco, USA), Glucose-Peptone-Yeast Agar (GPYA) composed of (g/L): 25.0 glucose, 3.0 yeast extract, 2.0 peptone, 1.0 K_2HPO_4, 1.0 KH_2PO_4, 0.25 $MgSO_4 \cdot 7H_2O$, and 10.0 agar on mycelial growth have been examined. Prepared media were autoclaved at 121°C for 20 min. *H. myxotricha* was transferred from stored cultures to PDA Petri dishes and cultured at 26±1°C to obtain mycelial colonies. Mycelial plugs 8-mm were cut from at the mycelial active growth stage using a sterile borer and used as inoculum. Cultivation on solid media was carried out at temperature 26±2 °C in the dark. The mycelia radial growth (RGR, mm/day) was measured daily till the colony covered the full plate and calculated according to the method proposed by WEIS et al. (1999) using the following formula:

$$\mathrm{RGR} = \frac{(R2 - R1)}{(t2 - t1)}$$

where:
RGR – radial growth rate
R – radius of the colony [mm]
t – time [days].

The growth rate (cm/day) was determined according to the formula:

$$\text{Growth rate} = \frac{\text{colony diameter on the last day [cm]}}{\text{number of day's menasurement was taken after inoculation}}$$

Mycelial characteristics on the agar surfaces were recorded. The texture of colonies was performed according to the scale described by STALPERS (1978). The mycelial density was evaluated as follows: very scanty (+), scanty (2+), moderate (3+), abundant (4+), very abundant (5+).

Effect of temperature, pH on mycelial growth

100 ml of liquid glucose-peptone-yeast (GPY) medium in each 250-mL Erlenmeyer flasks was inoculated with three mycelial plugs of 8 mm

diameter cut from the Petri dishes with PDA using a sterile borer in the mycelial active growth stage. To determine the influence of temperature, the inoculated flasks with *H. myxotricha* were incubated at different temperatures (15, 20, 25, 30°C) in incubators for 14 days. To assess the effect of pH level, the inoculated flasks were incubated for 14 days at 25±1°C at different pH levels (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0). The medium was adjusted to different pH levels used with the addition of 1M NaOH or HCl. After the incubation period, mycelium was separated from the medium by filtration through Whatman's filter paper No. 4, washed with distilled water, dried to constant weight at 105°C.

Effect of incubation time on mycelial growth and antimycotic activity

To study the influence of incubation time, the inoculated 250 mL Erlenmeyer flasks containing 100 mL of GPY nutrient medium and three discs of *H. myxotricha* were incubated at 25°C for different durations (7, 14, 21, 28, 35, and 42 days). After incubation period of cultivation, mycelium was removed, washed with distilled water, dried to constant weight as mentioned above.

To assess the effect of incubation period (14 and 21 days) on antimycotic activity the culture liquid was mixed with previously prepared and cooled to 40°C PDA nutrient medium, in a ratio of 1 : 1. The resulting mixture (a culture liquid with mycelium residues that did not grow on the surface but in the middle of the medium) was poured into Petri dishes with a diameter of 90 mm. After the medium was solidified, one disk of tested pathogenic fungus was introduced into the center of the Petri dish. Control growth of pathogenic fungus was maintained with adding of distilled water to the PDA medium. The observations on colony diameter of each fungus were registrited after the control Petri plates were completely filled with the mycelial growth of the respective fungus. The percent inhibition in mycelial growth was calculated by VINCENT (1947) formula:

$$I = \frac{C-T}{C} \cdot 100$$

where:
I – percent inhibition in colony growth
C – colony growth diameter in control
T – colony growth diameter in treatment.

Effect of carbon and nitrogen sources on mycelial growth and antimycotic activity

To assess the influence of carbon and nitrogen sources was chosen glucose-asparagine medium consisted of (g/L): 10.0 glucose, 0.4 asparagine, 1.0 KH_2PO_4, 0.5 $MgSO_4 \cdot 7H_2O$ (BISKO and KOSMAN 1988). Monosaccharides (arabinose, dextrose, galactose, glucose, fructose, xylose), disaccharides (lactose, maltose, sucrose), polysaccharide (soluble starch) and mannitol were used as carbon sources. For each of them, 4 g/L of C (pure carbon per liter calculated as the percentage of the carbon element in a molecule) were added individually to the medium to replace glucose. Different amounts of glucose (10–55 g were dissolved in 1000 mL of the medium) were used to assess the most suitable for mycelial growth carbon source concentration.

Different nitrogen sources (ammonium nitrate, ammonium sulphate, L-asparagine, peptone, sodium nitrate, yeast extracts, and urea) were used for the study. For each of them, 0.21 g/L of N (pure nitrogen per liter calculated as the percentage of nitrogen element in a molecule) was added individually to the medium to replace asparagine. Different amounts of yeast extracts (0.5–2.5 g were dissolved in 1000 mL of the medium) were used to assess the most suitable for mycelial growth nitrogen source concentration. 100 mL of medium in 250-ml Erlenmeyer flasks was inoculated with three mycelial plugs (8 mm diameter) of *H. myxotricha* and was incubated at 25°C for 7 days. After the incubation period, mycelial growth as well as antimycotic activity were determined as mentioned above.

Statistical analysis

The experimental results were expressed as means ± SEM (standard error of the mean) of triplicates. Statistical analysis was performed using Fisher's *F*-test. The data was analyzed with Excel statistical functions using the Microsoft Office XP software, Statistical Package for Social Sciences, version 11.5 (SPSS Inc., Chicago, 2002). Differences at $P \leq 0.05$ were considered to be significant.

Results and Discussion

The use of fungi is very actually and perspective trend due to their wide range of applications in different modern areas. The cultural and morphological properties of the vegetative mycelium of fungi are one of the integral and important parts of a comprehensive study of species in cul-

ture that can be used as additional criteria for solving taxonomic and biotechnological purposes (BUCHALO et al. 2011, BADALYAN et al. 2015, MYKCHAYLOVA et al. 2019, 2021). The determination of the optimal medium is the simplest and most essential tool for the introduction and as well as for improving the cultivation of fungi in culture.

Used media had a strong effect on colony morphology (Figure 1, Table 1) and growth of studied fungus (Table 1). The radial growth, average growth rate, texture, color, form, surface, margin, density of the culture mat, and its reverse color were taken into account as diagnostic macromorphological characteristics of *H. myxotricha* cultivated on various media (Table 1). *H. myxotricha* displayed a significant growth in all media used. However, the WA medium was the most suitable for mycelial growth of *H. myxotricha*.

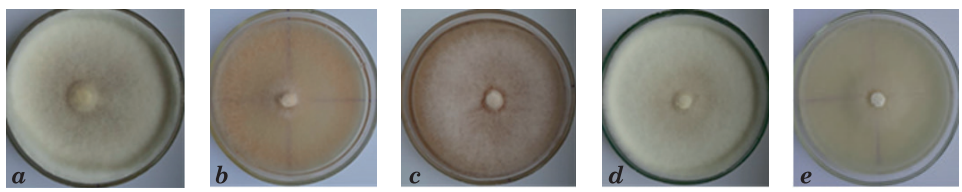

Fig. 1. Mycelial colonies of *H. myxotricha* on 9 days of cultivation on different media: *a* – WA – beer wort agar; *b* – PDA – potato dextrose agar; *c* – MEA – malt extract agar; *d* – GPYA – glucose-peptone-yeast agar; *e* – CZA – Czapek dox agar

Table 1

Colony morphology and growth of *H. myxotricha* cultivated on various media

Medium	Mycelial characteristics					Growth		
	texture	color	form/surface/ margin	density	reverse	mycelial growth	radial growth, [mm/day]	growth rate, [cm/day]
WA	felty	off-white	circular/ flat/entire	5+	unchanged	abundant	18.2±0.5	2.2±0.1
PDA	downy	white	circular/ flat/entire	3+	light brown	regular	10.7±0.3	1.5±0.1
MEA	downy	white	circular/ raised/entire	4+	unchanged	regular	8.8±0.4	1.3±0.2
GPYA	felty	off-white	circular/ flat/entire	5+	unchanged	abundant	12.2±0.2	1.8±0.1
CZA	silk thread, quickly aggregating	white	circular/ flat/entire	+	unchanged	scarce	11.2±0.1	1.5±0.0

Media: WA – beer wort agar; PDA – potato dextrose agar; MEA – malt extract agar; GPYA – glucose-peptone-yeast agar; CZA – Czapek dox agar. The mycelial density: very scanty (+); scanty (2+); moderate (3+); abundant (4+); very abundant (5+)

Biometric characteristics of the hyphae, clamp of *H. myxotricha* and crystals shown in Figure 2. The mycelium is unstained, contains inclusions of fat. Vegetative mycelia are septated, branched, of different thickness from 2.5 µm to 12.7 µm, anastomosed, with conspicuous clamp-connections (giant, single, pairs, whorls, oval, almond-shared), irregular hyphal loops (mycelial rings or trap) and crystals. Calcium oxalate crystal formation is quite a widespread phenomenon of wood-rotting basidiomycetes that may potential importance in the oxalate carbonate pathway (GUGGIARI et al. 2011). Therefore, several aspects of the microscopic characteristics of *H. myxotricha* mycelium can be useful in distinguishing between *Hohenbuehelia* taxa as well as in quality control of mycelial cultures by fungi cultivation for biotechnological purposes.

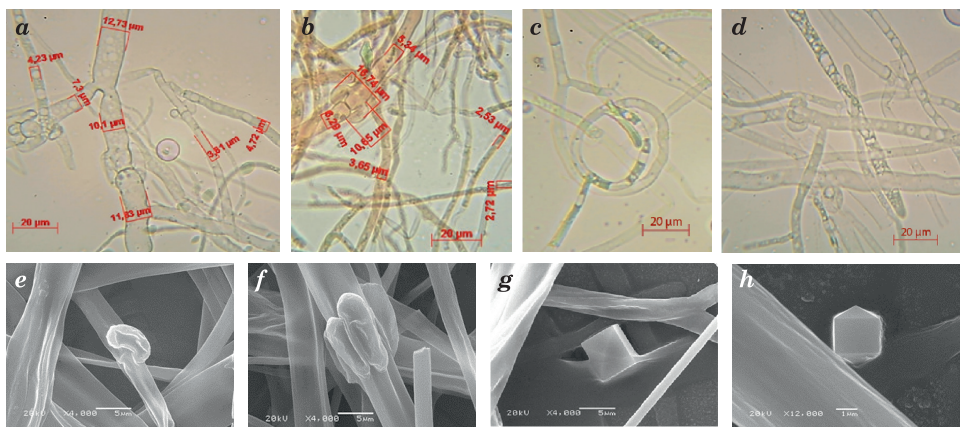

Fig. 2. Hyphal morphology of *H. myxotricha*: *a*, *b* – biometric characteristics of the hyphae; *c* – hyphal loops; *d–f* – hyphal growth of mycelium and clamp-connections; *g*, *h* – crystals. Bar = 20 µm (*a–d*) light microscopy; 5 µm (*e–g*) and 1 µm (*h*) scanning electron microscopy

The growth conditions can have significant impacts on the biomass and valuable metabolite production. And the determination a suitable fungi growth temperature is an important starting point for its cultivation *in vitro*. Basidiomycetes can grow in a wide range of temperatures, and *H. myxotricha* grew at all temperatures used. Our temperature investigations shown that *H. myxotricha* is a mesophilic fungus. The best mycelial growth (10.2 ±0.1 g/L) was observed at 25°C (Figure 3). This temperature was optimal for *Hohenbuehelia petaloides*, strain Hp 831 (ZHU et al. 2007). However, for other strain KACC 500040 of *H. petaloides* maximal mycelial growth was found at 30°C (YOO et al. 2001).

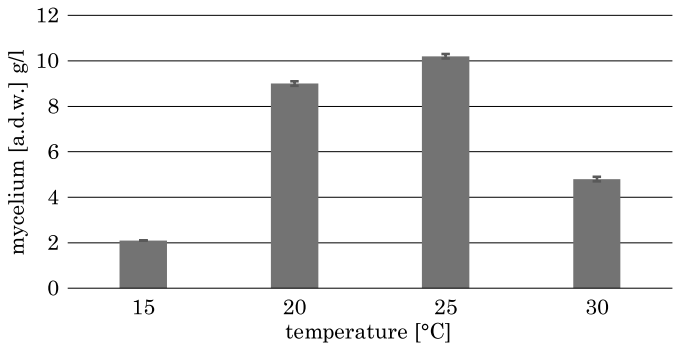

Fig. 3. Effect of temperature on mycelial growth of *H. myxotricha*

It is known that fungi have multiple mechanisms which allows them to adapt to pH changes. *H. myxotricha* grown in a wide range of initial pH levels (Figure 4). Suitable initial pH level of 4.5 provided maximal mycelial growth of this fungus at 11.6 ± 0.0 g/L. For mycelial growth of *H. petaloides* established other optimal pH levels: 6.0 (Yoo et al. 2001), and 6.0–7.0 (Zhu et al. 2007).

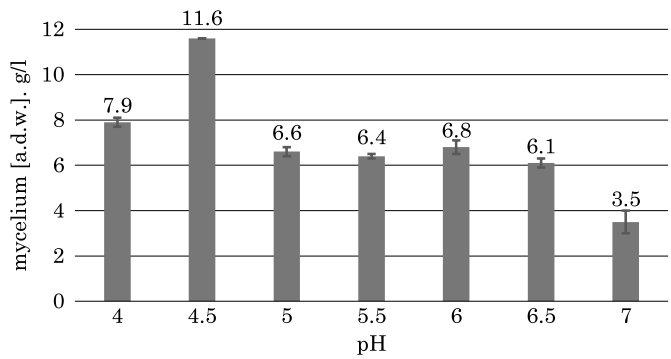

Fig. 4. Effect of pH on mycelial growth of *H. myxotricha*

The conditions for the correct time incubation are key to further understanding the dynamics of biomass production. The incubation period of 14 days was optimal for the best mycelial growth (9.4±0.3 g/L) of *H. myxotricha* (Figure 5). It should be noted that pH of cultivation medium was at constant level 4.0 starting up 7 to 42 days. This tendency points that fungus can change environment pH level close to its optimal parameter (Figure 4). It is in line with the capacity to control extracellular pH level that is an important attitude of fungal physiology.

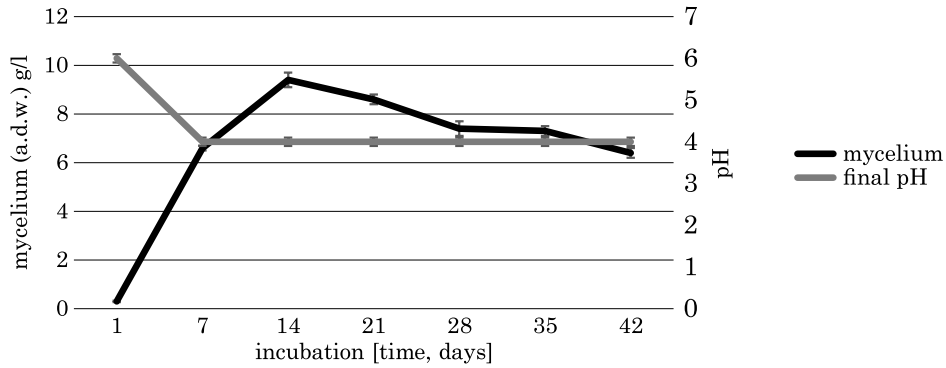

Fig. 5. Effect of incubation time on mycelial growth of *H. myxotricha*

It is well known that carbon sources are one of the most important nutrients required for the growth of living microorganisms. The presence of strong fermentative complex of *H. myxotricha* provided this fungus to assimilated all tested carbon sources at different level (Figure 6).

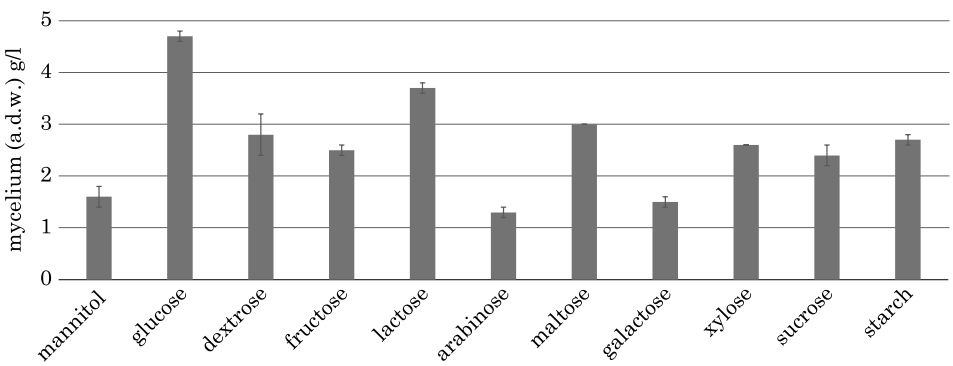

Fig. 6. Effect of carbon sources on mycelial growth of *H. myxotricha*

Among the eleven C-sources, glucose supported the best mycelial growth (4.7±0.1 g/L) of *H. myxotricha*, followed by lactose (3.7±0.1 g/L). The optimal concentration of glucose was 30 g/L, since we did not observe a statistically significant difference by further its increasing in the nutritional medium (Figure 7). Stimulatory effect of glucose may be explained that it is the main respiratory substrate and due to the ease with which this monosaccharide can metabolized to produce cellular energy (PAPASPYRIDI et al. 2012). While arabinose, galactose and mannitol were the most unfavorable C-sources. Dextrine (in concentration of 10%), fructose (in concentration of 10%) and lactose stimulated greater mycelial growth of *H. petaloides* (Yoo et al. 2001). Sodium carboxymethylcellulose was the most favorable for the growth of other strain of *H. petaloides* (ZHU et al. 2007).

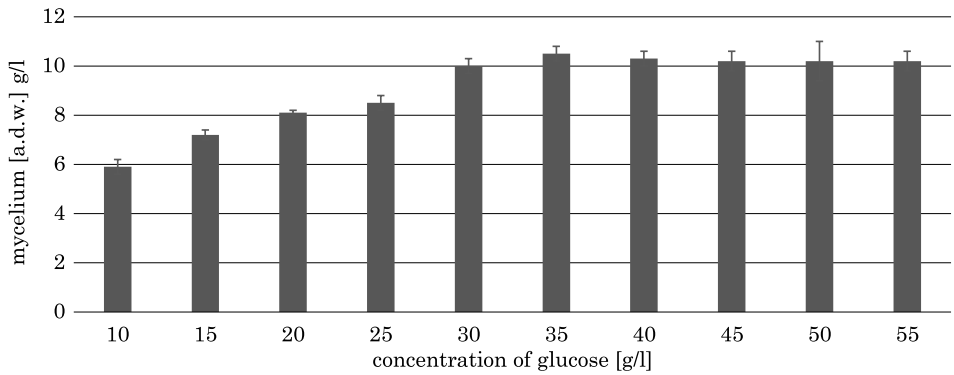

Fig. 7. Effect of glucose concentration on mycelial growth of *H. myxotricha*

The nitrogen sources have been shown to be essential to attitudes of fungi development and growth. All the six nitrogen sources tested were found suitable for investigated fungus growth (Figure 8). However, its capability to utilize various forms of nitrogen was differs. The most suitable nitrogen was yeast extracts that provided the highest mycelial yield 4.3±0.3 g/L.

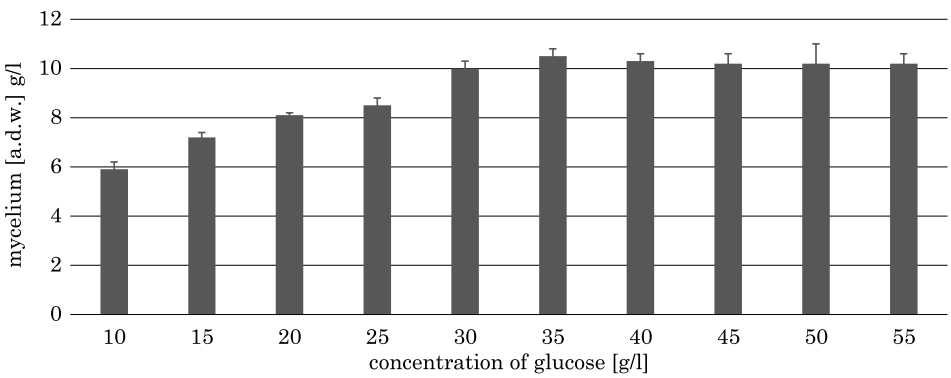

Fig. 8. Effect of nitrogen sources on mycelial growth of *H. myxotricha*

This effectivity of yeast extract can be explained due to its complexity composition like amino acids, carbohydrates, proteins, minerals, and vitamins. Yeast extract the best enhanced mycelial growth of *H. myxotricha* at concentration of 2 g/L (Figure 9). General, organic nitrogen sources were more suitable for *H. myxotricha* growth then inorganic N-sources. Tryptone, soytone, malt extract and also yeas extract (at concentration of 0.4%) were favorable for mycelial growth of *H. petaloides* (YOO et al. 2001). While peptone was the major nitrogen sources that supported the best mycelial growth of other strain of *H. petaloides* (ZHU et al. 2007).

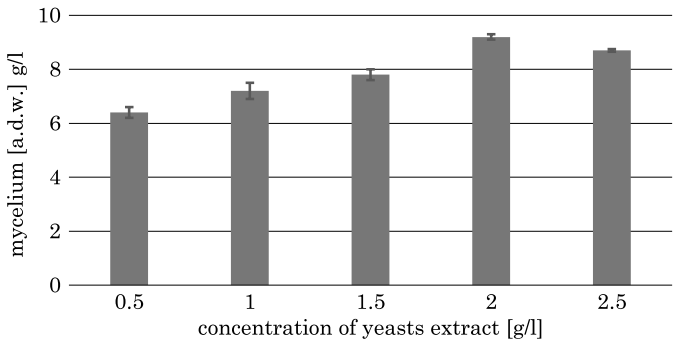

Fig. 9. Effect of yeast extract concentrations on mycelial growth of *H. myxotricha*

Fungal metabolites contributed the development of active substances in the modern pharmaceutical industry as well as promising agents to improve human and animal health. Our previously results showed that the ethanolic extract of *H. myxotricha* mycelia possessed strong antimicrobial potential, particularly antifungal activity (KRUPODOROVA et al. 2022). Metabolite's productions depend on different factors that vary among species, and also strains. The variation of growing condition can lead to significantly increased formation of a metabolites of interest. Incubation period affected the display of antimycotic activity of *H. myxotricha* (Figure 10).

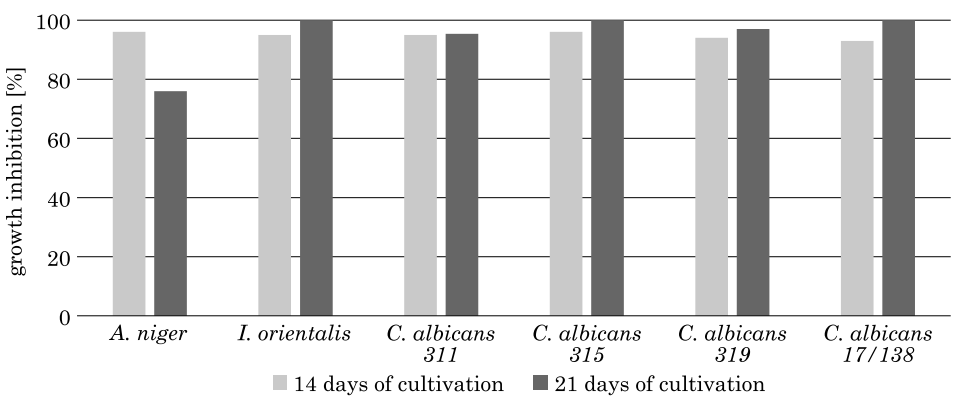

Fig. 10. Effect of incubation periods on antimycotic activity of *H. myxotricha*

It could be found that incubation time have in impact on the production on antimycotic metabolites of *H. myxotricha*. After 14 days of cultivation the antimycotic activity of *H. myxotricha* was significantly more effective against *A. niger* compared to other cultivation period. Increasing of incubation period to 21 days possessed maximal growth inhibition of *I. orientalis*, *C. albicans* 315, 319, 17/138. Suppression of *C. albicans* 311

growth was established at the same level (95%) regardless of the duration *H. myxotricha* cultivation. Mention above observation is in line with other studies (DYAKOV et al. 2011, SAZANOVA et al. 2013, BARAKAT and SADIK 2014, POPOVA 2015, SILVA et al. 2016, KRUPODOROVA et al. 2022) which reported that antimicrobial activity of Basidiomycetes (*Cantharellus cibarius* Fr., *Flammulina velutipes* (Curtis) Singer, *Fomitopsis betulina* (Bull.) B.K. Cui, *Lentinula edodes* (Berk.) Pegler, *Lentinus tigrinus* (Bull.) Fr., *Pleurotus ostreatus* (Jacq.) P. Kumm., *P. pulmonarius* (Fr.) Quél., *Pholiota lenta* (Pers.) Singer, *Rhodocollybia maculata* (Alb. & Schwein.) Singer) can varied depending on the duration of their cultivation time at different stage of their development.

Variation of nutritional conditions influenced also the production of antifungal secondary metabolites of *H. myxotricha* (Figure 11, Figure 12).

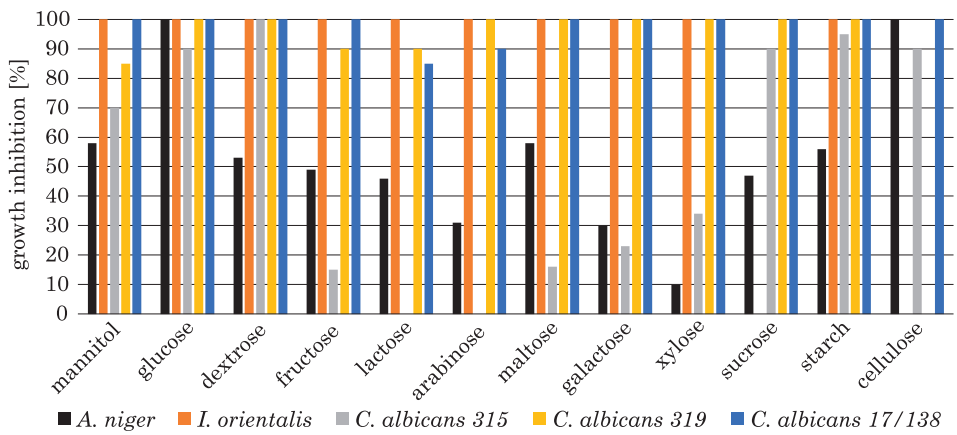

Fig. 11. Effect of carbon sources on antimycotic activity of *H. myxotricha*

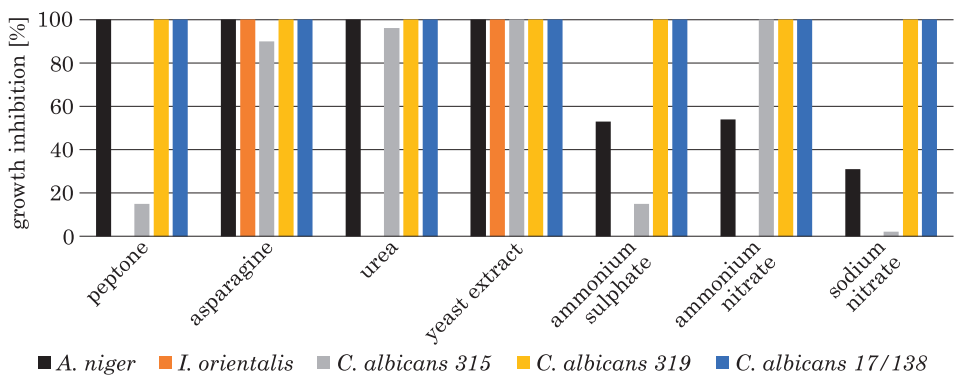

Fig. 12. Effect of nitrogen sources on antimycotic activity of *H. myxotricha*

The activity significantly varied depend of used carbon sources, the inhibition ranged from 10 to 100 % (Figure 11). It should be noted that we didn't observed activity against *C. albicans* 319 in case of cellulose application, against C. *albicans* 315 by present in the medium lactose or arabinose, and against *I. orientalis* by using of cellulose and sucrose. The most suitable for antimycotic metabolites promotion was glucose. Starch and dextrose inhibited growth of pathogenic fungi also good. The revealed need for the selection of optimal carbon sources to increase the antimicrobial activity is in agreement with previous reports (BARAKAT and SADIK 2014, POPOVA 2015, SILVA et al. 2016). Maltose was optimal for suppression of *A. niger* growth in experiment with *Pleurotus ostreatus* (BARAKAT and SADIK 2014). Growth inhibition of *Candida* spp. supported the presence in culture medium glucose and fructose in case of cultivation *P. ostreatus* (BARAKAT and SADIK 2014) and *Cantharellus cibarius* (POPOVA 2015), respectively. While glucose and starch by *P. pulmonarius* cultivation were suitable carbon sources for inhibition of *C. albicans* growth (SILVA et al. 2016).

The different effect of nitrogen sources on antimycotic activity of *H. myxotricha* was found (Figure 12). The activity greatly varied depending on the presence of nitrogen sources and the inhibition ranged from 2 to 100%, and similar dependence was also found previously for *Pleurotus ostreatus* submerged cultivation (VAMANU 2012). The most demanding under the conditions of the nitrogen experiment was *I. orientalis*. However, the most antimycotic activity of studied fungus against all used fungi was stimulated by yeast extract. Ammonium nitrate also good promoted growth suppression of all *C. albicans* strains. In general, organic nitrogen sources were more appropriated for antimycotic activity of *H. myxotricha* then inorganic N-sources. Ammonium sulfate, resulted in obtaining a mycelium *P. ostreatus* has shown the most pronounced antimicrobial effect (VAMANU 2012).

Conclusion

The results of our study demonstrated that *Hohenbuehelia myxotricha* can be successful growth at different conditions in culture. This is the first report concerting to detailed information about the cultural and morphological properties of the vegetative mycelium of *H. myxotricha* and on effects of basic nutritional requirements for its growth and some metabolites production. The growth conditions can have significant impacts on the colony morphology, mycelial growth and antimycotic compounds production. Additionally, abilities to *H. myxotricha* grow at different media,

pH levels, temperatures, as well as the capabilities of using wide ranges of C- and N- sources allow this fungus to adapt to changing conditions. This study has shown that maximal mycelia of *H. myxotricha* can be established after 14 days of liquid static cultivation at 25°C, pH 4.5, glucose at 30 g/L concertation, yeast extract at 2 g/L concentration. The ability to increase and ensure a controlled level of antimycotic activity of *H. myxotricha* indicates the viability of biotechnological and pharmaceutical applications of this species of fungus. Incubation period and nutritional needs affected the display of antimycotic activity of *H. myxotricha*. The optimal period for growth inhibition of *Aspergilus niger* was 14 days while 21 days of cultivation was more appropriated for growth inhibitions of *Saccharomycetales* used. The most suitable for promotion of antimycotic metabolites were glucose and yeast extract. In general, obtained results add to our knowledge of the environmental features of studied fungus and its secondary metabolites for axenic cultivation. Further work is needed to isolation and identification of the active compounds responsible for antimycotic activity that can provide new potential perspective compounds for modern pharmacology.

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Competing Interests

The authors declare that they have no competing interests.

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References

Angeli P., Scandurra S. 2012. *A rare member of the family Pleurotaceae collected at Palermo. Hohenbuehelia myxotricha*. Micol. Veget. Medit., 27(1): 3–11.

Badalyan S.M., Shnyreva A.V., Iotti M., Zambonelli A. 2015. *Genetic resources and mycelial characteristics of several medicinal polypore mushrooms (Polyporales, Basidiomycetes)*. Int. J. Med. Mushr., 17(4): 371–384, https://doi.org/10.1615/intjmedmushrooms.v17.i4.60.

Bala N., Aitken E.A.B., Cusack A., Steadman K.J. 2012. *Antimicrobial potential of australian macrofungi extracts against foodborne and other pathogens*. Phytother. Res., 26(3): 465–469, https://doi.org/10.1002/ptr.3563.

Barakat O.S., Sadik M.W. 2014. *Mycelial growth and bioactive substance production of Pleurotus ostreatus in submerged culture*. Int. J. Curr. Microbiol. App. Sci., 3(4): 1073–1085.

Bijeesh C., Manoj K.A., Pradeep C.K., Vrinda K.B. 2019. *A new species of Hohenbuehelia (Pleurotaceae) from India*. Phytotaxa, 420(1): 056–064, https://doi.org/10.11646/phytotaxa.420.1.3.

Bisko N.A., Kosman E.G. 1988. *Stock growth of Pleurotus ostreatus (Jacq.: Fr.) Kumm. on media with different carbon and nitrogen sources*. Mycology and Phytopathology, 22(6): 516–519.

Bisko N.A., Lomberg M.L., Mykchaylova O.B., Mytropolska N.Yu. 2021. *IBK Mushroom Culture Collection. Version 1.2*. The IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany, https://doi.org/10.15468/dzdsqu accessed via GBIF.orgon 15.04.2021.

Buchalo A.S., Wasser S.P., Mykchaylova O.B., Bilay V.T., Lomberg M. 2011. *Taxonomical significance of microstructures in pure cultures of macromycetes*. In: *Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products (ICMBMP7)*, pp. 50–57.

Consiglio G., Setti L., Thorn R.G. 2018. *New species of Hohenbuehelia, with comments on the Hohenbuehelia atrocoerulea – Nematoctonus robustus species complex*. Persoonia, 41: 202–212, https://doi.org/10.3767/persoonia.2018.41.10.

Dyakov M.Y., Kamzolkina O.V., Shtaer O.V., Bis'ko N.A., Poedinok N.L., Mikhailova O.B., Tikhonova O.V., Tolstikhina T.E., Vasil'eva B.F., Efremenkova O.V. 2011. *Morphological characteristics of natural strains of certain species of basidiomycetes and biological analysis of antimicrobial activity under submerged cultural conditions*. Microbiology, 80(2): 274–285, https://doi.org/10.1134/S0026261711020044.

Guggiari M., Bloque R., Aragno M., Verrecchia E., Job D., Junier P. 2011. *Experimental calcium-oxalate crystal production and dissolution by selected wood-rot fungi*. International Biodeterioration & Biodegradation, 65(6): 803–809, https://doi.org/10.1016/j.ibiod.2011.02.012.

Kirk P.M., Cannon P.F., Minter D.W., Stalpers J.A. 2008. *Dictionary of the fungi. 10th edition*. CAB International, Wallingford, UK.

Koziak A.T.E., Cheng K.C., Thorn R.G. 2007. *Phylogenetic analyses of Nematoctonus and Hohenbuehelia (Pleurotaceae)*. Canadian Journal of Botany, 85(8): 762–773, https://doi.org/10.1139/B07-083.

Krupodorova T.A., Barshteyn V.Yu. 2015. *Alternative substrates for higher mushrooms mycelia cultivation*. J. BioSci. Biotechnol., 4(3): 339–347.

Krupodorova T., Barshteyn V., Sevindik M. 2022. *Antioxidant and antimicrobial potentials of mycelial extracts of Hohenbuehelia myxotricha grown in different liquid culture media*. Bio-Technology, 103(1):19–28.

Krupodorova T.A., Barshteyn V.Yu., Zabeida E.F., Pokas E.V. 2016. *Antibacterial activity of macromycetes mycelia and culture liquid*. Microbiology and Biotechnology Letters, 44(3): 246–253, http://dx.doi.org/10.4014/mbl.1603.03003.

Krupodorova T.A., Ivanova T.S., Barshteyn V.Yu. 2014. *Screening of extracellular enzymatic activity of macrofungi*. J. Microbiol. Biotech. Food Sci., 3(4): 315–318.

Li X., Wang L., Wang Z. 2015. *Radioprotective activity of neutral polysaccharides isolated from the fruiting bodies of Hohenbuehelia serotina*. Physica Medica, 31(4): 352–359, https://doi.org/10.1016/j.ejmp.2015.02.004.

LI X., WANG L., WANG Z. 2017. *Structural characterization and antioxidant activity of polysaccharide from Hohenbuehelia serotina*. Int. J. Biol. Macromol., 98: 59–66, https://doi.org/10.1016/j.ijbiomac.2016.12.089.

LI X.Y., WANG Z.Y., WANG L., WALID E., ZHANG H. 2012. *In vitro antioxidant and anti-proliferation activities of polysaccharides from various extracts of different mushrooms*. Int. J. Mol. Sci., 13(5): 5801–5817, https://doi.org/10.3390/ijms13055801.

LIANG X., HUA D., WANG Z., ZHANG J., ZHAO Y., XU H., LI Y., GAO M., ZHANG X. 2013. *Production of bioethanol using lignocellulosic hydrolysate by the white rot fungus Hohenbuehelia* sp. *ZW-16*. Ann. Microbiol., 63: 719–723, https://doi.org/10.1007/s13213-012-0524-6.

LUBIAN C., KUHN O.J., PORTZ R.L., AGUSTINHA A.M., STANGARLIN J.R. 2021. *Biological control of Meloidogyne javanica in bean plants by Hohenbuehelia spp. and Trichoderma koningiopsis*. Arq. Inst. Biol., 88: 1–9, https://doi.org/10.1590/1808-1657000772019.

LUBIAN C.C., MARTINHA D.D., PORTZ R.L., SILVA F.A.G.S., CORTEZ V.G., MISSIO V. 2018. *Daily indexes for predation and growth of nematophagous mushrooms species of Hohenbuehelia (Pleurotaceae) on Panagrellus redividus*. J. Agric. Sci., 10(3): 276–289, https://doi.org/10.5539/jas.v10n3p276.

MENTRIDA C.S. 2016. *Species delimitation and phylogenetic analyses of the genus Hohenbuehelia in central Europe*. Master's Thesis, University of Wien, Wien.

MYKCHAYLOVA O., LOMBERG M., BISKO N. 2019. *Verification and screening of biotechnologically valuable macromycetes species in vitro*. In: *Development of modern science. The experience of European countries and prospects for Ukraine* (monograph / edited by authors). 3rd ed. – Riga, Latvia: "Baltija Publishing", pp. 354–375.

MYKCHAYLOVA O., LOMBERG M., TSAPKO S., KRASINKO V. 2021. *Morphological characteristics of the culture Clathrus archeri (Phallaceae, Basidiomycota)*. Pol. J. Natur. Sc., 36(3): 283–298.

QUATTELBAUM D., CARNER G.R. 1980. *A technique for preparing Beaveria* spp. *for scanning electron microscopy*. Canadian Journal of Botany, 58: 1700–1703, https://doi.org/10.1139/b80-198.

PAPASPYRIDI L.M., ALIGIANNIS N., TOPAKAS E., CHRISTAKOPOULOS P., SKALTSOUNIS A.L., FOKIALAKIS N. 2012. *Submerged fermentation of the edible mushroom Pleurotus ostreatus in a batch stirred tank bioreactor as a promising alternative for the effective production of bioactive metabolites*. Molecules, 17(3): 2714–2724, https://doi.org/10.3390/molecules17032714.

POPOVA T.P. 2015. *Investigations on antimicrobial activity in vitro of liquid cultures of Cantharellus cibarius*. Int. J. Curr. Microbiol. App. Sci., 4(3): 674–683.

REALE J. 2018. *The impact of a fungus-feeding nematode (Aphelenchoides sp.) on decomposition of trembling aspen wood by various wood-decay fungi*. Bachelor Thesis, Lakehead University Thunder Bay, Ontario, Canada.

SANDARGO B., THONGBAI B., STADLER M., SURUP F. 2018a. *Cysteine-derived pleurotin congeners from the nematode-trapping Basidiomycete Hohenbuehelia grisea*. J. Nat. Prod., 81: 286–291, https://doi.org/10.1021/acs.jnatprod.7b00713.

SANDARGO B., THONGBAI B., PRADITYA D., STEINMANN E., STADLER M., SURUP F. 2018b. *Antiviral 4-Hydroxypleurogrisein and antimicrobial pleurotin derivatives from cultures of the nematophagous basidiomycete Hohenbuehelia grisea*. Molecules, 23(10): 2697. https://doi.org/10.3390/molecules23102697.

SAZANOVA K.V., USATOVA V.S., KICHEVA A.A., ANANIEVA E.P., PSURTSEVA N.V. 2013. *Screening of Basidiomycetes from the LE-BIN culture collection for antifungal activity*. In: Yan Maximov (ed.), *Proceedings of the 2nd International Academic Conference "Applied and Fundamental Studies"*, 11–18, St. Louis, Missouri, USA.

SILVA B.P., ABRAHÃO J., PERALTA R.M. 2016. *Effects of carbon sources and time of cultivation on the antimicrobial activities of intra and extracellular extracts of Pleurotus pulmonarius cultured in submerged sonditions*. Int. J. Curr. Microbiol. App. Sci., 5(11): 97–105. http://dx.doi.org/10.20546/ijcmas.2016.511.011.

STALPERS J.A. 1978. *Identification of wood-inhabiting Aphyllophorales in pure culture*. *Studies in Mycology, 16*, Centraalbureau voor Schimmelcultures, Baarn.

THORN R., MONCALVO J., REDDY C., VILGALYS R. 2000. *Phylogenetic analyses and the distribution of nematophagy support a monophyletic Pleurotaceae within the polyphyletic pleurotoid-lentinoid fungi*. Mycologia, 92(2): 241–252, https://doi.org/10.2307/3761557.

VAMANU E. 2012. *In vitro antimicrobial and antioxidant activities of ethanolic extract of lyophilized mycelium of Pleurotus ostreatus PQMZ91109*. Molecules, 17(4): 3653–3671, https://doi.org/10.3390/molecules17043653.

VINCENT J.M. 1947. *Distortion of fungal hyphae in the presence of certain inhibitors*. Nature, 159, https://doi.org/10.1038/159850b0.

WEIS A.L., SOLOMKO E.F., BUCHALO A.S., WASSER S.P., MITROPOLSKAYA N.YU., GRIGANSKYI A.P., GOROVITS E.L. 1999. *Cultural study and Illudin S production of medicinal mushroom Omphalotus olearius (DC.: Fr.) Fay. (Agaricales s.l.) from Israel*. Int. J. Med. Mushr., 1(1): 93–103, https://doi.org/10.1615/IntJMedMushrooms.v1.i1.80.

YOO K.H., KIM J.H., SEOK S. 2001. *Studies on the cultural characteristics of Hohenbuehelia petaloides*. The Korean Journal of Mycology, 29(1): 52–60.

ZHANG R., ZHAO L., WANG H., NG T.B. 2014. *A novel ribonuclease with antiproliferative activity toward leukemia and lymphoma cells and HIV-1 reverse transcriptase inhibitory activity from the mushroom, Hohenbuehelia serotina*. International Journal of Molecular Medicine, 33(1): 209–214, https://doi.org/10.3892/ijmm.2013.1553.

ZHU W., HE X., WANG M. 2007. *Effect of selected culture parameters on the growth of Hohenbuehelia petaloides Mycelium*. Acta Edulis Fungi, 1: 49–53.

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A PHYLOGENETIC COMPARISON AND CLASSIFICATION OF LMCO SEQUENCES RETAINED FROM SOIL METAGENOME*

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Key words: laccase-like gene, metagenome, phylogenetic tree.

Abstract

Laccases (EC 1.10.3.2) belong to the family of multicopper oxidases (MCOs) and have the ability to oxidise various phenolic and nonphenolic compounds. In recent years, interest in bacterial laccases has increased because they have more favourable reaction features when compared to fungal laccases, such as their thermostability, alkaline pH, short acquisition time and simple overexpression system, making them highly desirable for industry. There is increasing molecular evidence that laccase-like multicopper oxidases (LMCOs) are widely distributed in bacteria. In this study, a total of 228 sequences of 3-domain LMCOs from a metagenome were used for phylogenetic analysis. A BLAST analysis and the reconstruction of the phylogenetic tree allowed to distinguish several LMCO sequence clusters, such as *Alphaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes* and a cluster of unclassified sequences. The results of the phylogenetic analysis also suggest that the bacterial class of *Deltaproteobacteria* may play an important role in the lignin degradation process.

Introduction

Laccases (EC 1.10.3.2) belong to the family of multicopper oxidases (MCOs), which are able to oxidise various phenolic and non-phenolic compounds by converting an oxygen molecule into water, with or without the presence of various mediators (CAÑAS and CAMARERO 2010, GIARDINA et al. 2010). These enzymes are classified as monomeric enzymes that have three cupredoxin-like domains and four characteristic histidine-rich cop-

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per-binding sites in the first and third domains. However, recent studies have shown that some laccases from the phylum *Actinobacteria* can form a separate group of MCOs with two domains (FERNANDES et al. 2014). In addition, the cupredoxin superfamily consists of many other oxidase enzymes, such as manganese oxidase and ascorbate oxidase. Due to their slightly different biochemical properties and imprecisely defined biological functions, it also seems justified to distinguish the LMCO-laccase-like multi-copper oxidases from the MCOs (BRANDER et al. 2014).

Laccases have been known since the 19th century when they were first discovered in the lacquer of the Japanese tree *Toxicodendron vernicifluum* (YOSHIDA 1883). Due to their oxidative abilities, laccases and laccase-like LMCOs have become sought after in the textile, pulp and paper and food industries. They are also used in the development of biosensors and biofuel cells and can be used as medical diagnostic tools and in bioremediation. The industry mainly uses laccase from fungi, especially white rot fungi such as *Trametes versicolor* (SHRADDHA et al. 2011). However, the industrial extraction of laccases from fungi is hampered by the long fermentation time, the low laccase yield and their applicability only under mesophilic and acidic reaction conditions (VISWANATH et al. 2014). In recent years, bacterial laccases have gained increasing interest due to their more favourable reaction features when compared to fungal laccases. Their thermostability, alkaline pH, short acquisition time and the ease with which they can be cloned and expressed have made them highly desirable in industry (WANG and ZHAO 2016b).

The first bacterial laccase was found in the bacterium *Azospirillum lipoferum* associated with plant roots (GIVAUDAN et al. 1993). Since then, laccases have been found mainly in gram-positive and gram-negative bacteria (CHAUHAN et al. 2017), and a new laccase-like enzyme was developed by screening after it was isolated directly from bacterial strains (SIROOSI et al. 2016, REZAEI et al. 2017). Heterologous expression of laccase-encoding genes from bacterial strains was also successfully implemented (MATHEWS et al. 2016, SUN et al. 2017). Several bacterial laccases have already been used on an industrial scale, particularly in the fields of wood, biofuels, paper, textiles, fine chemicals and food (ZERVA et al. 2020).

The laccase and laccase-like oxidation reaction of MCOs, releasing water as a by-product, is extremely tempting for industrial waste-free technologies. However, its implementation is hampered by the high cost of obtaining large quantities of the active enzyme with the desired substrate specificity and by the fact that relatively few bacterial laccases have been characterised to date, reinforcing the need to search for new laccases (IHSSEN et al. 2015, SHARMA et al. 2018).

Metagenomics is a valuable tool to discover new LMCOs, and soil seems to be an ideal source for their identification. Studies on soil bacterial communities carried out in 150 forest and 150 grassland soils have revealed the complexity and variability of the microbiological consortium in these environments, suggesting a potential richness of bacterial laccases (KAISER et al. 2016, YANG et al. 2018). In turn, culture-independent metagenomics is a powerful tool for discovering new enzymes from uncultured microorganisms and for gaining knowledge about the entire microbiological community through the direct extraction and cloning of DNA (HANDELSMAN 2004). The metagenomics library can be searched in two different approach. In the first, fuctional-besed approach, screening is based on the detection of expression products, which facilitates the identification of completely new enzymes. However, the probability of identifying a gene of interest depends mainly on the host-vector system and its abundance in the metagenome. It is a major challenge to discover a new enzyme using this approach, so the number of clones that have the desired activity is extremely low. The second approach involves screening metagenomic libraries based on the sequence of a target gene. Although this approach may somewhat limit the scope of novelty, bioinformatic analysis based on searching for phylogenetic markers or other similar sequences in exclusively existing public databases is warranted – especially when studying a heterogeneous group of enzymes such as bacterial laccase and LMCO (BERINI et al. 2017). The use of the metagenome to screen laccase has been little explored (KIMURA and KAMAGATA 2016, AUSEC et al. 2017, YUE et al. 2017), mainly due to the limitations of screening methods mentioned above. Nevertheless, metagenomics remains an attractive approach to identify and study the diversity of laccases and LMCO genes in the environment.

The aim of this study was the phylogenetic analysis of LMCOs genes from the forest soil metagenome library. This bioinformatic analysis allows us to distinguish the laccase-like coding fragments of different bacterial taxa and to reveal the species diversity of laccases in soil. This knowledge could be useful to understand the role of laccase in soil bioremediation or provide a way to predict the full gene sequence of LMCO, its expression and characterisation of enzyme activity for industry in the future.

Material and Methods

DNA isolation, DOP-PCR fragments library construction and sequencing

In previous studies, two soil samples (forest and fen) collected in April 2009 from areas within the city of Olsztyn (Poland) were characterized for multicopper oxidase activity based on FLOCH et al. (2007), and the soil samples were used for DNA library construction. The DOP-PCR reaction was performed to amplify laccase-like gene fragments of about 1200 bp in size (AUSEC et al. 2011a, ZIELIŃSKA and ADAMCZAK 2013). Briefly, the procedure involved isolation of metagenomic DNA using a GeneMATRIX Soil DNA Purification Kit (EURx, Gdansk, Poland), followed by the DOP-PCR reaction performed with degenerate primers specific for conservative laccase sites: Cu1AF 5'ACM WCB GTY CAY TGG CAY GG3' and Cu4R 5'TGC TCV AGB AKR TGG CAG TG3'(AUSEC et al. 2011a) with the following protocol: an initial denaturation at 94ºC for 3 min, 30 cycles of 30 s at 94ºC, 30 s at 48ºC, 1 min at 72ºC, ending with 5 min at 72ºC, 25 ml reactions contained 2.5 ml of each primer (20 μM), 1 ml of dNTP (40 μM each), 3 ml of $MgCl_2$ (25 mM), approximately 200 ng of DNA template, 5 ml of PCR buffer and 1 U of Taq DNA polymerase (GoTaq ® Flexi DNA Polymerase, Promega, Madison, Wisconsin USA).

The amplified DNA fragments were cloned into the pGEM-T vector and a metagenome library was prepared in *E coli* JM109 cells following the manufacturing protocol (Promega, Madison, Wisconsin, USA). Recombinant pGEM-T plasmids were isolated using the Plasmid Mini AX Kit (A&A Biotechnology, Gdansk, Poland) and inserts were amplified using the GoTaq Colourless Master Mix Kit (Promega, Madison, Wisconsin, USA) with primer T7 5'TAATACGACTCACTATAGGG3' and primer SP6 5'TATTTAGGTGACACTATAG3' according to the following protocol: an initial denaturation at 94ºC for 3 min, 30 cycles of 30 s at 94ºC, 30 s at 50ºC, 1 min at 72ºC, ending with 5 min at 72ºC, 50 ml reactions contained 5.0 ml of each primer (10 μM), approximately 250 ng of DNA template, 25 ml of PCR 2X Colourless GoTaq® Reaction Buffer each containing 400 μM dNTP, 3mM $MgCl_2$ and Taq DNA polymerase.

DNA fragments were sequenced from both ends using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on a ABI PRISM 3730 DNA analyser (Applied Biosystems, Foster City, CA). All sequences were manually proofread and assembled using DNA Baser v4.36.0. The sixteen metagenome sequences isolated from one of the forest soil samples were used for computational analysis in this study.

Sequences and Phylogenetic Analysis

For this work, the Polish soil LMCO sequences were stored in GenBank under accession numbers MN558914-MN558929, and one sequence artifacts was excluded from this study. The sequences with accession numbers: HM045759-HM045777, HQ286736-HQ286789, which were isolated from bog and fen soil samples using a non-culturing method, were also used in this study (AUSEC et al. 2011a). A total of eighty-six sequences potentially encoding LMCOs and derived from soil metagenome libraries were used for further computational analysis. These sequences were used for a similar search for sequences from the NCBI database (https://www.ncbi.nlm.nih.gov/) using BLASTn with a non-redundant nucleotide database, BLASTX and BLASTp with non-redundant protein sequences or a reference protein sequence database.

Sequence manipulation and phylogenetic tree reconstruction was carried out using two methods. In the first case, sequences from the Polish soil metagenome library and similar sequences from the NCBI database were used for phylogenetic tree reconstruction (about two hundred sequences). The nucleotide sequences containing LMCOs (LMCO sequences from the Polish soil metagenome and homologous sequences from the database) were translated into amino acid sequences using Geneious Prime (KEARSE et al. 2012). The sequences were then trimmed to contain two Cu-binding regions (cbr II and cbr III) and a nucleotide sequence occurring between them. The trimmed sequences were aligned using the software MUSCLE 3.8.425 (EDGAR et al. 2004). Based on the alignment, Bayesian analysis was performed using MrBayes 3.2.1 (RONQUIST and HUELSENBECK 2003). The MCMC algorithm was run for 5,000,000 generations (sampling every 500) with four incrementally heated chains. The first 25% of the trees were discarded as burn-in. The remaining trees were used to generate the consensus tree.

For the second phylogenetic tree reconstruction, nucleotide sequences from the Polish soil metagenome were translated using the Translate tool-ExPASy (https://web.expasy.org/translate/) and trimmed together with LMCO sequences from the database and from bog and fen soils (AUSEC et al. 2011a) trimmed to contain two Cu-binding regions, as had been done previously, and aligned as amino acid sequences using ClustalW implemented in MEGA7 v. 7.021 (KUMAR et al. 2016). MEGA7 v. 7021 software was used for sequence manipulation and phylogenetic reconstruction, and the tree was inferred from the protein sequence alignment. The evolutionary history was inferred using the Maximum Likelihood method based on the model of Whelan and Goldman (LE and GASCUEL 2008). An initial tree was first built using a Neighbor-Joining method and

its branch lengths were adjusted to maximise the likelihood of the dataset for this tree topology under the desired evolutionary model. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with gaps and missing data were eliminated.

The reference sequences of MCOs with the accession numbers, used in the phylogenetic analysis were as follows: PZN23754.1, RIK29390.1, WP_104023078.1, TML46446.1, PZR04526.1, WP_018614744.1, TAK25694.1, AKU93578.1, TMM19967.1, WP_137260238.1, WP_141196114.1, WP_136580480.1, WP_013768667.1, TMB38487.1, WP_123122544.1, WP_013891894.1., TMI72789.1, WP_139795072.1, MAY01904.1, TFG53223.1, RTM09228.1, RPI54963.1, WP_053239053.1, WP_044985630.1, WP_012233433.1, WP_129347183.1, WP_116036636.1, WP_086086790.1, WP_093734497.1, WP_004212985.1, WP_037491563.1, WP_035530640.1, WP_107978106.1, WP_080919611.1, WP_071900668.1, WP_015345738.1, WP_087868494.1, WP_141900028.1, WP_080522328.1, WP_050726212.1, WP_081950100.1, WP_093038291.1, WP_100095845.1, WP_067560161.1, WP_028464233.1, WP_012563321.1, WP_089851963.1, WP_040402904.1, WP_007602744.1, WP_009503858.1, WP_068241745.1, WP_102248111.1, WP_119461512.1, WP_109518985.1, WP_027238842.1, WP_094301476.1, WP_100161006.1, WP_119682026.1, WP_076695202.1, WP_029375169.1, WP_013901958.1, WP_046135170.1, WP_114910975.1, WP_048878272.1, WP_109923174.1, WP_094407154.1, WP_035228630.1, WP_062761965.1, WP_124964904.1, WP_103257142.1, WP_077546320.1, WP_071796151.1, WP_132474996.1, WP_141850878.1, WP_133034171.1, WP_119776634.1, HBD97996.1, WP_020404328.1, WP_070796186.1, WP_009021358.1, WP_121881975.1, WP_011642144.1, WP_109261381.1, WP_104832397.1, WP_009800481.1, WP_095158341.1, WP_053550716.1, WP_119376372.1, WP_060090832.1, WP_022698546.1, WP_022693166.1, WP_058178341.1, WP_031296247.1, WP_131153852.1, WP_091462888.1, WP_124235827.1, WP_119286241.1, WP_139795588.1, WP_116570295.1, WP_083962369.1, WP_093943049.1, WP_133787361.1, WP_083711762.1, WP_108046919.1, WP_130461760.1, WP_114958078.1, WP_119274986.1, WP_112661640.1, WP_140943417.1, WP_128779026.1, WP_073631571.1, WP_092863561.1, WP_073052225.1, WP_131834895.1, RPI54963.1, WP_136918250.1, WP_138380708.1, WP_012240709.1, WP_146645271.1, WP_129581229.1, WP_104023078.1, WP_136934632.1, WP_147205331.1, WP_146645271.1, WP_018614744.1, WP_136580480.1, WP_013768667.1, WP_023765434.1, WP_135908040.1, WP_053239053.1, WP_116036636.1, WP_013768667.1, WP_143177680.1, WP_015345738.1, WP_146979921.1, WP_073684142.1, WP_083812524.1, WP_046362600.1, WP_067983101.1, WP_078981544.1, WP_101368345.1, WP_062285860.1, WP_135116536.1, WP_114073056.1,

WP_071100875.1, WP_113682993.1, WP_019747846.1, WP_130461760.1, WP_148798198.1, WP_131153852.1, WP_111472834.1, WP_125483648.1, WP_068016701.1, WP_123490970.1, WP_086134294.1, WP_013031520.1, WP_006927672.1, WP_073377490.1, WP_086091162.1, WP_116460059.1, WP_086086790.1, WP_131569804.1, WP_119461512.1, WP_145809072.1, WP_110909913.1, WP_053206700.1, WP_130025932.1, WP_012173860.1, WP_102071196.1, WP_011633152.1, WP_106859220.1, WP_129206354.1, WP_082125471.1, WP_008974103.1, WP_006927672.1, WP_116717539.1, WP_073377490.1, WP_012173860.1, WP_116460059.1, WP_026014869.1, WP_148474672.1, WP_035353113.1, WP_026447326.1, WP_035353113.1, WP_024537120.1, WP_011633152.1, WP_013519563.1, WP_015056770.1, WP_095605214.1, WP_076441808.1, WP_047639815.1, WP_139717470.1, WP_123769215.1, WP_128739836.1.

Conserved domain analysis

A CD-search tool was used to find conserved multicopper oxidase domains in the protein sequences (https://www.ncbi.nlm.nih.gov/). The Pfam database is a large collection of protein families, each represented by multiple sequence alignments and Hidden Markov Models (HMMs) (https://pfam.xfam.org/) (MARCHLER-BAUER et al. 2017, EL-GEBALI et al. 2019). SMART (Simple Modular Architecture Research Tool) was used as a second approach for protein domain search (http://smart.embl-heidelberg.de/) (LETUNIC and BORK 2018).

Results and Discussion

Motif/Domain analysis

In previous studies, sequences were isolated from the soil metagenome by specific primers for laccase-like genes (AUSEC et al. 2011b, ZIELIŃSKA and ADAMCZAK 2013). In this study, sixteen fragments from a soil sample, each about 1200 bp in length, were used for bioinformatic analysis. The sequences could be translated into protein sequences without stop codons. The amino acid sequences derived from the soil metagenome DNA were used to identify protein domains. Identification of the conserved domains by CD-Search tools against the CCD database showed that eleven sequences (Lac3D4, Lac3D5, Lac3D8, Lac3D10, Lac3D30, Lac3D23, Lac3D25, Lac3D18, Lac3D32, Lac3D33, Lac3D15) contained the superfamily domain Sulf (COG2132) typical of multicopper oxidase with three cupredoxin domains, indicating that they belonged to the fragments of laccase genes.

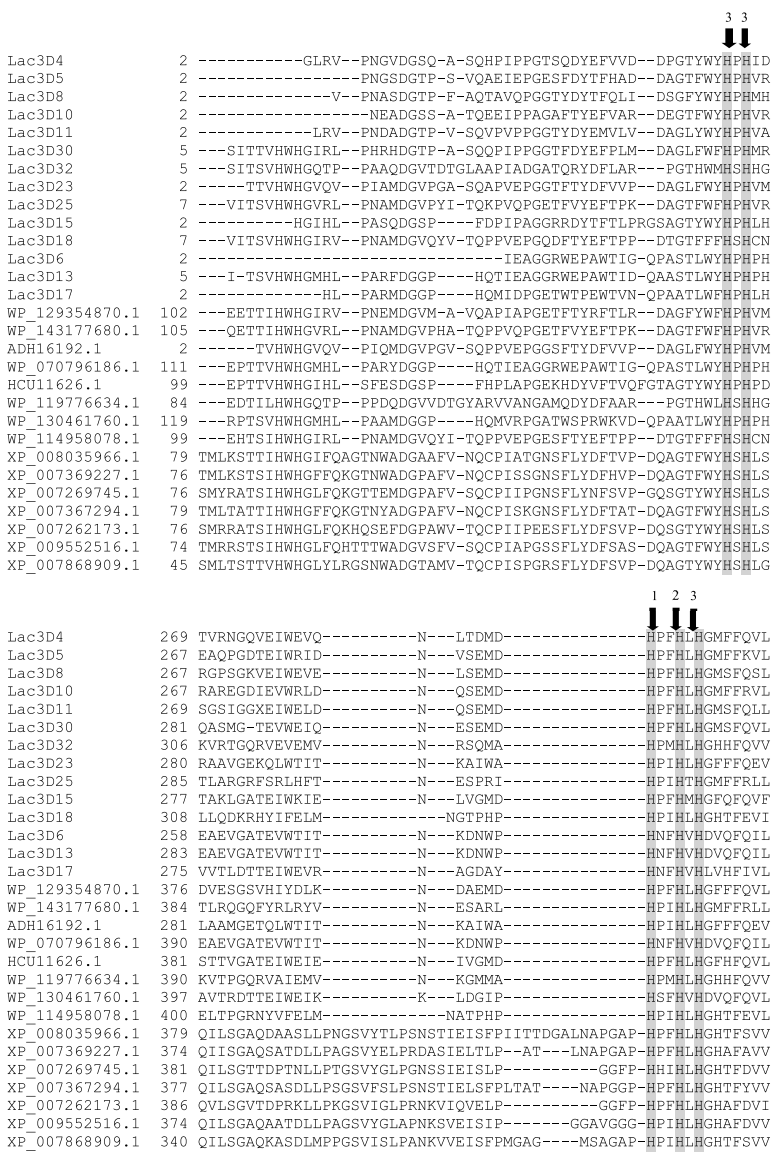

Fig. 1. Fragments of protein sequences alignment containing a two Cu-binding region (cbr II, cbr III) of sequences from Polish soil metagenome (Lac3D4-32), bacterial laccase: *Sorangium cellulosum* (WP_129354870.1), *Cystobacter ferrugineus* (WP_143177680.1), putative laccase partial uncultured bacterium (DH16192.1), *Rhodococcus erythropolis* (WP_070796186.1), *Gemmatimonadetes bacterium* (HCU11626.1), *Oleomonas sp.* K1W22B-8 (WP_119776634.1), *Plantactinospora* sp. CNZ321 (WP_130461760.1), *Rhizobiaceae bacterium* CPCC 101076 (WP_114958078.1); fungal laccases: *Trametes versicolor* FP-101664 SS1 (XP_008035966.1), *Dichomitus squalens* LYAD-421 SS1 (XP_007369227.1), *Fomitiporia mediterranea* MF3/22 (XP_007269745.1), *Dichomitus squalens* LYAD-421 SS1 (XP_007367294.1), *Fomitiporia mediterranea* MF3/22 (XP_007262173.1), *Heterobasidion irregulare* TC 32-1 (XP_009552516.1), *Gloeophyllum trabeum* ATCC 11539 (XP_007868909.1). Arrows and accompanying numbers indicate the amino acid residues for Cu-binding and the type of each ligand Cu. The histidine in copper-binding residues was marked on gray. The position of the first residue of each fragment in the polypeptide chain is indicated.

The sequences Lac3D6, Lac3D13 and Lac3D17 showed similarity to the multicopper oxidase domain belonging to the PRK10965 superfamily (cl35978). The domain of the cupredoxin superfamily domain (Cl19115) was identified for the sequence Lac3D33, while the domain of the copper_ res_A superfamily (cl36914) was found in the sequence Lac3D12.

However, searching for protein domains using the CD-Search tools against the Pfam database allowed the identification of the cupredoxin superfamily domain (cl19115) for all sequences analysed. All analysed sequences, with the exception of Lac3D33, showed significant similarity to Cu-oxidase_2 (pfam07731). The Cu oxidase (pfam00394) domain was identified in four sequences (Lac3D5, Lac3D8, Lac3D10 and Lac3D 11). The three domains Cu-oxidase_2 (PF07731), Cu oxidase (PF00394) and Cu-oxidase_3 (PF07732) were identified by the software SMART in all sequences except sequences in which two domains were found (Lac3D 33 – domains PF00394 and PF07732; Lac3D13, Lac3D17 – domains PF07731, PF07732). Regardless of the method used to identify the domains, domains/motifs typical of MCOs were detected in each of the sequences analysed. Alignment of the amino acid sequences using MEGA7 software revealed that the Lac12 sequences did not contain a particular motif characteristic of the copper-binding site typical of laccase, and therefore this sequence was excluded from further analysis. The alignment of the remaining amino acid sequences made it possible to distinguish two copper-binding sites (Cu-binding region; cbr II, cbrIII) characteristic of laccase enzymes. These copper-binding regions are involved in the formation of the active enzyme centre, which contains four copper atoms divided into types 1-3. These three types are partially present in the sequences alignment (Figure 1 Type 1-1 his, type 2 and 3-4 his). The copper-binding region designated cbr III (Figure 1) is typical for laccase *sensu stricto* from fungi and was detected in 14 analysed sequences. The sequences derived from the Polish soil metagenome can therefore be classified as coding laccase genes or rather as laccase-like MCOs, according to the classification of HOEGGER et al. (2006), which is based on research into the diversity of bacterial laccase-like MCO genes.

The results of a domain analysis confirmed the usefulness of the selected tools such as CD-Search and SMART for identifying LMCO domains of genes isolated from a metagenome. CD-Search uses BLAST for a quick scan of a set of precalculated position-specific scoring matrices (PSSMs) with a protein query, and although the BLAST database search method is still the most widely used (GUPTA et al. 2017), it provides less specific results. In turn, the software SMART, which implements methods using probabilistic models called Profile Hidden Markov Models (pHMMs)

and was used in this study together with a Pfam profile database, has been shown to be more specific for identifying Cu-binding regions. Although the pHMMs model of AUSEC et al. (2011a) has been successfully used for searching databases and identifying laccase genes, it appears to be of little use for the analysis of bacterial LMCOs from metagenome data. The pHMMs-based search of Global Ocean Survey data yielded numerous hits for prokaryotic laccases that closely matched the copper-binding regions of the models (277 and 847 sequences showed similarities to laccase-like sequences with two and three domains, respectively), but only 33% of the putative laccases with three domains could be assigned to bacteria (AUSEC et al. 2011a).

Sequence analysis of LMCO from the Polish soil metagenome

BLASTn analysis of the Polish soil metagenome sequences showed that only five sequences in the database had significant similarities, i.e. Lac3D6 and Lac3D13 showed identity with *Rhodococcus* spp. (99% identity), Lac3D12 with *Maricaulis maris* (71% identity), while Lac3D23 and Lac3D18 showed identity with uncultured bacteria (76% identity). To see how limited the database is in this area of knowledge, note that the Entrez database has 2.5 billion records at the time of writing (SAYERS et al. 2019). A BLASTx search for protein sequences made it possible to characterise the tested sequences in more detail and to qualify them as proteins belonging to multicopper oxidase or copper oxidase from cultured and uncultured bacteria. The two sequences were associated with the gene encoding multicopper oxidase from *Rhodococcus* ssp. (*Actinobacteria*) (Lac3D6 – 100%, Lac3D13 – 99%), which is consistent with the results of BLASTN. This species is present in the soil, which was confirmed in the research carried out by AUSEC et al. (2011a), and the LMCOs from the genus *Rhodococcus* show interesting properties. For example, the actinomycete *Rhodococcus ruber* (designated C208; EC1.10.3.2.) was found to be able to recycle and degrade polyethylene at a high temperature (70°C), allowing recycling in view of the growing waste problem (SANTO et al. 2013).

The five sequences from the soil metagenome (Lac3D32, Lac3D15, Lac3D17, Lac3D18, Lac3D23) were associated with the gene encoding multicopper oxidase from *Oleomonas* sp., the bacterium *Gemmatimonadetes, Plantactinospora* sp. and the bacterium and uncultured bacterium clone, with a positive amino acid hit rate of over 58%. Six sequences were affiliated to a multicopper oxidase from *Sorangium cellulosum* with an amino acid hit rate below 40% in a BLASTX and BLASTp search. AUSEC et al. (2011a) came to similar conclusions. They found seven sequences

showing similarity to a multicopper oxidase from *Sorangium cellulosum*, but with a positive amino acid hit rate of about 60%. Bioinformatic analysis conducted in 2011 (AUSEC et al. 2011b) revealed the high diversity of bacterial genes for laccase-like enzymes and found that *Sorangium cellulosum* So ce 56 has eight genes in its chromosomes, with a two-domain laccase in each genome, and that the other enzymes are the three-domain laccases. Similarly, as mentioned earlier, two sequences of LMCO genes were obtained from *Rhodococcus* ssp., as mentioned earlier, which could also be related to the presence of multiple genes in this bacterial genus, as, for example, seven genes were detected in *Rhodococcus erythropolis* and five genes in *Rhodococcus opacus* and *Rhodococcus jostii* (AUSEC et al. 2011b).

Multiple laccase genes have been confirmed to be found in many organisms, including bacteria, fungi and plants, and this phenomenon is thought to be related to biological function (JANUSZ et al. 2020). For example, in the filamentous ascomycete *Gaeumannomyces graminis var. tritici*, three laccase genes (LAC1, LAC2 and LAC3) are able to oxidise or reduce polyphenolic compounds such as lignin or melanin precursors, depending on the redox potential of the environment (LITVINTSEVA and HENSON 2002). In addition, a multifunctional laccase gene family in cotton (*Gossypium* spp.) has been shown to play an important role in cotton fibre development (BALASUBRAMANIAN et al. 2016). However, it has been suggested that bacterial laccase-like genes are more involved in laccase activity than their fungal counterparts (CHAUHAN et al. 2017). The studies on multiple homologous laccase-encoding genes of *Achromobacter xylosoxidans* HWN16, *Citrobacter freundii* LLJ 16 and *Pleurotus nebrodensis* have shown that these genes might be responsible for the properties of laccases, such as optimal temperature, pH, thermal stability, pH stability or increased enzymatic activity under the influence of certain concentrations of fluoride (YUAN et al. 2016,UNUOFIN et al. 2019). However, the exact function of all bacterial laccase genes discovered so far has not yet been elucidated, as this requires time-consuming studies using an analysis of the overexpressed target genes, as was the case for the *Cot*A laccases from *Bacillus subtilis* or the recently characterised novel laccase gene (Lcc1) from *Ganoderma tsugae* (JIN et al. 2018). Nevertheless, LMCOs genes from multiplate bacteria have previously been linked to pigment synthesis, oxidation of phenolic compounds, sporulation, UV and H2O2 resistance, Cu^{2+} resistance and morphogenesis (CHAUHAN et al. 2017).

In summary, the sequence analysis of the LMCO genes suggests that most of the metagenome-derived partial genes belonged to unknown bacterial species. And several of the LMCO sequences isolated from

Polish soil metagenome, bog and fen soils (AUSEC et al. 2011b) showed similarity to the LMCO gene of *Sorangium cellulosum*. This could be evidence that these LMCO genes belong to a single bacterial group that is strongly associated with the lignin degradation process regardless of the environment.

Phylogenetic analysis of LMCO sequences from the Polish soil metagenome

To identify and assess the biodiversity of LMCO genes in environmental samples, several primers based on copper-binding regions (cbr) conserved in both bacterial and fungal LMCO have been designed (CHAUHAN et al. 2017). The oligonucleotide primer Cu2R was first designed for the detection of cbr II from basidiomycete LMCOs (LUIS et al. 2004). The first primer Cu1AF aligns to the sequence encoding two histidines of cbr that have enabled the detection of bacterial LMCOs and was designed by KELLNER et al. (2008). These primers proved useful in the detection of LMCO genes from the DNA of various bacterial taxa by amplifying a fragment of 140 bp (KELLNER et al. 2008). In contrast, the CuR4 primer design within the cbr IV region of LMCO sequences with three domains demonstrated the richness of diversity of LMCOs with 2 and 3 domains in soils. And the combination of Cu1AF and CuR4 primers with fractionated fragment sequencing allowed the detection of LMCOs with a sequence length of 1200 bp, which is almost the total length of the LMCO gene (AUSEC et al. 2011a). This strategy was used to obtain LMCO genes for the phylogenetic analysis presented in this article. However, the diversity of the LMCO amino acid sequence proved to be rather large to allow a logical alignment. This problem was also noted by other authors, and HOEGGER et al. (2006) performed the alignment of 350 multicopper oxidases (MCOs) from fungi, insects, plants and bacteria, but the sequences were trimmed to the most conserved parts of the sequences. However, this restriction also reduced the resolution of the phylogenetic analysis. In this work, the studies focused exclusively on bacterial LMCOs, and the sequences were trimmed to include two Cu-binding regions (cbr II and cbr III), but the intervening sequence was retained, which is a novelty compared to the method used by other authors.

For the preliminary phylogenetic tree reconstruction, all fifteen sequences of LMCO from the Polish soil metagenome and reference sequences from the NCBI database were used. The Bayesian phylogenetic method was chosen to distinguish the LMCO sequences isolated from soil samples.

Fig. 2. Phylogenetic tree obtained by Bayesian inference analysis of 110 LMCOs sequences from Polish soil metagenome and sequences from GenBank in NCBI, within two Cu-binding regions (cbr II and cbr III). Node numbers represent Bayesian posterior probability values

This statistical method is closely related to the Maximum Likelihood method, but could be a faster method to assess support for trees than Maximum Likelihood bootstrapping. However, in the preliminary study, it was not possible to obtain a phylogenetic tree with a good Bayesian posterior probability value (data not shown). To circumvent this problem, the Lac3D12 sequence with its similar reference sequences was excluded for further analysis, so that this sequence does not belong to the true laccase gene. Finally, a phylogenetic analysis of fourteen sequences from the Polish soil metagenome classified as laccase coding genes was performed, together with ninety-six reference sequences obtained from GenBank in NCBI. Most of the sequences from the Polish soil sample were affiliated to a specific cluster with a good Bayesian posterior probability value (> 0.9) – Figure 2.

The affiliation to *Actinobacteria* (NCBI:txid1760) was determined for three sequences, namely Lac3D6, Lac3D13 and Lac3D17. In turn, the sequences Lac3D25 and Lac3D32 were assigned to two different clades belonging to the *Alphaproteobacteria* cluster (NCBI:txid28211). Most sequences were identified as belonging to the *Deltaproteobacteria* cluster (NCBI:txid28221), but Lac3D25 and the six following sequences – Lac3D5, Lac3D10, Lac3D14, Lac3D8, Lac3D30, Lac3D11 – were grouped in different clades. Interestingly, these six sequences of LMCOs that showed homology to *Sorangium cellulosum* in the BLAST search were assigned to the *Deltaproteobacteria* cluster (NCBI:txid28221) but were not merged in the phylogenetic analysis (Figure 2). The sequence Lac3D23 showed the highest similarity to the unclassified *Acidobacteria* (NCBI:txid1978231) when the sequences BLAST were aligned against the non-redundant database.

Phylogenetic analysis of LMCO sequences from three soil metagenomes

Analysis of the 16S rRNA gene is commonly used to assess species diversity, but the recent study using a concatenated protein phylogeny has shown that this approach is not unreliable (PARKS et al. 2018). Therefore, phylogenetic tree reconstruction based on the alignment of the LMCO amino acid of the soil metagenome was applied in this study. This approach resulted in a classification of the LMCO sequences of the soil metagenome into individual bacterial classes and revealed some trends in the bacterial diversity of soils, which are familiar with the results of other authors (WANG et al. 2016a, YUN et al. 2016).

In the preliminary study, the fifteen LMCOs sequences from the Polish soil metagenome and seventy-two LMCOs sequences isolated by AUSEC et al. (2011a) and similar sequences from the GenBank database were

used for the phylogenetic analysis. The Maximum likelihood methods was used for phylogenetic reconstruction. Likelihood-based methods aim to find the best topology by maximising the likelihood function with respect to the topology and branch lengths by comparing the posterior probabilities for the different possible topologies (SVENNBLAD et al. 2006). However, when aligning all LMCO sequences, two groups of LMCO sequences were distinguished, one of which belonged to the "true" laccases with 3 domains and was used for further analysis (data not shown).

The phylogenetic tree was reconstructed for 132 LMCOs from the Polish soil metagenome, for 3-domain LMCOs from bog and fen soil (AUSEC et al. 2011a) and for sequences from the GenBank database in NCBI obtained by a BLAST search against reference protein sequences (Figure 3). Cluster membership was confirmed for most sequences from the Polish soil metagenome when they were compared with the phylogenetic tree reconstructed by Bayesian inference analysis. Only for the sequence Lac3D23 were the results different, and this sequence showed the highest similarity to the assigned cluster *Deltaproteobacteria* (NCBI:txid28221). The difference in sequence affiliation of Lac3D23 was likely due to the sequence having the highest similarity (amino acid hit rate 71%) to multicopper oxidase from unclassified *Acidobacteria* (RPI54963.1), which was newly identified and not included in the reference protein sequence database. This false affiliation was not found for other sequences. Therefore, all sequences derived from the Polish soil metagenome, as well as those obtained by AUSEC et al. (2011a), were checked against a non-redundant database by a BLAST search. In addition, studies on different bacteria with lignin-degrading potentials isolated from two cabbage species showed that LMCOs from both *Proteobacteria* (NCBI:txid1224) and *Actinobacteria* (NCBI:txid1760) had high similarities (85.00-100.00%), suggesting that these genes might be from the same ancestor (WANG et al. 2016a). Therefore, it is difficult to clearly determine the affiliation of this sequence.

Among the LMCO sequences obtained by AUSEC et al. (2011a), a large cluster of 17 sequences, for which no references were identified, was also listed and linked to the sequences of laccases from various *Proteobacteria* (NCBI:txid1224) using BLASTx (Figure 3). The phylogenetic analysis presented in this paper made it possible to match a part of the sequence to be assigned at the class level, and two clusters of *Alphaproteobacteria* (NCBI:txid28211) (I, II) and *Deltaproteobacteria* (NCBI:txid28221) (I, III) and one cluster of *Gammaproteobacteria* (NCBI:txid1236) (I) were highlighted (Figure 3). Similar to the study by AUSEC et al. (2011a), a large cluster of sequences was indicated to have similarity to *Acidobacteria* (NCBI:txid57723) LMCO (Figure 2). Four sequences were assigned to the

Fig. 3. Unrooted phylogenetic tree of the 132 amino acid sequences of LMCOs obtained from Polish forest soil metagenome, bog and fen metagenome (AUSEC et al. 2011a) and sequences from GenBank in NCBI. Tree was bootstrapped using 1000 replications

Bacteroidetes (NCBI:txid976) cluster, which contradicts the results of AUSEC et al. (2011a). Three sequences of LMOC resembled the laccase-like genes of *Actinobacteria* (NCBI:txid201174), although they were previously affiliated to *Verrucomicrobia* (NCBI:txid74201) (Figure 3) (AUSEC et al. 2011a). Of all sequences identified as LMCO, 7.57% were not assigned to any bacterial phylum, as confirmed by BLASTx and BLASTp analysis, and the sequences had amino acid similarity below 40%. The differences in the

phylogenetic analysis result from the fact that the GenBank NCBI database has been enriched in recent years, especially with the sequences belonging to the uncultured bacterium, which is due to the metagenomics studies (FERRER et al. 2010). A new approach using a concatenated protein phylogeny as the basis for a bacterial taxonomy that conservatively removes polyphyletic groups and normalises taxonomic ranks based on relative evolutionary divergence has resulted in changes to the Genome Taxonomy Database, i.e. 58% of the 94,759 genomes have had changes made to the existing taxonomy. These changes have not omitted the phylum *Proteobacteria* (NCBI:txid1224), within which the subdivision into the subphylum delta/epsilon has been formed, containing the class *Deltaproteobacteria* (NCBI:txid28221). There were also changes within the FCB group, *Bacteroidetes* (NCBI:txid976) and *Actinobacteria* (NCBI:txid1760) (PARKS et al. 2018).

Interestingly, several LMCO sequences from Polish bog and fen metagenomes were assigned to the same clusters of *Deltaproteobacteria* (NCBI:txid28221) and *Alphaproteobacteria* (NCBI:txid28211), which might indicate the particular role of these bacteria in the soil environment and in the bioremediation process. This hypothesis was confirmed by the research carried out by WILHELM et al. (2019). The authors use metagenomic and quantitative stable isotope probes to identify and characterise the functional properties of lignin, cellulose and hemicellulose degrading fungi and bacteria in coniferous forest soils. Overall, high bacterial degradation of the model lignin substrate was found, particularly by gram-negative bacteria from the *Alphaproteobacteria* (NCBI:txid28211) and *Deltaproteobacteria* (NCBI:txid28221) groups. The existing evidence for bacterial lignin degradation by alpha and gamma-proteobacteria shows that it is necessary to characterise bacterial populations and determine their role in different soil environments in order to better understand the processes controlling the degradation of lignocellulose in situ.

Knowledge of the potential impact on soil bacterial diversity is also desirable so that the bacteria and their metabolites desired by industry can be identified in the future. A recent study highlighted that possible climate and land use changes (soil type, carbon source, precipitation patterns) affect the abundance and diversity of soil bacteria (BICKEL and OR 2020). A detailed study demonstrated the dominance of individual bacterial classes in relation to pH and soil type in karst areas, with surface soils dominated by *Acidobacteria* (NCBI:txid57723), *Verrucomicrobia* (NCBI:txid74201) and *Planctomycetes* (NCBI:txid203682). In turn, *Nitrospirae* (NCBI:txid40117), *Gemmatimonadetes* (NCBI:txid142182), *Firmicutes* (NCBI:txid1239) and *Chloroflexi* (NCBI:txid200795) occurred only in

cave sediments, while *Actinobacteria* (NCBI:txid1760) and *Proteobacteria* (NCBI:txid1224) dominated in weathered rock and drip water, respectively (YUN et al., 2016). Metagenomic analysis of 16S rRNA genes revealed that seven groups, including *Alphaproteobacteria* (NCBI:txid28211), *Gammaproteobacteria* (NCBI:txid1236), *Deltaproteobacteria* (NCBI:txid28221), *Betaproteobacteria* (NCBI:txid28216), *Acidobacteria* (NCBI:txid57723), *Actinobacteria* (NCBI:txid1760) and *Bacteroidetes* (NCBI:txid976) from the sediment of the polymetallic nodule fields dominated and consisted mainly of unclassified, uncultured bacteria (WANG et al. 2010).

It is also interesting that the LMCO sequences showing similarity to the laccase of *Bacteroidetes* were assigned to two different clusters, but too few sequences were analysed to draw further conclusions.

In this study, the universal primers Cu1AF and CuR4 were used to identify LMCO genes, but the entire biodiversity of LMCO genes could not be captured. This was described by FERNANDES et al. (2014), where primers specific for LMCO from actinomycetes belonging to LccED superfamilies I and K were designed and amplified LMCO fragments from isolates not recognised by the above primers, and where the fragments obtained were correctly assigned to the predicted superfamily. Another primer pair was designed by WANG et al. (2016a). This allowed for the first time the amplification of the LMCO gene from several genera, including *Massila*, which was detected in high expression using real-time PCR (qRT-PCR), confirming coal as a good seed bank. A new specific PCR primer pair targeting the two conserved copper-binding regions of the LMCO two-domain of *Streptomyces* was also developed. Most of the sequence clusters obtained in eight distinct clades are homologous with *Streptomyces* two-domain LMCO genes, but the sequences of clades III and VIII do not match any reference sequence of known *Streptomyces* (LU et al. 2014).

The studies described above and presented in this article have shown how limited the GenBank database is, especially for the taxa of the *Deltaproteobacteria*. For example, the sequence of LMCO from *Sporangium cellulosum* (*Deltaproteobacteria*) was most frequently identified as similar to the metagenome-derived LMCO sequences identified by various authors (AUSEC et al. 2011a; WANG et al. 2010, WANG et al. 2016a).

Soil metagenomics studies, which involve the isolation of soil DNA and the preparation and screening of clone libraries, can provide a cultivation-independent assessment of the largely untapped genetic reservoir of soil microbial communities. This approach has already led to the identification of new laccases (CHAUHAN et al. 2017, BERINI et al. 2018, KUMAR et al. 2018).

Conclusions

In this study, a total of 228 3-domain laccase-like multicopper oxidase LMCO sequences, including 66 sequences from the metagenome, were used for phylogenetic analysis. A BLAST analysis and phylogenetic tree reconstruction allowed distinguishing several LMCO sequence clusters belonging to different bacterial classes, such as *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and a cluster of unclassified sequences. The results of the phylogenetic analysis also suggest that bacteria from the class of *Deltaproteobacteria* may play a significant role in the lignin degradation process. Trimming sequences to obtain two Cu-binding regions (cbr II and cbr III) and an intervening nucleotide sequence has been shown to be a good method for phylogenetic analysis of metagenome-derived LMCO sequences. However, since the limited information in the database could lead to false affiliations, extensive research using "omic" techniques should be conducted for a more comprehensive characterisation of bacterial laccase in different soil types.

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References

Ausec L., Berini F., Casciello C., Cretoiu M.S., Van Elsas J.D., Marinelli F., Mandić-Mulec I. 2017. *The first Acidobacterial laccase-like multicopper oxidase revealed by metagenomics shows high salt and thermo-tolerance*. Appl. Microbiol. Biotechnol., 101(15): 6261–6276.

Ausec L., Dirk Van Elsas J., Mandic-Mulec I. 2011a. *Two- and three-domain bacterial laccase-like genes are present in drained peat soils*. Soil Biol. Biochem., 43(5): 975–983.

Ausec L., Zakrzewski M., Goesmann A., Schlüter A., Mandic-Mulec I. 2011b. *bioinformatic analysis reveals high diversity of bacterial genes for laccase-like enzymes*. PLoS ONE, 6(10): e25724.

Balasubramanian V.K., Rai K. M., Thu S. W., Hii M.M., Mendu V. 2016. *Genome-wide identification of multifunctional laccase gene family in cotton (Gossypium* spp.*); expression and biochemical analysis during fiber development*. Sci Rep., 29(6): 1–16.

Berini F., Casciello C., Marcone G.L., Marinelli F. 2017. *Metagenomics: novel enzymes from non-culturable microbes*. FEMS Microbiol. Lett., 364(21): 1–19.

Berini F., Luka M.V., Ausec L., Rosini E., Tonin F., Pollegioni P., Mandić-Mulec I. 2018. *Isolation and characterization of a heterologously expressed bacterial laccase from the anaerobe Geobacter metallireducens*. Appl. Microbiol. Biotechnol., 102(5): 2425–2439.

Bickel S., Or D. 2020. *Soil bacterial diversity mediated by microscale aqueous-phase processes across biomes*. Nat. Commun., 116(1): 1–9.

Brander S., Mikkelsen J.D., Kepp K.P. 2014. *Characterization of an alkali- and halide-resistant laccase expressed in E. coli: cota from Bacillus clausii*. PloS One, 9(6): 99402–99412.

Cañas A.I., Camarero S. 2010. *Laccases and their natural mediators: biotechnological tools for sustainable eco-friendly processes*. Biotechnol Adv., 28(6): 694–705.

Chauhan P.S., Goradia B., Saxena A. 2017. *Bacterial laccase: recent update on production, properties and industrial applications*. 3 Biotech., 7(5): 323–343.

Edgar R.C. 2004. *MUSCLE: multiple sequence alignment with high accuracy and high throughput*. Nucleic Acids Research., 32(5): 1792–1797.

El-Gebali S., Mistry J., Bateman A., Eddy S.R., Luciani A., Potter S.C., Qureshi M., Richardson L.J., Salazar G.A., Smart A., Sonnhammer E.L.L., Hirsh L., Paladin L., Piovesan D., Tosatto S.C.E., Finn R.D. 2019. *The pfam protein families database in 2019*. Nucleic Acids Res., 8(47): 427–432.

Fernandes T., Silveira W., Passos F., Zucchi T. 2014. *Laccases from Actinobacteria – what we have and what to expect*. Adv. Microbiol., 4(4): 285–296.

Ferrer M., Beloqui A., Golyshin P.N. 2010. *Screening metagenomic libraries for laccase activities*. In: *Metagenomics. Methods in Molecular Biology (Methods and Protocols)*. Eds. W. Streit, R. Daniel, vol 668. Humana Press, Totowa, New Jork.

Floch C., Alarcon-Gutiérrez E., Criquet S. 2007. *ABTS assay of phenol oxidase activity in soil*. J. Microbiol. Methods, 71: 319–324.

Giardina P., Faraco V., Pezzella C., Piscitelli A., Vanhulle S., Sannia G. 2010. *Laccases: a never-ending story*. Cell. Mol. Life Sci., 67 (3): 369–385.

Givaudan A., Effosse A., Faure D., Potier P., Bouillant M.L., Bally R. 1993. *Polyphenol oxidase in azospirillum lipoferum isolated from rice rhizosphere:evidence for laccase activity in non-motile strains of Azospirillum lipoferum*. FEMS Microbiol. Lett., 108(2): 205–210.

Gupta V., Gupta N., Capalash N., Sharma P. 2017. *Bio-prospecting bacterial diversity of hot springs in northern himalayan region of India for laccases*. Indian J. Microbiol., 57(3): 285–291.

Handelsman J. 2004. *Metagenomics: application of genomics to uncultured microorganisms*. Microbiol. Mol. Biol. Rev., 68(4): 669–685.

Hoegger P.J., Kilaru S., James T.Y., Thacker J.R., Kües U. 2006. *Phylogenetic comparison and classification of laccase and related multi-copper oxidase protein sequences*. FEBS Journal, 273(10): 2308–2326.

Ihssen J., Reiss R., Luchsinger R., Thöny-Meyer L., Richter M. 2015. *Biochemical properties and yields of diverse bacterial laccase-like multicopper oxidases expressed in Escherichia coli*. Sci. Rep., 5: 1–13.

Janusz G., Pawlik A., Świderska-Burek U., Polak J., Sulej J., Jarosz-Wilkołazka A., Paszczyński A. 2020. *Laccase properties, physiological functions, and evolution*. Int. J. Mol. Sci., 21(3): 966–991.

Jin W., Li J., Feng H., You S., Zhang L., Norvienyeku J., Hu K., Sun S., Wang Z. 2018. *Importance of a laccase gene (lcc1) in the development of Ganoderma tsugae*. Int. J. Mol. Sci., 19(2): 471–487.

Kaiser K., Wemheuer B., Korolkow V., Wemheuer F., Nacke H., Schöning I., Schrumpf M., Daniel R. 2016. *Driving forces of soil bacterial community structure, diversity, and function in temperate grasslands and forests*. Sci. Rep., 6(1): 1–12.

Kearse M., Moir R., Wilson A., Stones-Havas S., Cheung M., Sturrock S., Buxton S., Cooper A., Markowitz S., Duran C. 2012. *Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data*. Bioinformatics, 28(12): 1647–1649.

Kellner H., Luisc P., Zimdarsa B., Kieseld B., Buscot F. 2008. *Diversity of bacterial laccase-like multicopper oxidase genes in forest and grassland Cambisol soil samples*. Soil Biol. Biochem., 40(3): 638–648.

Kimura N., Kamagata Y. 2016. *A thermostable bilirubin-oxidizing enzyme from activated sludge isolated by a metagenomic approach*. Microbes Environ., 31(4): 435–441.

Kumar S., Stecher G., Tamura K. 2016. *Mega7: molecular evolutionary genetics analysis version 7.0 for bigger datasets*. Mol. Biol. Evol., 33(7): 1870–1874.

KUMAR M., VERMA S., GAZARA R.K., KUMAR M., PANDEY A., VERMA P.K., THAKUR I.S. 2018. *Genomic and proteomic analysis of lignin degrading and polyhydroxyalkanoate accumulating β-proteobacterium Pandoraea sp. ISTKB*. Biotechnol. Biofuels, 11(154): 1–23.

LE S.Q., GASCUEL O. 2008. *An Improved General Amino Acid Replacement Matrix*. Mol. Biol. Evol, 25(7): 1307–1320.

LETUNIC I., BORK P. 2018. *20 years of the SMART protein domain annotation resource*. Nucleic Acids Res., 46(4): 493–496.

LITVINTSEVA A.P., HENSON J.M. 2002. *Cloning, characterization, and transcription of three laccase genes from Gaeumannomyces graminis var. tritici. the take-all fungus*. Appl. Environ. Microbiol., 68(3): 1305–1311.

LUIS P., WALTHER G., KELLNER H., MARTIN F., BUSCOT F. 2004. *Diversity of laccase genes from basidiomycetes in a forest soil*. Soil Biol. Biochem., 36 (7): 1025–1036.

LU L., ZENG G., FAN G., ZHANG J., CHEN A., CHEN M., JIANG M., YUAN Y., WU H., LAI, M., HEA Y. 2014. *Diversity of two-domain laccase-like multicopper oxidase genes in Streptomyces* spp.*: identification of genes potentially involved in extracellular activities and lignocellulose degradation during composting of agricultural waste*. Appl. Environ. Microbiol., 80(11): 3305–3314.

MARCHLER-BAUER A., BO Y., HAN L., HE J., LANCZYCKI C.J., LU S., CHITSAZ F., DERBYSHIRE M.K., GEER R.C., GONZALES N.R., GWADZ M., HURWITZ D.I., LU F., MARCHLER G.H., SONG J.S., THANKI N., WANG Z., YAMASHITA R.A., ZHANG D., ZHENG C., GEER L.Y., BRYANT S.H. 2017. *Cdd / Sparcle: functional classification of proteins via subfamily domain architectures*. Nucleic Acids Res., 4(45): 200–203.

MATHEWS S.L., SMITHSON C.E., GRUNDEN A.M. 2016. *Purification and characterization of a recombinant laccase-like multi-copper oxidase from Paenibacillus glucanolyticus SLM1*. J. Appl. Microbiol., 121(5): 1335–1345.

PARKS D.H., CHUVOCHINA M., WAITE D. W., RINKE C., SKARSHEWSKI A., CHAUMEIL P.A., HUGENHOLTZ P. 2018. *A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life*. Nat. Biotechnol., 36(10): 996–1004.

REZAEI S., SHAHVERDI A.R., FARAMARZI M.A. 2017. *Isolation, one-step affinity purification, and characterization of a polyextremotolerant laccase from the halophilic bacterium aquisalibacillus elongatus and its application in the delignification of sugar beet pulp*. Bioresour. Technol., 230(1): 67–75.

RONQUIST F., HUELSENBECK J.P. 2003. *MrBayes 3: Bayesian phylogenetic inference under mixed models*. Bioinformatics. 19(12): 1572–1574.

SANTO M., WEITSMAN R., SIVAN A. 2013. *The role of the copper-binding enzyme-laccase-in the biodegradation of polyethylene by the actinomycete Rhodococcus ruber*. Int. Biodeterior. Biodegrad., 84: 204–210.

SAYERS E.W., AGARWALA R., BOLTON E.E., BRISTER J.R., CANESE K., CLARK K., CONNOR R., FIORINI N., FUNK K., HEFFERON T., HOLMES J.B., KIM S., KIMCHI A., KITTS P.A., LATHROP S., LU Z., MADDEN T.L., MARCHLER-BAUER A., PHAN L., SCHNEIDER V.A., SCHOCH C.L., PRUITT K.D., OSTELL J. 2019. *Database resources of the National Center for Biotechnology Information*. Nucleic Acids Res., 8(47): 23–28.

SHARMA B., DANGI A.K., SHUKLA P. 2018. *Contemporary enzyme based technologies for bioremediation: A Review*. J. Environ. Manage., 210(1): 10–22.

SHRADDHA A., SHEKHER R., SEHGAL S., KAMTHANIA M., KUMAR A. 2011. *Laccase: microbial sources, production, purification, and potential biotechnological applications*. Enzyme Res., 4(4): 217–861.

SIROOSI M., AMOOZEGAR M. A., KHAJEH K. 2016. *Purification and characterization of an alkaline chloride-tolerant laccase from a halotolerant bacterium, Bacillus* sp. *strain WT*. J. Mol. Catal. B Enzym., 134(1): 89–97.

SUN J., ZHENG M., LU Z., LU F., ZHANG C. 2017. *Heterologous production of a temperature and ph-stable laccase from bacillus vallismortis fmb-103 in Escherichia coli and its application*. Process Biochem., 55(1): 77–84.

Svennblad B., Erixon P., Oxelman B., Britton T. 2006. *Fundamental differences between the methods of maximum likelihood and maximum posterior Probability in phylogenetics*. Systematic Biology, 55(1): 116–121.

Unuofin J.O., Moubasher H.A., Okoh A.I., Nwodoa U.U.2019. *Production of polyextremotolerant laccase by Achromobacter xylosoxidans HWN16 and Citrobacter freundii LLJ16*. Biotechnol Rep (Amst)., 22(1): 1–9.

Viswanath B., Rajesh B., Janardhan A., Kumar A.P., Narasimha G. 2014. *Fungal laccases and their applications in bioremediation*. Enzyme Res., 8(1): 1–21.

Wang C.S., Liao L., Xu H.X., Xu X.W., Wu M., Zhu L.Z. 2010. *Bacterial diversity in the sediment from polymetallic nodule fields of the Clarion-Clipperton Fracture Zone*. J. Microbiol., 48(5): 573–585.

Wang L., Nie Y., Tang Y.Q., Song X.M., Cao K., Sun L.Z., Wang Z.J., Wu Z.L. 2016a. *Diverse bacteria with lignin degrading potentials isolated from two ranks of coal*. Front Microbiol., 7(1): 1–14.

Wang T.N., Zhao M. 2016b. *A simple strategy for extracellular production of CotA laccase in Escherichia coli and decolorization of simulated textile effluent by recombinant laccase*. Appl. Microbiol. Biotechnol., 101(2): 685–696.

Wilhelm R.C., Singh R., Eltis L.D., Mohn W.W. 2019. *Bacterial contributions to delignification and lignocellulose degradation in forest soils with metagenomic and quantitative stable isotope probing*. ISME Journal, 13(2): 413–429.

Yang Q., Zhang M., Zhang M., Wang C., Liu Y., Fan X., Li H. 2018. *Characterization of a novel, cold-adapted, and thermostable laccase-like enzyme with hightolerance for organic solvents and salt and potent dye decolorization ability, derived from a marine metagenomic library*. Front. Microbiol., 5(9): 1–9.

Yoshida H. 1883. *Chemistry of lacquer (Urushi) Part 1*. J. Chem. Soc., 43(1): 472–486.

Yuan X., Tian G., Zhao Y., Zhao L., Wang H., Ng T.B. 2016. *Biochemical characteristics of three laccase isoforms from the basidiomycete pleurotus nebrodensis*. Molecules., 21(2): 203–218.

Yue, Q., Yang, Y., Zhao, J., Zhang L., Xu L., Chu, X., Liu X., Tian J., Wu N. 2017. *Identification of bacterial laccase cueO mutation from the metagenome of chemical plant sludge*. Bioresour. Bioprocess., 4(48): 1–8.

Yun Y., Wang H., Man B., Xiang X., Zhou J., Qiu X., Duan Y., Engel A.S. 2016. *The relationship between pH and bacterial communities in a single karst ecosystem and its implication for soil acidification*. Front Microbiol., 7(1): 1–16.

Zerva A., Simić S., Topakas E., Nikodinovic-Runic J. 2020. *Applications of microbial laccases: patent review of the past decade (2009–2019)*. Catalysts, 9(12): 1023–1048.

Zielińska M., Adamczak M. 2013. *Zastosowanie metody DOP-PCR do identyfikacji genów lakaz – oksydoreduktaz. In: Na pograniczu chemii i biochemii*. Eds. H. Korniak, J. Barciszewski. Wydawnictwo Naukowe Uniwersytetu im. Adama Mickiewicza, Poznań, pp. 309–317.

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EFFECTS OF FEEDING FREQUENCY ON SILVER RASBORA (*RASBORA ARGYROTAENIA*) FRY GROWTH

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Key words: aquaculture, domestication, fish feeding, basic breeding parameters, production.

Abstract

As a newly domesticated fish species, silver rasbora (*Rasbora argyrotaenia*) farming needs more basic knowledge about culture procedures. Feeding management is basic crucial information in the farming system of many species. This study purposes to evaluate the growth and feed efficiency of silver rasbora fry reared under different feeding frequencies. The feeding treatments of fish were divided into 4 different feeding frequency treatments (1, 2, 3, and 4 times daily), each treatment consisted of 5 replicates with 20 fish. Fish were fed ad satiation. The effects of feeding frequency on growth were determined by calculating the parameters namely body weight gain, biomass gain, specific growth rate, and feed conversion ratio. Based on the results, different feeding frequency has a significant effect ($P < 0.05$) on all parameters. The growth was higher with increasing feeding frequency; the highest growth was occurred on the most frequently feeding. The best growth was observed in 4 times daily feeding (final body weight: 0.45 ± 0.01 g/fish, bodyweight gain: 0.19 ± 0.00 g/fish, biomass gain: 5.58 ± 0.09 g, specific growth rate: 1.34 ± 0.06 %/day); meanwhile, feed conversion ratio decrease and showed the lowest value on 4 times daily feeding frequency treatment (1.56 ± 0.04). Referring to the growth parameters and the lowest feed conversion ratio was obtained for fish fed 4 times daily. It can be concluded that feeding 4 times daily as optimum feeding frequency in the present study resulted in better growth and feed conversion ratio during the rearing of silver rasbora.

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Introduction

Silver rasbora (*Rasbora argyrotaenia*); belong to the family Danionidae (Cypriniformes order) with maximum length 12 cm; naturally distributed in Philippines, Malaysia, Cambodia, Thailand, and Indonesia (ARYANI 2015, KUSUMA et al. 2017, CAPULI and BAILLY 2022). As newly domesticated species in Indonesia, silver rasbora has economic values for consumption and ornamental fish (ADAWIYAH et al. 2019, HERAWATI et al. 2018). Silver rasbora wild captures still become the main fulfillment of the market demands because aquaculture is limited (BUDI et al. 2020, ROSADI et al. 2014). Silver rasbora as under development aquaculture species needs more basic information about its farming procedures to overcome overfishing in the wild (SZMYT et al. 2013, BUDI et al. 2020, SALMATIN et al. 2021).

Feeding management is basic crucial information in the farming of many species (BEN et al. 2016, TIAMIYU et al. 2018). A good feeding practice involves providing an efficient feedstuff at the right time, amounts, and form for the optimal fish growth (OKOMODA et al. 2019). Overfeeding and underfeeding lead to inefficient production. Overfeeding cause a significant reduction in water quality (decreasing dissolved oxygen and increasing ammonia content), reducing feed growth and utilization, and increased susceptibility to infection because of stress due to poor water quality (DWYER et al. 2002, NG et al. 2000, SCHNAITTACHER et al. 2005). On the other hand, underfeeding has a direct impact on the production period because growth slows down due to the fish population are partially starved (BOOTH et al. 2008, KÜÇÜK et al. 2014, MIHELAKAKIS et al. 2002, OH and MARAN 2015).

Application of optimal feeding regimes can optimize the production period, maximize utilization of feed, improve the rearing media (water qualities), and increase the uniformity of fish size until harvest (DWYER et al. 2002, OH et al. 2013, OH and MARAN 2015, SILVA et al. 2007, ZHOU et al. 2003). These regimes vary for different species, size/age/stadia, the composition of feed, and rearing media (CHO et al. 2003, LEE et al. 2000, WANG et al. 1998, XIE et al. 2011). Previous study about feeding frequencies have shown effect on growth, survival, and social interactions on fry and juvenile of some species namely African catfish (*Clarias gariepinus*) (OKOMODA et al. 2019), Korean rockfish (*Sebastes schlegeli*) (LEE et al. 2000), gibel carp (*Carassius auratus gibelio*) (ZHOU et al. 2003), yellowtail flounder (*Limanda ferruginea*) (DWYER et al. 2002), gilthead sea bream (*Sparus aurata*) (MIHELAKAKIS et al. 2002), and rock bream (*Oplegnatus*

faciatus) (OH and MARAN 2015). Meanwhile, studies on the effect of feeding frequency on silver rasbora have never been conducted.

This study aims to evaluate the growth and feeding efficiency of silver rasbora fry reared under different feeding frequencies.

Materials and Methods

Origin of fish and acclimation

This study was conducted from February to March 2019 in the laboratory and aquaculture facilities of Airlangga University, Banyuwangi Campus (East Java, Indonesia). This research was conducted under the oversight and approved by Marine Sciences Faculty of Airlangga University (based on assignment letter from Dean of Marine Sciences Faculty of Airlangga University, 1851/UN3.1.16/PPd/2018).

The fry were obtained from silver rasbora breeding program in aquaculture facilities of Airlangga University, Banyuwangi Campus. All fish were transferred to 20 glass aquaria (volume 60 l; 30 fish/aquaria) and acclimated for 1 week. Fish were fed on commercial dry pellets (PF-1000, Prima Feed™, Indonesia; size 1.3-1.7 mm; crude protein 39-41%; crude lipid 5%; moisture 6%; ash 15%; water 15%) during acclimation twice daily on ad satiation.

The parameters of water quality were monitored daily during experiment including temperature (25–28 °C), pH (7.6–8), dissolved oxygen (6.0–6.7 mg/l), total dissolved solid (143–203 mg/l), and total ammonia nitrogen (0.033–0.058 mg/l).

Experimental design

The feeding trials of fish were divided into 4 different feeding frequency treatments consisted 5 replicates with 20 fish (initial body weight can see in Table 1). Feeding frequency trials were carried out are 1 (T1), 2 (T2), 3 (T3), and 4 (T4) times daily. Fish were fed ad satiation. Schedule of feeding time can be seen in Table 2. All feeding frequency trial groups were used commercial dry pellets (same as the diet in the acclimation period) on ad satiation, no excess of feed in all aquariums. Experiments were carried out for 40 days. Every 10 days, fish body weight was measured.

Table 1

The initial average body weight (BW_i, mean ± SD, n = 5) of silver rasbora (*Rasbora argyrotaenia*) fry

Parameters	Treatment			
	T1	T2	T3	T4
BW_i (g/fish)	0.26 ± 0.01	0.26 ± 0.01	0.27 ± 0.02	0.26 ± 0.01

Tabel 2

Feeding time of silver rasbora (*Rasbora argyrotaenia*) fry with different feeding frequency treatments

Treatment	Feeding schedule			
T1	08.00-09.00	–	–	–
T2	08.00-09.00	17.00-18.00	–	–
T3	08.00-09.00	12.30-13.30	17.00-18.00	–
T4	08.00-09.00	11.00-12.00	14.00-13.00	17.00-18.00

T1, T2, T3, and T4 are 1, 2, 3, and 4 times daily feeding frequency treatment.

Observed parameters

The effects of feeding frequency on growth were determined by calculating the parameters namely body weight gain (BWG), biomass gain (BG), specific growth rate (SGR), and feed conversion ratio (FCR) with the following formula:

$$BWG\ [g/fish] = (BW_f - BW_i)$$
$$BG\ [g] = (N_f\, BW_f - Ni\ BW_i)$$
$$SGR\ [\%/day] = (\ln BW_f - \ln BW_i) / t)$$
$$FCR = FC/(N_f\, BW_f - Ni\ BW_i)$$

where:

BW_i and BW_f – the initial average body weight [g] and the final average body weight [g]
N_i and N_f – the initial number and the final number of larvae
t – the experiment duration in days
FC – amount of feed consumption during experiment.

A total 20 grams of feed was weighed at the beginning of the study and the remaining feed was weighed at the end of the study, the FC value was the difference between the weight of the initial feed and the rest of the feed.

Data analysis

The distribution and homogeneity of data were analyzed; all data have normal distribution and homogeneity of variances. Then, data were analyzed statistically by analysis of variances (ANOVA) test at 95% confi-

dence level and continued with Duncan Multiple Range Test (DMRT) using SPSS 17.0 software.

Results

The growth of silver rasbora fry reared with different feeding frequency showed in Table 3. Based on the data, different feeding frequency has significant effect ($P < 0.05$) on all parameters. At the end of study, the average of final body weight (BW_f), body weight gain (BWG), biomass gain (BG), specific growth rate (SGR), and feed consumption (FC) showed a similar tendency between treatments; increase sequentially from treatment T1 to T4. Feed consumption (FC) not significantly different on treatment T1, T2, and T3 ($P > 0.05$); meanwhile, feed conversion ratio (FCR) decrease and showed lowest value on T4 (1.56 ± 0.04).

The growth was higher with increasing of feeding frequency; the highest growth was occurred on the most frequently feeding. The best growth was observed in T4 (Bw_f: 0.45 ± 0.01 g/fish, BWG: 0.19 ± 0.00 g/fish, BG: 5.58 ± 0.09 g, SGR: 1.34 ± 0.06 %/day).

Table 3

The final average body weight (BW_f), body weight gain (BWG), biomass gain (BG), specific growth rate (SGR), feed consumption (FC), feed conversion ratio (FCR), and survival rate (SR) of silver rasbora (*Rasbora argyrotaenia*) fry reared with different feeding frequency for 40 days

Parameters	Treatment			
	T1	T2	T3	T4
BWG [g/fish]	$0.06^{c} \pm 0.01$	$0.12^{b} \pm 0.02$	$0.13^{b} \pm 0.03$	$0.19^{a} \pm 0.00$
BG [g]	$1.86^{c} \pm 0.23$	$3.54^{b} \pm 0.68$	$3.90^{b} \pm 0.76$	$5.58^{a} \pm 0.09$
SGR [% /day]	$0.53^{c} \pm 0.08$	$0.92^{b} \pm 0.18$	$1.00^{b} \pm 0.22$	$1.34^{a} \pm 0.06$
FC [g]	$4.42^{c} \pm 0.53$	$7.68^{b} \pm 0.41$	$8.08^{ab} \pm 0.55$	$8.76^{a} \pm 0.21$
FCR	$2.41^{a} \pm 0.42$	$2.23^{a} \pm 0.39$	$2.11^{a} \pm 0.26$	$1.56^{b} \pm 0.04$
SR [%]	100	100	100	100

Values (mean ± SD, $n = 5$) with different superscripts in the same row are significantly different ($P < 0.05$). T1, T2, T3, and T4 are 1, 2, 3, and 4 times dialy feeding frequency treatment.

Discussion

The increased growth and improvement feeding efficiency of fish due to increased feeding frequency were demonstrated by several previous studies (Oh and Maran 2015, Okomoda et al. 2019, Wang et al. 2007). In

the current study, we were able to reveal that the growth (showed by value of BWG, BG, and SGR; Table 2) and FCR of silver rasbora fry significantly decreased until four meals per day.

The results showed that the optimum feeding frequency for silver rasbora fry was occurred in higher feeding frequency treatment (4 time/day) – Table 2, because at this treatment the fish had higher value of BWG, BG, and SGR; but lower FCR compared with other less feeding frequency treatment. The optimal feeding frequency for growth of fish varies widely depending on species and size (OH and MARAN 2015). For example, Korean rockfish (*Sebastes schlegelii*) juveniles (initial weight 5.7 g) with feeding frequency 1 time/day grew faster and converted feed more efficient than 1 time/2 days of feeding (LEE et al. 2000). On the other hands, the optimal feeding frequency of juvenile yellowtail flounder (Limanda ferruginea) with 6.8 g initial weight had 2 times/day (DWYER et al. 2002), juvenile hybrid sunfish (male *Lepomis macrochirus* × female *Lepomis cyanellus*) with 7.4 g initial weight had 3 times/day (WANG et al. 1988), post-larvae ayu (*Plecoglossus altivelis*) with 0.15 g initial weight had 4 times/day (CHO et al. 2003), and juvenile gibel carp (*Carassius auratus gibelio*) with 3.0 g initial weight had 24 times/day (ZHOU et al. 2003).

Fish fed more frequently eat a larger amount of food than those fed at lower frequencies, but the individual feed consumption size was smaller (DWYER et al. 2002). Fish achieve this by improving their stomach volume and becoming hyperphagic (ROUHONEN et al. 1998). The digestibility ratio is related to the frequency of meals depending on the volume of the stomach (GRAYTON and BEAMISH 1977, JOBLING 1983, ROUHONEN et al. 1998), the interval of meals (BISWAS et al . 2010, LIU 1999), and the rate of gastric emptying (LEE et al. 2000). Data regarding daily feed intake patterns will provide important information regarding the timing of feeding and the amount of feed provided at each feed to increase the growth of silver rasbora.

In present study, FCR was affected by feeding frequency is similar with other previous research of Asian sea bass (*Lates calcarifer*) (SALAMA 2008), *Clarias gariepinus* (ADEROLU et al. 2021, JAMABO et al. 2015), and Black Sea trout (*salmo trutta labrax*) (BAŞÇINAR et al. 2007). In line with growth parameters, best FCR was occurred in most of frequently feeding (4 times/day feeding frequency). Similar tendency of relation between FCR and growth that affected by feeding frequency was also occurred in previous study in some species, for example Asian sea bass (*Lates calcarifer*) (SALAMA 2008), hybrid sunfish (male *Lepomis macrochirus* × female *Lepomis cyanellus*) (WANG et al. 1988), Australian snapper (*Pagrus auratus*) (TUCKER et al. 2006).

Referring to the growth parameters and the lowest FCR were obtained for fish fed 4 times daily, although further studies should be conducted to investigate the effect of more frequent feeding. It can be concluded that feeding 4 times daily as optimum feeding frequency in the present study resulted in better growth and FCR during the rearing of silver rasbora.

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Refferences

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

Aderolu A.Z., Seriki B.M., Apatira A.L., Ajaegbo C.U. 2010. *Effects of feeding frequency on growth, feed efficiency and economic viability of rearing African Catfish (Clarias Gariepinus, Burchell 1822) Fingerlings and Juveniles*. African J. Food Sci., 4(5): 286–90.

Aryani N. 2015. *Native species in Kampar Kanan River, Riau Province Indonesia*. Int. J. Fish. Aquat. Sci., 2(5): 213–216.

Başçinar N., Çakmak, E. Çavdar Y., Aksungur N. 2007. *The effect of feeding frequency on growth performance and feed conversion rate of black sea trout (Salmo trutta labrax Pallas, 1811)*. Turkish J. Fish. Aquat. Sci., 17: 13–17.

Biswas, A., Kazushige I., Takii K. 2010. *Feeding interval and photoperiod influence the growth performance of striped knifejaw, Oplegnathus fasciatus*. Aquac. Res., 41(10): 517–523.

Booth M.A., Tucker B.J., Allan G.L., Fielder D.S. 2008. *Effect of feeding regime and fish size on weight gain, feed intake and gastric evacuation in juvenile Australian snapper Pagrus auratus*. Aquaculture, 282(1–4): 104–110.

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

Capuli E.E., Bailly N. 2022. *Rasbora argyrotaenia (Bleeker, 1849), Silver rasbora*. FishBase, https://www.fishbase.in/summary/Rasbora-argyrotaenia.html.

Cho S.H., Lim Y.S., Lee J.H., Lee J.K., Park S., Lee S.M. 2003. *Effects of feeding rate and feeding frequency on survival, growth, and body composition of ayu post-larvae plecoglossus altivelis*. J. World Aquac. Soc., 34: 85–91.

Dwyer K.S., Brown J.A., Parrish C., Lall S.P. 2002. *Feeding frequency affects food consumption, feeding pattern and growth of juvenile yellowtail flounder (Limanda ferruginea)*. Aquaculture, 213: 279–292.

Grayton B.D., Beamish F.W.H. 1977. *Effects of feeding frequency on food intake, growth and body composition of rainbow trout (Salmo gairdneri)*. Aquaculture, 11: 159–172.

Herawati T., Yustiati A., Nurhayati A., Mustikawati R. 2018. *Proximate composition of several fish from Jatigede Reservoir in Sumedang district, West Java*. in: IOP Conference Series: Earth and Environmental Science, pp. 1–7.

Jamabo N.A., Fubara R.I., Dienye H.E. 2015. *Feeding frequency on growth and feed conversion of Clarias Gariepinus (Burchell, 1822) Fingerlings*. Int. J. Fish. Aquat. Stud., 3: 353–356.

JOBLING M. 1983. *Effect of feeding frequency on food intake and growth of Arctic charr, Salvelinus alpinus* L. J. Fish Biol., 23: 177–185.

KÜÇÜK E., AYDIN I., POLAT H., EROLDOĞAN O.T., ŞAHIN T. 2014. *Effect of feeding frequency on growth, feed efficiency and nutrient utilization of juvenile flounder (Platichthys flesus luscus).* Aquac. Int., 22: 723–732.

KUSUMA W.E., SAMUEL P.D., WIADNYA D.G.R., HARIATI A.M., KUMAZAWA Y. 2017. *Complete mitogenome sequence of Rasbora argyrotaenia (Actinopterygii: Cyprinidae).* Mitochondrial DNA Part B Resour., 2: 373–374.

LEE S.M., HWANG U.G., CHO S.H. 2000. *Effects of feeding frequency and dietary moisture content on growth, body composition and gastric evacuation of juvenile Korean rockfish (Sebastes schlegeli).* Aquaculture, 187: 399–409.

LIU F.G. 1999. *Effect of feeding regimen on the food consumption, growth, and body composition in hybrid striped bass Morone saxatilis × M. chrysops*. Fish. Sci., 65: 513–519.

MIHELAKAKIS A., TSOLKAS C., YOSHIMATSU T. 2002. *Optimization of feeding rate for hatchery-produced juvenile gilthead sea bream Sparus aurata*. J. World Aquac. Soc., 33: 169–175.

NG W.K., Lu K.S., HASHIM R., ALI A., 2000. *Effects of feeding rate on growth, feed utilization and body composition of a tropical bagrid catfish*. Aquac. Int., 8: 19–29.

OH S.Y., KIM M.S., KWON J.Y., MARAN B.A.V. 2013. *Effects of feed restriction to enhance the profitable farming of blackhead seabream Acanthopagrus schlegelii schlegelii in sea cages*. Ocean Sci. J., 48: 263–268.

OH S.Y., MARAN B.A.V. 2015. *Feeding frequency influences growth, feed consumption and body composition of juvenile rock bream (Oplegnathus fasciatus).* Aquac. Int., 23: 175–184.

OKOMODA V.T., AMINEM W., HASSAN A., MARTINS C.O. 2019. *Effects of feeding frequency on fry and fingerlings of African catfish Clarias gariepinus*. Aquaculture, 511.

Okwuosa Obinna B., Nwachukwu V., Agha Etuu P., Enyinnaya C. 2016. *The impact of feeding frequency on the growth and survival of Clarias gariepinus fingerlings fed at different percentage body weight in four phases*. Int. J. Fish. Aquat. Stud., 4(5): 173–178.

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

RUOHONEN K., VIELMA J., GROVE D.J. 1998. *Growth and food utilisation of rainbow trout (Oncorhynchus mykiss) fed low-fat herring and dry diets enriched with fish oil*. Aquaculture, 163: 275–283.

SALAMA A.J. 2008. *Effects of different feeding frequency on the growth, survival and feed conversion ratio of the Asian sea bass Lates calcarifer juveniles reared under hypersaline seawater of the Red Sea*. Aquac. Res., 39: 561–567.

SALMATIN N., MUBARAK A.S., BUDI D.S. 2021. *Egg diameter of female silver rasbora (Rasbora argyrotaenia) at different gonadal maturity stage*. IOP Conf. Ser. Earth Environ. Sci., 718: 012066.

SCHNAITTACHER G., KING V.W., BERLINSKY D.L., 2005. *The effects of feeding frequency on growth of juvenile Atlantic halibut, Hippoglossus hippoglossus* L. Aquac. Res., 36: 370–377.

SILVA C.R., GOMES L.C., BRANDAO F.R. 2007. *Effect of feeding rate and frequency on tambaqui (Colossoma macropomum) growth, production and feeding costs during the first growth phase in cages*. Pol. J. Natur. Sc., 28: 471–483.

SZMYT M., GORYCZKO K., GRUDNIEWSKA J., LECK A.M., WIŚNIEWSKA A.M., WOZNIAK M. 2013. *Preliminary result of Europan grayling (Thymallus Thymallus* L.*) fry rearing to the autumn fingerlings stage*. Aquaculture, 264: 135–139.

TIAMIYU L.O., OKOMODA V.T., AGBO H.E. 2018. *The effect of different feeding rates and restriction on the growth performance of Clarias gariepinus*. Iran. J. Fish. Sci., 17: 840–847.

TUCKER B.J., BOOTH M.A., ALLAN G.L., BOOTH D., FIELDER D.S. 2006. *Effects of photoperiod and feeding frequency on performance of newly weaned Australian snapper Pagrus auratus*. Aquaculture, 258: 514–520.

WANG N., HAYWARD R.S., NOLTIE D.B. 1998. *Effect of feeding frequency on food consumption, growth, size variation, and feeding pattern of age-0 hybrid sunfish*. Aquaculture, 165: 261–267.

WANG Y., KONG L.J., LI K., BUREAU D.P. 2007. *Effects of feeding frequency and ration level on growth, feed utilization and nitrogen waste output of cuneate drum (Nibea miichthioides) reared in net pens*. Aquaculture, 271: 350–356.

XIE F., AI Q., MAI K., XU W., MA H. 2011. *The optimal feeding frequency of large yellow croaker (Pseudosciaena crocea, Richardson) larvae*. Aquaculture., 311: 162–167.

ZHOU Z., CUI Y., ZIE S., ZHU X., LEI W., XUE M., YANG Y. 2003. *Effect of feeding frequency on growth, feed utilization, and size variation of juvenile gibel carp (Carassius auratus gibelio)*. J. Appl. Ichthyol., 19: 244–249.

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FRAGMENTOMIC ANALYSIS OF BIOPEPTIDES *IN SILICO* RELEASED FROM MILK PROTEINS*

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Key words: bioactive peptides, BIOPEP-UWM database, milk proteins, in silico hydrolysis, fragmentomics.

Abstract

The fragmentomic-assisted method was employed to predict the biological potential of peptides derived from milk proteins hydrolyzed by papain and bromelain. Firstly, protein sequences were acquired from the BIOPEP-UWM database and then hydrolyzed by the above enzymes using a BIOPEP-UWM tool called "Enzyme(s) action". The released peptides were defined as parent peptides and further analyzed for the presence of shorter peptidic regions with documented bioactivity as well as their likelihood to be bioactive.

The results revealed the bioactive potential of the released parent peptides. β-Casein was found as the best source of biopeptides. Although this finding is consistent with literature data, the new parent peptide i.e., PVQPFTESQSLTLTDVENLHLPPLLLQSWMHQPHQPLPPTVMFPPQ SVLSLSQSK, produced by the action of bromelain might be considered as a new strategic zone due to the presence of multi-active regions. Some parent peptides theoretically produced from milk proteins turned out to be fully bioactive. Despite the usefulness of the tools for peptide bioactivity prediction, the critical thinking while planning the application of such data in future experiments would thus appear to be a worthwhile line of inquiry.

Introduction

It is well known that food affects our well-being (ROZIN et al. 1999) due to the presence of, i.a., proteins, sugars, fats, and vitamins that are respon-

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sible for the regulation of body functions (CARREIRO et al. 2016**)**. The first serve several crucial functions in all biological processes as, e.g., catalysts, transporters, immune protectors, growth and differentiation controllers (BERG et al. 2002). Proteins are additionally sources of biologically active (bioactive) peptides (BHANDARI et al. 2020), i.e., non-active fragments encrypted in protein sequences, which serve various biological functions after enzymatic release and interactions with appropriate body receptors, including e.g., reduction of blood pressure, reduction of glucose and cholesterol levels as well as immunomodulating, antioxidative, antibacterial, antithrombotic etc. effects (MOHANTY et al. 2016). According to the scientific reports, milk proteins derived from different animal species represent the richest sources of biopeptides (IWANIAK et al. 2020a).

Once the computers had been invented, they were used for data storage. The popularity of Internet facilitated the remote access to scientific data (DATE 2005), including databases (CHANPUT et al. 2010). Databases useful in food science contain information about nutritional characteristics of individual foods, meals, diets, dishes and food-derived compounds (MARCONI et al. 2018). They are repositories of the molecules, like e.g., peptides, carbohydrates, enzymes etc. The first include the databases of biologically active peptides, like BIOPEP-UWM, EROP-Moscow or AHTPDB. They provide the sequences of biopeptides with various bioactivities (first two) and peptides exhibiting the antihypertensive effect (the latter one) (MINKIEWICZ et al. 2019).

Depending on the nature of a molecule, databases are the core of bio- and cheminformatics. These both disciplines deal with the elaboration of databases for categorization and data storage as well as employment of computer technologies for data analysis (IWANIAK et al. 2019). The coupled use of computer programs that are suitable to study biomolecules from foods and databases enables the bioinformatic-assisted approach. It is the one of the three approaches employed in food science to analyze biopeptides derived from food proteins. The second one is called a classical approach and relies on biological material (protein or food protein source) selection, its hydrolysis, identification of peptides in hydrolysates, and assessment of biological activity of both hydrolysate and peptides. Finally, the combination of the above methodologies is defined as hybrid/integrated approach, which helps getting more insights on the nature of biopeptides. Regardless the approach applied, each of them has its own pros and cons (IWANIAK et al. 2019).

According to RIEDER et al. (2010), potency is a key quality attribute of a biological material. The potential of a molecule can be determined using different bioassays (RIEDER et al. 2010). One of the attractive approaches

to determine the biological potency of a compound is by computing its similarity (both chemical and structural) to the molecule with a known bioactivity (PERIWAL et al. 2020). This similarity-assisted concept is consistent with the fragmentomic idea of research introduced by ZAMYATNIN et al. (2009). Briefly, it relies on the rule according to which the presence of shorter motifs with a known biological function in the fragment with an unknown bioactivity may decide about the potency of the whole molecule (ZAMYATNIN et al. 2009).

Many peptides derived from food proteins were studied using classical or hybrid approach (IWANIAK et al. 2019). The latter one enabled identifying a biopeptide sequence in the hydrolysate and then predicting its bioactivity based on the peptide search in specific peptide databases. If there were no such sequences in the specific database, it was presumed that peptide might not be bioactive (IWANIAK et al. 2020a). Thus, the aim of this study was to apply a fragmentomic-assisted approach for the prediction of the biological potency of bioactive peptides *in silico* released from bovine milk proteins using papain and bromelain.

Materials and Methods

The following protein sequences derived from bovine milk *(Bos taurus)* were acquired from the BIOPEP-UWM database (https://biochemia.uwm.edu.pl/biopep-uwm/; MINKIEWICZ et al. 2019) and then analyzed: α_{s1}-casein, genetic variant B (ID 1087); α_{s2}-casein, genetic variant A (ID 1090); β-casein, genetic variant A1 (ID 1097); κ-casein, genetic variant A (ID 1117); α-lactabumin, genetic variant B (ID 1115); β-lactoglobulin, genetic variant A (ID 1116); and serum albumin (ID 1729). The numbers in brackets denote the accession numbers of these sequences in the BIOPEP-UWM database providing the following data: the full sequence of the protein, the number of amino acid residues, references, cross-references to other databases, and additional information (if possible).

The analyses of the above-mentioned proteins involved: their hydrolysis simulation using bromelain (EC 3.4.22.32) and papain (EC 3.4.22.2), followed by the fragmentomic analysis of potentially released peptides (defined as parent peptides). Theoretical hydrolysis of milk proteins was computed using the BIOPEP-UWM option called "Enzyme(s) action" available in the tab entitled "Analysis". The predicted proteolysis was performed by selecting one out of the three possible options, i.e., one protein sequence: one enzyme at one computation. Finally, the fragmentomic analysis involved the prediction of potential bioactivity of the theoretically

released peptides. It was achieved by calculating the Score parameter for each parent peptide predicting the presence of shorter motifs with known bioactivities in the released peptides. The Score was computed using PeptideRanker program (http://distilldeep.ucd.ie/PeptideRanker/; MOONEY et al. 2012). All computations were carried out in April-June 2021.

Results and Discussion

Loads of articles concerning the discovery of new biologically active peptides are published every year. The Web of Science database (https://apps.webofknowledge.com/WOS_GeneralSearch; accession date: 22 April 2021] showed 2,384 records when typing the following words: "bioactive peptides", "foods", and "proteins" in search via "topic". It concerned the period of 1991–2021. More detailed statistics showed that the number of the released articles in 1991–1999 varied between 1 (1991) and 6 (1999). Years 2000 (12) – 2009 (56) provided more papers on this issue (see in brackets). In the next decade, there were 72 (2010) and 320 (2019) articles, and finally 390 publications were released in 2020. In turn, 62 papers were published between January and April 2021. Such a dynamics in the research concerning the bioactive peptides shows that, despite the new information appearing on a daily basis, it is rather impossible to predict all possible sequences in the protein of interest. Such an amount of data also requires the regular update of databases (UDENIGWE 2014) used for the so-called in silico analyses. Thus, although bovine milk proteins have been known as the source of biopeptides for decades (CAPRIOTTI et al. 2016), their continued analyses are found reasonable.

Peptides theoretically released from bovine milk proteins using papain and bromelain are shown in Table 1 and Table 2, respectively. When analyzing the results, the main attention was paid to the released sequences that were composed of at least 4 amino acids. They were called parent peptides. A similar terminology was applied by IWANIAK et al. (2020a) who harnessed the fragmentomic approach to study the presence of bitter-tasting motifs in peptides (i.e., parent peptides) released from a milk protein concentrate hydrolyzed by different proteolytic enzymes, including the two above. As potential products of milk proteins' hydrolysis by papain and bromelain, di- and tripeptides were excluded from our studies. The fragmentomic analysis of milk protein sequences was carried out using a BIOPEP-UWM tool called the profile of potential biological activity of protein. It is defined as the type (understood as bioactivity) and location of a peptide in a protein chain (MINKIEWICZ et al. 2019). This function is

available in the BIOPEP-UWM database and enables inserting shorter fragments instead of the protein. "Technically", this analysis shows the exact matches of some sequential motifs in a sample (i.e., parent peptide in this case). In the case of potentially released dipeptides, the exact match would show the same sequence, whereas in the case of tripeptides, it would be the same sequence and/or dipeptide match. This means that the shorter the parent sequence is, the smaller number of bioactive motifs can be found in it. This regularity is also applicable to the substrate (protein and/or parent peptide sequence) as the relatively short substrate sequences produce a smaller number of peptides than the longer ones (IWANIAK et al. 2020a). It needs to be noted that we did not include in our analysis the repetitions of bioactive motif in the parent sequence because, regardless of the number of motif repetitions, it would show the same bioactivity and the value of PeptideRanker Score.

Table 1

Peptides theoretically released from bovine milk proteins due to the action of papain

Substrate for hydrolysis: α-lactalbumin	
Parent peptide	Encrypted bioactive motif
MMSFVSLLLVG	**MM**$^{\text{ACEi, DPP4i,AOX}}$; **SF**$^{\text{ACEi, DPP4i}}$; LV/**LL** $^{\text{DPP4i,sti-glu}}$; **LLL**$^{\text{sti-sub}}$; SL $^{\text{DPP4i}}$; VG$^{\text{ACEi, DPP4i}}$
ILFH	**LF**$^{\text{ACEi}}$; IL $^{\text{ACEi, sti-glu}}$
QLTK	QL/LT/TK $^{\text{DPP4i}}$
VSLPE	**LP**/SL/VS $^{\text{DPP4i}}$
WVCTTFH	**TF**$^{\text{ACEi, DPP4i}}$; **WV**/TT $^{\text{DPP4i}}$
DTQA	QA $^{\text{DPP4i}}$;TQ$^{\text{ACEi, DPP4i}}$
IVQNNDSTE	NN/QN/ND/VQ $^{\text{DPP4i}}$;IVQ/ST $^{\text{ACEi}}$; TE$^{\text{ACEi, DPP4i}}$;IV $^{\text{sti-glu}}$
LFQINNK	**LF**/NK $^{\text{ACEi}}$; **FQ**$^{\text{ACEi, DPP4i}}$; IN/QI/NN $^{\text{DPP4i}}$
IWCK	**WC** $^{\text{DPP4i}}$; **IW**$^{\text{ACEi, DPP4i}}$
DDQNPH	NP/QN/DQ $^{\text{DPP4i}}$; **PH**$^{\text{ACEi, DPP4i}}$
FLDDDLTDDIMCVK	**FL/IM** $^{\text{DPP4i}}$; LT/TD/VK $^{\text{DPP4i}}$
ILDK	IL$^{\text{ACEi, DPP4i, sti-glu}}$
LCSE	SE$^{\text{sti-sub}}$
LDQWLCE	**WL**$^{\text{ACEi, DPP4i}}$; **QW**/DQ $^{\text{DPP4i}}$
Substrate for hydrolysis: β-lactoglobulin	
CLLLA	**LL** $^{\text{DPP4i,sti-glu}}$; **LLL**$^{\text{sti-sub}}$; LA$^{\text{ACEi, DPP4i,ubi}}$
LTCG	LT $^{\text{DPP4i}}$
LIVTQTMK	**TW**/LIVTQ$^{\text{ACEi}}$; LI $^{\text{DPP4i,sti-glu}}$, IV $^{\text{sti-glu}}$; MK/TM/QT/TQ/VT $^{\text{DPP4i}}$
LDIQ$^{\text{K(score = 0.11)}}$	IQ $^{\text{DPP4i}}$; LDIQK/QK$^{\text{ACEi}}$
SDISLLDA	**LL** $^{\text{DPP4i,sti-glu}}$; SL $^{\text{DPP4i}}$; DA$^{\text{ACEi}}$

Cont. Table 1

PTPE	TP/PT[ACEi, DPP4i]
ILLQK	**LL** [DPP4i,sti-glu]; IL[ACEi, DPP4i, sti-glu]; LQ/QK[ACEi]
VLVLDTDY	DY[ACEi, reg-ion]; LVL[ACEi]; VL/LV [DPP4i, sti-glu]; TDY [AOX]; TD [DPP4i]
LLFCME	**LF/LLF**[ACEi]; **LL** [DPP4i,sti-glu]; ME [ACEi, DPP4i]
QSLA	SL/QS [DPP4i]; LA[ACEi, DPP4i,ubi]
CQCLVR	LVR[ACEi]; VR [ACEi, DPP4i]; LV [DPP4i, sti-glu]
VDDE	VD [DPP4i]
LPMH	**PM/LP/MH** [DPP4i]
LSFNPTQLE	**FN/**NP/QL[DPP4i i]; **SF/**PT/TQ [ACEi, DPP4i]
Substrate for hydrolysis: κ-casein	
SFFLVVTILA	**FFL**[ACEi]; **FL** [DPP4i]; **SF** [ACEi, DPP4i]; IL[ACEi, DPP4i,sti-glu]; LA[ACEi, DPP4i,ubi]; LV [DPP4i,sti-glu]; TI/VV/VT [DPP4i]
LTLPFLG	**PF/FL/LP/**TL/LT [DPP4i]; **LPF/LG** [ACEi]
QNQE	QN/NQ/QE [DPP4i]
QPIR	**PI/**QP [DPP4i]; IR [ACEi, DPP4i,AOX,i-ren, CaMPDEi]
IPIQ[Y(score = 0.36)]	**PI/**IPI/IPIQY/QY/IQ [DPP4i]; **IP** [ACEi, DPP4i]; IQY[ACEi, AOX, ab]
VLSR	VL [DPP4i,sti-glu]
LINNQFLPY	**FL/QF/LP/PY/**IN/NN/NQ [DPP4i]; LI [DPP4i,sti-glu]
QILQWQVLSNTVPA	**QW/WQ/PA/**QI/NT/QV/TV [DPP4i]; IL [ACEi, DPP4i, sti-glu];VP [ACEi, DPP4i]; LQ [ACEi]; VL [DPP4i, sti-glu]
SCQA	QA [DPP4i]
QPTTMA	**MA/**QP/TM/TT [DPP4i]; PT [ACEi, DPP4i]
LSFMA	**SF** [ACEi, DPP4i]; MA [DPP4i]
IPPK	**PP/IP** [ACEi, DPP4i]; **IPP** [ACEi]; PK [DPP4i]; **PPK** [ACEi, at]
NQDK[(score = 0.07)]	NQDK[at]; NQ/QD [DPP4i]
IPTINTIA	**IP/**PT/IA[ACEi, DPP4i];IN/TI/NT [DPP4i]
PTSTPTTE	TP/PT/TE[ACEi, DPP4i]; TS/TT [DPP4i]; ST [ACEi]
STVA	ST[ACEi]; VA/TV [DPP4i]
DSPE	**SP** [DPP4i]
SPPE	**PP**[ACEi, DPP4i]**/SP** [DPP4i]
INTVQVTSTA	TSTA[AOX]; ST/VQV[ACEi]; IN/TA/NT/TS/QV/VQ/TV/VT [DPP4i]
Substrate for hydrolysis: β-casein	
LNVPG	**PG**[ACEi, DPP4i, anm, at, reg-sto]; LN/VP[ACEi, DPP4i]; NV [DPP4i]
SLSSSE	SL [DPP4i];SSS[sti-sub]; SE[reg-ion]
SITR	SI/TR [DPP4i]
FQSE	**FQ**[ACEi, DPP4i]; QS [DPP4i]; SE[sti-glu]
QQQTE	QQ/OT [DPP4i]; TE[ACEi, DPP4i]

Cont. Table 1

LQDK	LQ^{ACEi}; QD ^{DPP4i}
QTQSLVY	VY/TQ$^{ACEi,\ DPP4i}$; LVY^{ACEi}; LV $^{DPP4i,\ sti\text{-}glu}$; VY^{AOX}; SL/QS/QT ^{DPP4i}
PFPG	**FP**$^{ACEi,\ DPP4i}$; **PF** ^{DPP4i}; **PG**$^{ACEi,\ DPP4i,\ anm,\ at,\ reg\text{-}sto}$
NSLPQNIPPLTQT PVVVPPFLQPE	**PF/FL/PPL/FLQP/LP/LPQNIPPL/**QP/SL/IPPLTQTPV/ VV/IPPLTQT-PV/PV/LQ/LT/QN/QT^{DPP4i}; **PP/PL/IP/**LQP/PQ/VP/TQ^{ACEi,DPP4i}; **IPP/VVVPPF/VPP/**TPVVVPPFLQP/SLPQN/TP/ NIPPLTQTPV/VVPP/ LTQTPVVVPPF^{ACEi};VVV^{acan}
MPFPK	**FP**^{ACEi,DPP4i}; **PF/MP/**PK^{DPP4i}
PVQPFTE	TE^{ACEi,DPP4i}; **PF/**QP/PV/VQ^{DPP4i}
SQSLTLTDVE	**VE**^{ACEi,DPP4i}; LTLTDVE^{ACEi}; SL/TL/LT/QS/TD^{DPP4i}
LPPLLLQSWMH	**WM/PL/PP**^{ACEi,DPP4i}; **SW/PPL/LP/MH/**QS^{DPP4i}; **LPP/LQSW/**LQ^{ACEi}; **LLL**$^{sti\text{-}sub}$; **LL** $^{DPP4i,sti\text{-}glu}$
QPLPPTVMFPPQSVL-SLSQSK	**MF/FP/PP/PL/**PQ/PT^{ACEi,DPP4i}; VL$^{DPP4i,sti\text{-}glu}$; **PLP/LPP**^{ACEi}; **LP/**QP/SL/VM/QS/SK/SV/TV^{DPP4i}
VLPVPE	VL$^{DPP4i,sti\text{-}glu}$; **LP/**PV^{DPP4i}; VP^{ACEi,DPP4i}; VLP^{ACEi}
DMPIQA	**MP/PI/**QA/IQ^{DPP4i}; **DM**^{ACEi}
FLLY	**LL** $^{DPP4i,sti\text{-}glu}$; **FL**^{ACEi,DPP4i}; **LY**^{ACEi,AOX}; **LLY**^{ist}
QQPVLG	VL $^{DPP4i,sti\text{-}glu}$; **QQ/**QP/PV^{DPP4i}; **LG**^{ACEi,}
PFPIIV	**FP**^{ACEi,DPP4i}; II$^{DPP4i,sti\text{-}glu}$; IV$^{sti\text{-}glu}$; **PI/PF**^{DPP4i}; **FPIIV**^{ACEi}
Substrate for hydrolysis: α_{S2}-casein	
FFIFTCLLA	**LL** $^{DPP4i,sti\text{-}glu}$; LA$^{ACEi,\ DPP4i,ubi}$; **IF**^{ACEi}
NTME	ME^{ACEi,DPP4i}; TM/NT^{DPP4i}
VSSSE	SSS$^{sti\text{-}sub}$; SE$^{reg\text{-}ion}$; VS^{DPP4i}
SIISQE	II$^{DPP4i,sti\text{-}glu}$; SI/QE^{DPP4i}
INPSK	NP/PS/IN/SK^{DPP4i}
NLCSTFCK	**TF**^{ACEi,DPP4i}; NL^{DPP4i}; ST^{ACEi}
SSSE	SSS$^{sti\text{-}sub}$; SE$^{reg\text{-}ion}$
ITVDDK	VD/TV^{DPP4i}
INQFY	**QF/**IN/NQ^{DPP4i}; **FY**^{ACEi}
FPQY	**FP**^{ACEi,DPP4i}; PQ/QY^{DPP4i}
PIVLNPWDQVK	**PW**^{AOX,DPP4i}; **PWD**^{AOX}; IV$^{sti\text{-}glu}$; VL $^{DPP4i,\ sti\text{-}glu}$; LN/VK^{ACEi,DPP4i}; **WD/PL/**NP/DQ/QV^{DPP4i}; **LNP**^{ACEi}
VPITPTLNR	LN/TP/PT/VP^{ACEi,DPP4i}; **PI/**NR/TL/VPITPT^{DPP4i}
QLSTSE	SE$^{reg\text{-}ion}$; QL/TS^{DPP4i}; ST^{ACEi}
TVDME	**DM**^{ACEi}; **ME** ^{ACEi,DPP4i}; TV/VD^{DPP4i}
VFTK	**VF**^{ACEi,DPP4i}; TK^{DPP4i}
LNFLK	**NF/FL/**LN^{ACEi,DPP4i}; **LNF**^{ACEi} ; LK^{AOX}
LPQY	PQ^{ACEi,DPP4i}; **LP/**QY^{DPP4i}
PWIQPK	**PW**^{AOX,DPP4i}; **PWI**^{AOX}; IQP^{ACEi,DPP4i}; **WI/WIQP/**QP/IQ/PQ^{DPP4i}

Cont. Table 1

VIPY	**IP**^{ACEi,DPP4i}; **IPY**^{ACEi}; **PY/**VI^{DPP4i}
Substrate for hydrolysis: α_{S1}-casein	
LPQE	**LP/**PQ/QE^{DPP4i}
VLNE	VL $^{DPP4i,\ sti\text{-}glu}$; LN^{ACEi,DPP4i}; NE^{DPP4i}
NLLR	**LL** $^{DPP4i,\ sti\text{-}glu}$; **LR**^{ACEi}; **LLR**^{AOX}; NL^{DPP4i}
FFVA	VA^{DPP4i}
PFPQVFG	**FP/VF/**PQ^{ACEi,DPP4i}; **FG**^{ACEi}; **PF/**QV ^{DPP4i}
SISSSE	SSS$^{sti\text{-}sub}$; SE$^{reg\text{-}ion}$; SI ^{DPP4i}
IVPNSVE	VP/VE^{ACEi,DPP4i}; IV$^{sti\text{-}glu}$; PN/SV ^{DPP4i}
DVPSE	SE$^{reg\text{-}ion}$; VP^{ACEi,DPP4i}; PS ^{DPP4i}
QLLR	**LL** $^{DPP4i,sti\text{-}glu}$; **LR**^{ACEi}; **LLR**^{AOX}; QL^{DPP4i}
VPQLE	PQ/VP^{ACEi,DPP4i}; QL^{DPP4i}
IVPNSA	VP^{ACEi,DPP4i}; IV$^{sti\text{-}glu}$; PN^{DPP4i}
PMIG	**PM/**MI ^{DPP4i}; **IG**^{ACEi}
VNQE	NQ/QE/VN^{DPP4i}
QLDA	DA^{ACEi}; QL ^{DPP4i}
VPLG	VPL $^{DPP4i,anm,\ sti\text{-}sub,}$; **PL/**VP^{ACEi,DPP4i}; **LG**^{ACEi}; **PLG**$^{ACEi,\ op}$
PSFSDIPNPIG	**SF/IP**^{ACEi,DPP4i}; **IG**^{ACEi}; **PI/**NP/PN/PS ^{DPP4i}
TTMPL$^{W(score\ =\ 0.74)}$	**TTMPLW** $^{ACEi,,\ mod,\ op}$; **LW**^{ACEi,DPP4i,AOX}; **PLW**^{ACEi}; **MP/**TM/TT ^{DPP4i}
Substrate for hydrolysis: serum albumin	
WVTFISLLLLFSSA	**LL** $^{DPP4i,sti\text{-}glu}$; **LLL**$^{sti\text{-}sub}$; **TF**^{ACEi,DPP4i}; **LF/LLF**^{ACEi}; **WV/**SL/VT ^{DPP4i}
LVLIA	LI/VL/LV$^{DPP4i,sti\text{-}glu}$; IA^{ACEi,DPP4i}; LVL^{ACEi}
FSQY	QY ^{DPP4i}
LQQCPFDE	LQ/LQQ^{ACEi}; **PF/**QQ ^{DPP4i}
LVNE	NE/VN^{DPP4i}; LV$^{DPP4i,sti\text{-}glu}$
TCVA	VA ^{DPP4i}
TLFG	**LF/FG**^{ACEi}; TL ^{DPP4i}
CFLSH	**CF/FL**^{ACEi}; SH ^{DPP4i}
DDSPDLPK	**DLP**^{ACEi}; **LP/**SP/PK ^{DPP4i}
PDPNTLCDE	DP/PN/TL/NT ^{DPP4i}
VFQE	**FQ/VF**^{ACEi,DPP4i}; QE ^{DPP4i}
CCQA	QA ^{DPP4i}
CLLPK	**LL** $^{DPP4i,sti\text{-}glu}$; **LLP**^{ACEi}; **LP/**PK ^{DPP4i}
SIQK	QK^{ACEi}; SI/IQ ^{DPP4i}
WSVA	**WS/**VA/SV ^{DPP4i}
LSQK	QK^{ACEi}
LVTDLTK	LV$^{DPP4i,sti\text{-}glu}$; LT/TD/TK/VT ^{DPP4i}

Cont. Table 1

DLLE	**LL** $^{\text{DPP4i,sti-glu}}$
ICDNQDTISSK	DN/NQ/QD/SK/TI $^{\text{DPP4i}}$
PLLE	**LL** $^{\text{DPP4i,sti-glu}}$; **PL**$^{\text{ACEi,DPP4i}}$
NLPPLTA	**PP/PL**$^{\text{ACEi,DPP4i}}$; **LLP**$^{\text{ACEi}}$; **PPL/LP**/NL/LT/TA $^{\text{DPP4i}}$
SFLY	**LY**$^{\text{ACEi,AOX}}$
VSVLLR	**LL**/VL $^{\text{DPP4i,sti-glu}}$; **LR**$^{\text{ACEi}}$; **LLR**$^{\text{AOX}}$; SV/VS $^{\text{DPP4i}}$
DDPH	**PH**$^{\text{ACEi,DPP4i}}$; **DP** $^{\text{DPP4i}}$
STVFDK	**FDK**/ST$^{\text{ACEi}}$; **VF**$^{\text{ACEi,DPP4i}}$; TV $^{\text{DPP4i}}$
LVDE	LV$^{\text{DPP4i,sti-glu}}$; **VD** $^{\text{DPP4i}}$
PQNLIK	LI$^{\text{DPP4i,sti-glu}}$; PQ$^{\text{ACEi,DPP4i}}$; NL/QN$^{\text{DPP4i}}$
QNCDQFE	**QF**/QN/DQ $^{\text{DPP4i}}$
FQNA	**FQ**/NA/QN $^{\text{DPP4i}}$
LIVR	LI$^{\text{DPP4i,sti-glu}}$; IV$^{\text{sti-glu}}$; VR$^{\text{ACEi,DPP4i}}$; IVR$^{\text{ACEi}}$
VPQVSTPTLVE	PQ/TP/PT/VP/VE$^{\text{ACEi,DPP4i}}$; TL/QV/VS/$^{\text{DPP4i}}$; LV$^{\text{DPP4i,sti-glu}}$; LVE/ST$^{\text{ACEi}}$
CCTK	TK$^{\text{DPP4i}}$
MPCTE	TE$^{\text{ACEi,DPP4i}}$; **MP** $^{\text{DPP4i}}$
LSLILNR	IL $^{\text{DPP4i,sti-glu, ACEI}}$; LI$^{\text{DPP4i,sti-glu}}$; LN$^{\text{ACEi,DPP4i}}$; SL/NR$^{\text{DPP4i}}$
LCVLH	LH$^{\text{AOX,DPP4i}}$; VL$^{\text{DPP4i,sti-glu}}$
TPVSE	SE$^{\text{reg-ion}}$; TP$^{\text{ACEi,DPP4i}}$; PV/VS $^{\text{DPP4i}}$
CCTE	TE$^{\text{ACEi,DPP4i}}$
SLVNR	LV$^{\text{DPP4i,sti-glu}}$; SL/NR/VN$^{\text{DPP4i}}$
PCFSA	**CF**$^{\text{ACEi}}$
LTPDE	TP$^{\text{ACEi,DPP4i}}$; LT$^{\text{DPP4i}}$
LFTFH	**TF**$^{\text{ACEi,DPP4i}}$; LF$^{\text{ACEi}}$
DICTLPDTE	TE$^{\text{ACEi,DPP4i}}$; **LP**/TL$^{\text{DPP4i}}$
TVME	ME$^{\text{ACEi,DPP4i}}$; TV/VM $^{\text{DPP4i}}$
NFVA	**NF**$^{\text{ACEi,DPP4i}}$; VA$^{\text{DPP4i}}$
FVDK	VD$^{\text{DPP4i}}$
LVVSTQTA	LV$^{\text{DPP4i,sti-glu}}$; TQ$^{\text{ACEi,DPP4i}}$; ST$^{\text{ACEi}}$; TA/QT/VS/VV$^{\text{DPP4i}}$

ACEi – angiotensin converting enzyme inhibitor; DPP4i – dipeptydyl peptidase IV inhibitor; AOX – antioxidative; sti-glu – stimulating absorption of glucose; sti-sub – stimulating the release of vasoactive substances; ubi – activator of ubiquitin-mediated proteolysis; reg-ion – regulator of ion flow; i-ren – renin inhibitor, acan-anticancer; CaMPDEi – inhibitor CaMPDE (calmodulin--dependent cyclic nucleotide phosphodiesterase); ist – immunostimulating; mod – immunomodulating; ab – antibacterial; at – antithrombotic; anm – antiamnestic; op – opioid; reg-sto – regulator of stomach mucosal membrane action; **bold** – peptide with Score > 0.5; grey – peptide with confirmed bioactivity

Table 2

Peptides theoretically released from bovine milk proteins due to the action of bromelain

Substrate for hydrolysis: *α*-lactalbumin	
Parent peptide	Encrypted bioactive motif
MMSFVSLLLVG	MM^{ACEi,DPP4i,AOX}; **LL**/LV$^{DPP4i,sti\text{-}glu}$; **LLL**$^{sti\text{-}sub}$; **SF/**VG$^{ACEi,\ DPP4i}$; SL/VS^{DPP4i}
ILFHA	IL$^{ACEi,DPP4i,sti\text{-}glu}$; **LF**^{ACEi}; HA^{DPP4i}
EQLT$^{K(score\ =\ 0.05)}$	EQLTK^{ab}
CEVFRELK	**FR/VF/**EV$^{ACEi,\ DPP4i}$; LK/EL^{AOX}
VSLPEWVCTTFHTSG	**TF/EW/**EV$^{ACEi,\ DPP4i}$; **LP/WV/LPEWVCTTFH**/SL/HT/TS/VS/TT ^{DPP4i}; SG^{ACEi}
DTQA	TQ$^{ACEi,\ DPP4i}$; QA^{DPP4i}
IVQNNDSTEY	EY/TE$^{ACEi,\ DPP4i}$; IV$^{sti\text{-}glu}$; ST/IVQ^{ACEi}; NN/QN/ND/VQ^{DPP4i}
LFQINNK	**FQ**$^{ACEi,\ DPP4i}$; **LF**/NK^{ACEi}; NN/IN/QI^{DPP4i}
IWCK	**IW**$^{ACEi,\ DPP4i}$; **WC**^{DPP4i}
DDQNPHSSNICNI-SCDK	**PH**$^{ACEi,\ DPP4i}$; PHS^{AOX}; NP/HS/QN/DQ/QNPHSSNICN^{DPP4i}
FLDDDLTDDIMCVK	VK$^{ACEi,\ DPP4i}$; **FL/IM/**LT/TD^{DPP4i}
ILDK	IL$^{ACEi,\ DPP4i,\ sti\text{-}glu}$
LCSEK	SE$^{reg\text{-}ion}$; EK$^{ACEi,\ DPP4i}$
LDQWLCEK	**VL**/EK$^{ACEi,\ DPP4i}$; **QV**/DQ^{DPP4i}
Substrate for hydrolysis: β-lactoglobulin	
CLLLA	**LL**$^{DPP4i,sti\text{-}glu}$; **LLL**$^{sti\text{-}sub}$; LA$^{ACEi,\ DPP4i,ubi}$
LTCG	LT^{DPP4i}
LIVTQTMK	LI $^{DPP4i,\ sti\text{-}glu}$; IV $^{sti\text{-}glu}$; MK/TM/QT/TQ/VT^{DPP4i}; **TW/**LIVTQ^{ACEi}
LDIQ$^{K(score\ =\ 0.11)}$	LDIQK/QK^{ACEi}; IQ^{DPP4i}
SDISLLDA	**LL**$^{DPP4i,sti\text{-}glu}$; SL^{DPP4i}; DA^{ACEi}
PLRVY	VY^{ACEi,DPP4i,AOX}; **PL**$^{ACEi,\ DPP4i}$; **LR/**RVY^{ACEi}
VEELK	EE$^{sti\text{-}sub}$; LK/EL^{AOX}; VE^{ACEi}
PTPEG	TP/PT/EG$^{ACEi,\ DPP4i}$
DLEILLQK	**LL**$^{DPP4i,sti\text{-}glu}$; EI$^{ACEi,\ DPP4i}$; IL^{ACEi,DPP4i,AOX}; LQ/OK^{ACEi}
WENDECA	**WE/**ND$^{ACEi,\ DPP4i}$
LNENK	LN$^{ACEi,\ DPP4i}$; NE^{DPP4i}; NK^{ACEi}
VLVLDTDY	VL/LV$^{DPP4i,sti\text{-}glu}$; DY$^{ACEi,\ sti\text{-}ion,}$; TDY^{AOX}; TD^{DPP4i}; LVL^{ACEi}
LLFCMENSA	**LL**$^{DPP4i,sti\text{-}glu}$; ME$^{ACEi,\ DPP4i}$; **LLF/LF/**CMENSA^{ACEi}
EPEQSLA	LA$^{ACEi,\ DPP4i,ubi}$; EP/QS/SL^{DPP4i}
CQCLVRTPEVDDEA	LV$^{DPP4i,sti\text{-}glu}$; LVR/LVRT/EA^{ACEi}; TP/VR/EV$^{ACEi,\ DPP4i}$; VD^{DPP4i}

Cont. Table 2

LPMHIRLSF-NPTQLEEQCHI	IR$^{\text{ACEi, DPP4i,AOX,i-ren, CaMPDEi}}$; **SF**/PT/TQ$^{\text{ACEi, DPP4i}}$; **PM/FN/LP/MH**/NP/QL/HI$^{\text{DPP4i}}$; **MHIRL**$^{\text{AOX}}$; HIRL/HIR/LEE$^{\text{ACEi}}$; EE$^{\text{sti-sub}}$
Substrate for hydrolysis: κ-casein	
SFFLVVTILA	LA$^{\text{ACEi, DPP4i,ubi}}$; IL$^{\text{ACEi,DPP4i,sti-glu}}$; LV$^{\text{DPP4i,sti-glu}}$; **SF**$^{\text{ACEi, DPP4i}}$; TI/VT/VV$^{\text{DPP4i}}$; **FFL**$^{\text{ACEi}}$
LTLPFLG	**LPF/LG**$^{\text{ACEi}}$; **PF/FL/LP**/TL/LT$^{\text{DPP4i}}$
QEQNQEQPIRCEK	IR$^{\text{ACEi, DPP4i,AOX,i-ren, CaMPDEi}}$; **EK**$^{\text{ACEi, DPP4i}}$; **PI**/QP/QN/NQ/QE$^{\text{DPP4i}}$
DERFFSDK	**RF**$^{\text{ACEi}}$
IPIQY	**IP**$^{\text{ACEi, DPP4i}}$; IQY$^{\text{AOX,ACEi,ab}}$; **PI**/IPI/IPIQY/QY/IQ$^{\text{DPP4i}}$
VLSRY	VL$^{\text{DPP4i,sti-glu}}$; **RY**$^{\text{ACEi}}$
LINNQFLPY	LI$^{\text{DPP4i,sti-glu}}$; **FL/QF/LP/PY**/IN/NN/NQ$^{\text{DPP4i}}$
VRSPA	VR$^{\text{ACEi, DPP4i}}$; **PA/SP**$^{\text{DPP4i}}$; VRSP$^{\text{ACEi}}$
QILQWQVLSNTVPA	VL$^{\text{DPP4i,sti-glu}}$; IL$^{\text{ACEi,DPP4i,sti-glu}}$; **QW/WQ**/PA/QI/NT/QV/TV$^{\text{DPP4i}}$; **VP**$^{\text{ACEi, DPP4i}}$; LQ$^{\text{ACEi}}$
SCQA	QA$^{\text{DPP4i}}$
QPTTMA	PT$^{\text{ACEi, DPP4i}}$; **MA/**QP/TM/TT/PT$^{\text{DPP4i}}$
RHPHPHLSFMA	**SF/HP/PH**$^{\text{ACEi, DPP4i}}$; **RHPHP**$^{\text{AOX,ACEi}}$; **PHL/HPHL/**HPH$^{\text{AOX}}$; HL$^{\text{ACEi, DPP4i,AOX}}$; **MA/**RH$^{\text{DPP4i}}$; **PHPHLSF**$^{\text{chymi}}$
IPPK	**PP/IP** $^{\text{ACEi, DPP4i}}$; **IPP** $^{\text{ACEi}}$; PK $^{\text{DPP4i}}$; **PPK** $^{\text{ACEi, at}}$
NQDK$^{(\text{score} = 0.07)}$	NQDK$^{\text{at}}$; NQ/QD$^{\text{DPP4i}}$
TEIPTINTIA	**IP**/PT/IA/EI/TE$^{\text{ACEi, DPP4i}}$; IN/TI/NT$^{\text{DPP4i}}$; EIPT$^{\text{ab}}$
EPTSTPTTEA	TP/PT/TE$^{\text{ACEi, DPP4i}}$; EP/TS/TT $^{\text{DPP4i}}$; ST/EA$^{\text{ACEi}}$
VESTVA	VE$^{\text{ACEi, DPP4i}}$; VA/ES/TV$^{\text{DPP4i}}$; ST$^{\text{ACEi}}$
TLEDSPEVIESPPEIN-TVQVTSTA	**PP**/EI/EV$^{\text{ACEi, DPP4i}}$; TSTA$^{\text{AOX}}$; VQV/IE$^{\text{ACEi}}$; **SP/**IN/TL/ST/TA/VI/TS/NT/QV/ES/VQ/VT$^{\text{DPP4i}}$
Substrate for hydrolysis: *β*-casein	
RELEELNVPG	**PG**$^{\text{ACEi, DPP4i, anm, at, reg-sto}}$; LN/VP$^{\text{ACEi, DPP4i}}$; VE$^{\text{ACEi, DPP4i}}$; EL$^{\text{AOX}}$; NV$^{\text{DPP4i}}$; LEE$^{\text{ACEi}}$; EE$^{\text{sti-sub}}$
EIVESLSSSEESI-TRINK	EI/VE$^{\text{ACEi, DPP4i}}$; RI/SL/SI/IN/TR/ES$^{\text{DPP4i}}$; SSS/SE/EE$^{\text{sti-sub}}$; NK$^{\text{ACEi}}$; IV$^{\text{sti-glu}}$
FQSEEQQQTE-DELQDK	**FQ/**TE$^{\text{ACEi, DPP4i}}$; SE/EE$^{\text{sti-sub}}$; LQ$^{\text{ACEi}}$; EL$^{\text{AOX}}$; QS/QD/QQ/QT$^{\text{DPP4i}}$
IHPFA	**HP**$^{\text{ACEi, DPP4i}}$; **PF/FA**/IH$^{\text{DPP4i}}$
QTQSLVY	VY/TQ$^{\text{ACEi, DPP4i}}$; LVY$^{\text{ACEi}}$; LV $^{\text{DPP4i, sti-glu}}$; VY$^{\text{AOX}}$; SL/QS/QT $^{\text{DPP4i}}$
PFPG	**FP**$^{\text{ACEi, DPP4i}}$; **PF** $^{\text{DPP4i}}$; **PG**$^{\text{ACEi, DPP4i, anm, at, reg-sto}}$
PIHNSLPQNIPPLTQT-PVVVPPFLQPEVMG	**MG/PP/PL/IP**/LQP/LQ/PQ/TP/VP/TQ/EV$^{\text{ACEi,DPP4i}}$; **IPP/VVVPPF/VPP/**TPVVVPPFLQP/SLPQN/NIPPLTQTPV/VVPP/ LTQTPVVVPPF$^{\text{ACEi}}$; **PF/FL/PPL/FLQP/LP/LPQNIPPL/LPQNIPP/PI/**QP/VM/SL/IPPLTQTPV/PV/IH/LT/QN/QT/VV$^{\text{DPP4i}}$; VVV$^{\text{acan}}$

Cont. Table 2

EMPFP$^{K(score = 0.77)}$	**FP**$^{ACEi,\ DPP4i}$; **EMPFPK** ^{ACEi}; **PF/MP**/PK^{DPP4i}
FLLY	**LL** $^{DPP4i,sti\text{-}glu}$; **FL**^{ACEi,DPP4i}; **LY**^{ACEi,AOX}; **LLY**^{ist}
PFPIIV	**FP**^{ACEi,DPP4i}; II$^{DPP4i,sti\text{-}glu}$; IV$^{sti\text{-}glu}$; **PI/PF**^{DPP4i}; **FPIIV**^{ACEi}
QQPVLG	VL $^{DPP4i,sti\text{-}glu}$; **QQ**/QP/PV^{DPP4i}; **LG**^{ACEi}
PVQPFTESQSLT TDVENLHLP PLLLQSWMHQ PHQPLPPTVMFP PQSVLSLSQSK	**WM/MF/FP/PP/PL/PH**/PQ/PT/VE/TE^{ACEi,DPP4i}; **PF/SW/PPL/LP/MH//** VM/SL/NL/PV/TL/LT/QS/SK/SV/VQ/TD/ES/TV^{DPP4i}; **PLP/LPP/QSWM-HQPHQ/LQSW**/LHLP/NLHLP/LQ/TESQSLT/LTLTDVE^{ACEi}; **LLL**$^{sti\text{-}sub}$; **LL** $^{DPP4i,sti\text{-}glu}$; HL$^{ACEi,\ DPP4i,AOX}$; LH^{AOX,DPP4i}; LHL/PHQ^{AOX}; VL $^{DPP4i,sti\text{-}glu}$
VLPVPEK	VP/EK$^{ACEi,\ DPP4i}$; VLP^{ACEi}; **LP/**QT^{DPP4i}
PQRDMPIQA	PQ$^{ACEi,\ DPP4i}$; **DM/**PQR^{ACEi}; RDMPIQ^{AOX}; **MP/PI**/IQ/QA^{DPP4i}
PVRG	VR$^{ACEi,\ DPP4i}$; **RG**/PV^{DPP4i}
Substrate for hydrolysis: a_{S2}-casein	
FFIFTCLLA	**LL** $^{DPP4i,sti\text{-}glu}$; LA$^{ACEi,\ DPP4i,ubi}$; **IF**^{ACEi}
NTMEHVSSSE-ESIISQETY	ME$^{ACEi,\ DPP4i}$; II$^{DPP4i,sti\text{-}glu}$; TY^{DPP4i,AOX}; SSS$^{sti\text{-}sub}$; SE$^{reg\text{-}ion}$; EE$^{sti\text{-}sub}$; TM/SI/HV/NT/EH/VS/QE/ES/ET^{DPP4i}
INPSK	NP/PS/IN/SK^{DPP4i}
ENLCSTFCK	ST^{ACEi}; **TF**$^{ACEi,\ DPP4i}$; NL^{DPP4i}
EVVRNA	VR/EV$^{ACEi,\ DPP4i}$; RN/LA/VV^{DPP4i}
NEEEY	EY$^{ACEi,\ DPP4i}$; NE^{DPP4i}; EE/EEE$^{sti\text{-}sub}$
SSSEESA	SSS$^{sti\text{-}sub}$; SE$^{reg\text{-}ion}$; EE$^{sti\text{-}sub}$; ES^{DPP4i}
TEEVK	VK/EV/TE$^{ACEi,\ DPP4i}$; EE$^{sti\text{-}sub}$
ITVDDK	VD/TV^{DPP4i}
FPQY	**FP**^{ACEi,DPP4i}; PQ/QY^{DPP4i}
LNEINQFY	LN/EI$^{ACEi,\ DPP4i}$; **FY**^{ACEi}; **QF**/IN/NQ/NE^{DPP4i}
PIVLNPWDQVK	**PW**^{AOX,DPP4i}; **PWD**^{AOX}; IV$^{sti\text{-}glu}$; VL $^{DPP4i,\ sti\text{-}glu}$; LN/VK^{ACEi,DPP4i}; **WD/PL/**NP/DQ/QV^{DPP4i}; **LNP**^{ACEi}
VPITPTLNREQLST-SEENSK	TP/LN/PT/VP/^{ACEi,DPP4i}; SE$^{reg\text{-}ion}$; EE$^{sti\text{-}sub}$; **PI/**QL/NR/VPITPT/TL/ST/TS/^{DPP4i};
TVDMESTEVFTK	**VF**/ME/EV/TE^{ACEi,DPP4i}; **DM/**ST^{ACEi}; ES/VD/TK/TV^{DPP4i}
LTEEEK	EE/EEE$^{sti\text{-}sub}$; TE/EK^{ACEi,DPP4i}; LT^{DPP4i}
LPQY	PQ^{ACEi,DPP4i}; **LP/**QY^{DPP4i}
PWIQPK	**PW**^{AOX,DPP4i}; **PWI**^{AOX}; IQP^{ACEi,DPP4i}; **WI/WIQP**/QP/IQ/PQ^{DPP4i}
VIPY	**IP**^{ACEi,DPP4i}; **IPY**^{ACEi}; **PY/**VI^{DPP4i}
NRLNFLK	**NF/**RL/LN^{ACEi,DPP4i}; **LNF**^{ACEi}; **FL/**NR^{DPP4i}; LK^{AOX}
ISQRY	**RY**^{ACEi}
QHQK	QH^{ACEi}; QK^{DPP4i}

Cont. Table 2

Substrate for hydrolysis: a_{S1}-casein	
HPIK	**HP**^{ACEi}; **PI**^{DPP4i}
LPQEVLNENLLRFFVA	**FP/VF/**PQ^{ACEi,DPP4i}; **LL** $^{DPP4i,\ sti\text{-}glu}$; **LR**^{ACEi}; **LLR**^{AOX}; VL $^{DPP4i,\ sti\text{-}glu}$; VLNENLLR^{ab}; **RF**^{ACEi}; **LP/**NL/VA/QE/NE^{DPP4i}
PFPQVFG	PQ/LN/EV^{ACEi,DPP4i}; **FG**^{ACEi}; **PF/**QV ^{DPP4i}
VNELSK	EL^{AOX}; SK/VN/NE ^{DPP4i}
SESTEDQA	TE^{ACEi,DPP4i}; SE$^{reg\text{-}ion}$; ST^{ACEi}; QA/DQ/ES ^{DPP4i}
MEDIK	ME^{ACEi,DPP4i}
EMEA	ME^{ACEi,DPP4i}; EA^{ACEi}
ESISSSEEIVPNSVEQK	VP/EI/VE^{ACEi,DPP4i}; SE$^{reg\text{-}ion}$; EE$^{sti\text{-}sub}$; IV$^{sti\text{-}glu}$; SSS$^{sti\text{-}sub}$; QK^{ACEi}; PN/SI/SV/ES ^{DPP4i}
HIQK	HI/IQ/QK ^{DPP4i}
EDVPSERY	VP^{ACEi,DPP4i}; SE$^{reg\text{-}ion}$; **RY**^{ACEi}; PS ^{DPP4i}
LEQLLRLK	**RL**^{ACEi,DPP4i}; **LL** $^{DPP4i,\ sti\text{-}glu}$; **LR**^{ACEi}; **LLR/**LK^{AOX}; QL^{DPP4i}
VPQLEIVPNSA	PQ/VP/EI^{ACEi,DPP4i}; IV$^{sti\text{-}glu}$; PN/QL^{DPP4i}
TTMPLW$^{(score\ =\ 0.74)}$	**TTMPLW** $^{ACEi,,\ mod,\ op}$; **LW**^{ACEi,DPP4i,AOX}; **PLW**^{ACEi}; **MP/**TM/TT ^{DPP4i}
VPLG	VPL $^{DPP4i,anm,\ sti\text{-}sub,}$; **PL/**VP^{ACEi,DPP4i}; **LG**^{ACEi}; **PLG**$^{ACEi,\ op}$
QLDA	DA^{ACEi}; QL ^{DPP4i}
PSFSDIPNPIG	**SF/IP**^{ACEi,DPP4i}; **IG**^{ACEi}; **PI/**NP/PN/PS ^{DPP4i}
EERLHSMK	**RL**^{ACEi,DPP4i}; EE$^{sti\text{-}sub}$; LHS^{AOX}; LH^{AOX,DPP4i}; MK/HS ^{DPP4i}
EPMIG	**IG**^{ACEi}; **PM/MI/**EP^{DPP4i}
VNQELA	LA$^{ACEi,\ DPP4i,ubi}$; EL^{AOX}; NQ/VN/QE^{DPP4i}
PELFRQFY	PEL/EL^{AOX}; **FR**^{ACEi,DPP4i}; **LF/FY/LFR/LFRQ**^{ACEi}; **QF**^{DPP4i}
SENSEK	SE$^{reg\text{-}ion}$; **EK**$^{ACEi,\ DPP4i}$
Substrate for hydrolysis: serum albumin	
WVTFISLLLLFSSA	**LL** $^{DPP4i,sti\text{-}glu}$; **LLL**$^{sti\text{-}sub}$; **TF**^{ACEi,DPP4i}; **LF/LLF**^{ACEi}; **WV/**SL/VT ^{DPP4i}
LVLIA	LI/VL/LV$^{DPP4i,sti\text{-}glu}$; IA^{ACEi,DPP4i}; LVL^{ACEi}
VFRRDTHK	**FR/VF/RR**^{ACEi,DPP4i}; HK^{ACEi}; TH ^{DPP4i}
TCVA	VA ^{DPP4i}
SEIA	IA/EI^{ACEi,DPP4i}; SE$^{reg\text{-}ion}$
HRFK	**RF**^{ACEi}; HR^{DPP4i}
EEHFK	**HF/**EH^{DPP4i}; EE$^{sti\text{-}sub}$
LVLIA	LI/VL/LV$^{DPP4i,sti\text{-}glu}$; IA^{ACEi,DPP4i}; LVL^{ACEi}
FSQY	QY ^{DPP4i}
LQQCPFDEHVK	VK^{ACEi,DPP4i}; HK^{ACEi}; **PF/**LQQ/QQ/HV/EH^{DPP4i}

Cont. Table 2

LVNELTEFA	TE^{ACEi,DPP4i}; LV$^{DPP4i,sti\text{-}glu}$; **EF**$^{i\text{-}ren,\ CaMPDEi}$; EL^{AOX}; **FA/**LT/VN/NE^{DPP4i}
DESHA	HA/SH/ES^{DPP4i}
SLHTLFG	LH^{AOX,DPP4i}; LHT^{AOX}; **LF/FG**^{ACEi}; TL/FL/HT^{DPP4i}
DELCK	EL^{AOX}
SLRETY	TY^{AOX,DPP4i}; **LR**^{ACEi}; SL/ET^{DPP4i}
DCCEK	EK^{ACEi,DPP4i}
QEPERNECFLSHK	**CF/**HK^{ACEi}; **FL/**RN/EP/SH/QE/NE^{DPP4i}
DDSPDLPK	**DLP**^{ACEi}; **LP/**SP/PK ^{DPP4i}
PDPNTLCDEFK	**EF**$^{i\text{-}ren,\ CaMPDEi}$; DP/PN/TL/NT^{DPP4i}
RRHPY	**RR/HP**^{ACEi,DPP4i}; **PY/**RH^{DPP4i}
PELLY	PEL/EL^{AOX}; **LL** $^{DPP4i,sti\text{-}glu}$; **LLY**^{ist}; **LY**^{ACEi,AOX}
VFQECCQA	**FQ/VF**^{ACEi,DPP4i}; QA/QE^{DPP4i}
CLLPK	**LL** $^{DPP4i,sti\text{-}glu}$; **LLP**^{ACEi}; **LP/**PK ^{DPP4i}
IETMREK	EK^{ACEi,DPP4i}; IE^{ACEi}; **MR/**TM/ET^{DPP4i}
RQRLRCA	**RL**^{ACEi,DPP4i}; **LR**^{ACEi}
SIQK	QK^{ACEi}; SI/IQ ^{DPP4i}
WSVA	**WS/**VA/SV ^{DPP4i}
RLSQK	**RL**^{ACEi,DPP4i}; QK^{ACEi}
EFVEVTK	**EF**$^{i\text{-}ren,\ CaMPDEi}$; EV/VE^{ACEi,DPP4i}; TK/VT ^{DPP4i}
LVTDLTK	LV$^{DPP4i,sti\text{-}glu}$; LT/TD/TK/VT^{DPP4i}
ECCHG	HG^{ACEi}
DLLECA	**LL** $^{DPP4i,sti\text{-}glu}$
DDRA	RA$^{ACEi,\ DPP4i,ubi}$; DR^{DPP4i}
ICDNQDTISSK	DN/NQ/QD/SK/TI ^{DPP4i}
PLLEK	**LL** $^{DPP4i,sti\text{-}glu}$; **PL/**EK^{ACEi,DPP4i}; LEK^{ACEi}
SHCIA	IA^{ACEi,DPP4i}; SH^{DPP4i}
EVEK	EV/VE/EK^{ACEi,DPP4i}
IPENLPPLTA	**PL/PP/IP**^{ACEi,DPP4i}; **LPP**^{ACEi}; **PPL/LP/**NL/LT/TA^{DPP4i}
SFLY	**LY**^{ACEi,AOX}; **SF**^{ACEi}; **FL**^{DPP4i}
SRRHPEY	**RR/HP/**EY^{ACEi,DPP4i}; RH^{DPP4i}
VSVLLRLA	**RL**^{ACEi,DPP4i}; **LL/**VL$^{DPP4i,sti\text{-}glu}$; **LLR**^{AOX}; LA$^{ACEi,\ DPP4i,ubi}$; **LR**^{ACEi}; SV/VS^{DPP4i}
TLEECCA	EE$^{sti\text{-}sub}$; LEE^{ACEi}; TL^{DPP4i}
DDPHA	**PH**^{ACEi,DPP4i}; PHA^{AOX}; DP/HA^{DPP4i}

Cont. Table 2

STVFDK	**FDK**/ST^{ACEi}; **VF**^{ACEi,DPP4i}; TV ^{DPP4i}
HLVDEPQNLIK	HL$^{ACEi,\ DPP4i,AOX}$; LV$^{DPP4i,sti\text{-}glu}$; LI$^{DPP4i,sti\text{-}glu}$; PQ^{ACEi,DPP4i}; NL/EP/QN/VD^{DPP4i}
QNCDQFEK	EK^{ACEi,DPP4i}; **QF**/QN/DQ^{DPP4i}
FQNA	**FQ**/NA/QN ^{DPP4i}
LIVRY	**RY**^{ACEi}; VR^{ACEi,DPP4i}; LI$^{DPP4i,sti\text{-}glu}$; IV$^{sti\text{-}glu}$; VRY/IVR^{ACEi,}
VPQVSTPTLVEVSR-SLG	PQ/TP/PT/VP/EV/VE^{ACEi,DPP4i}; LV$^{DPP4i,sti\text{-}glu}$; **LG**/STE/LVE^{ACEi}; SL/TL/VS/QV ^{DPP4i}
TRCCTK	TR/TK^{DPP4i}
PESERMPCTEDY	TE^{ACEi,DPP4i}; SE$^{reg\text{-}ion}$; DY$^{ACEi,\ sti\text{-}ion,}$; **MP/RM/**ES^{DPP4i}
LSLILNRLCVLHEK	**RL**/LN/EK^{ACEi,DPP4i}; LHE^{AOX}; IL$^{ACEi,\ sti\text{-}glu}$; LI/VL$^{DPP4i,sti\text{-}glu}$; LH^{AOX,DPP4i}; SL/NR/HE^{DPP4i}
TPVSEK	TP/EK^{ACEi,DPP4i}; SE$^{reg\text{-}ion}$; PV/VS^{DPP4i}
CCTESLVNRRPCFSA	**RP/RR/**TE^{ACEi,DPP4i}; LV$^{DPP4i,sti\text{-}glu}$; **CF**^{ACEi}; SL/NR/VN/ES^{DPP4i};
LTPDETY	TP^{ACEi,DPP4i}; TY^{AOX,DPP4i}; LT/ET^{DPP4i}
FDEK	EK^{ACEi,DPP4i}
LFTFHA	**TF**^{ACEi,DPP4i}; **LF**^{ACEi}; HA^{DPP4i}
DICTLPDTEK	TE/EK^{ACEi,DPP4i}; **LP**/TL^{DPP4i}
LVELLK	**LL**/LV$^{DPP4i,sti\text{-}glu}$; LK/EL^{AOX}; VE^{ACEi,DPP4i}; LVE^{ACEi}
TEEQLK	TE^{ACEi,DPP4i}; LK^{AOX}; EE$^{sti\text{-}sub}$; QL^{DPP4i}
TVMENFVA	**NF**/ME^{ACEi,DPP4i}; VM/VA/TV^{DPP4i}
FVDK	VD^{DPP4i}
LVVSTQTA	LV$^{DPP4i,sti\text{-}glu}$; TQ^{ACEi,DPP4i}; ST^{ACEi}; TA/QT/VS/VV^{DPP4i}

ACEi – angiotensin converting enzyme inhibitor; DPP4i – dipeptydyl peptidase IV inhibitor; AOX – antioxidative; sti-glu – stimulating absorption of glucose; sti-sub – stimulating the release of vasoactive substances; ubi – activator of ubiquitin-mediated proteolysis; reg-ion – regulator of ion flow; i-ren – renin inhibitor; chymi – chymosin inhibitor; acan-anticancer; CaMPDEi – inhibitor CaMPDE (calmodulin-dependent cyclic nucleotide phosphodiesterase); ist – immunostimulating; mod – immunomodulating; ab – antibacterial; at – antithrombotic; anm – antiamnestic; op – opioid; reg-sto – regulator of stomach mucosal membrane action; **bold** – peptide with Score > 0.5; grey – peptide with confirmed bioactivity

All milk proteins were theoretically hydrolyzed using papain and bromelain. These enzymes were proven potent to produce bioactive peptides form foods in vitro (IWANIAK et al. 2020a). For example, papain was effective in producing ACE inhibitors and antioxidative peptides from gelatin-derived tilapia skin (CHOOPINHAM et al. 2015), whereas bromelain was applied to hydrolyze clam proteins to generate antibacterial peptides (ZAMBROWICZ et al. 2013). Moreover, papain and bromelain can be used as substitutes for rennet in the cheesemaking (ARLENE et al. 2015). They also

offer an alternative to animal-derived coagulants when taking into account ethic, religious, and economic concerns (AKTAYEVA et al. 2018). Additionally, papain and bromelain were tested during the hydrolysis of goat and bovine milks to produce peptidic ACE inhibitors (SHU et al. 2018). Thus, our predictions involving these enzymes might be suitable in the design of food rich in bioactive peptides. However, the scientists highlight that successful prediction of peptides' release from proteins depends on the regular update of the database with the new sequences and/or completing the information about the bioactivities of peptides and specificity of enzymes (UDENIGWE 2014).

SOŁOWIEJ et al. (2016) highlighted the role of casein as a valuable ingredient that may be incorporated to foods due to its functional properties, like e.g., consistency and fat emulsification ability. *In silico* hydrolysis of milk proteins with two enzymes also revealed that caseins were good sources of biopeptides. It especially concerned *β*-casein, being the richest source of parent peptides and, thus, abundant in bioactive motifs. These proteins produced the longest parent sequences (see above rules). Among them, the longest fragment (i.e. PVQPFTESQSLTLTDVENLHLPPLLLQ SWMHQPHQPLPPTVMFPPQSVLSLSQSK) was produced during the hydrolysis of *β*-casein with bromelain (Table 2). It consisted of 55 residues and contained 10 peptides with a dual bioactivity (ACE/DPP-IV inhibitors), 9 ACE inhibitors, 18 DPP-IV inhibitors, and 1 peptide with anticancer function. Multiple bioactive motifs found in the PVQPFTESQSLTLT-DVENLHLPPLLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK parent peptide suggest its potential to be another strategic zone of *β*-casein. So far, the term "strategic zone" concerned the region between the 60^{th} and 70^{th} residue (PFAQTQSLVYPFPGPIHNSL) of *β*-casein. According to the scientific reports, *β*-casein is the richest source of biopeptides, including its strategic zone, due to the presence of the motifs eliciting ACE-inhibiting, immunostimulating, and opioid effects (HAQUE and CHAND 2008). When looking at our results, the part of this strategic zone (PIHNSL-motif) was encrypted in another parent peptide i.e., PIHNSLPQNIPPLTQTPVVVP-PFLQPEVMG, being a product of *β*-casein hydrolysis with bromelain (Table 2). The PIHNSL-motif contained two sequences, namely IH and PI, acting as DPP-IV inhibitors.

Regardless of the enzyme applied, the function of the great majority of parent peptides that were released from all milk proteins remained unknown (Table 1 and Table 2). This dearth of data encouraged us to further employ the fragmentomic analysis of milk proteins hydrolyzed (*in silico*) by the two tested enzymes. It showed that the released parent peptides were abundant in the motifs with ACE and DPP-IV inhibiting activ-

ities. Briefly, these peptides are involved in the regulation of blood pressure and glucose level, respectively, thereby eliciting antihypertensive and antidiabetic effects (RIVERO-PINO et al. 2020). These motifs could be detected in the parent sequences due to their abundance in the BIOPEP-UWM database, which increases the probability of finding them encrypted in a parent peptide, especially when they are di- or tripeptides.

When looking at the structure (sequence) of parent peptides being the sources of ACE-inhibiting motifs, they were rich in amino acids with non-polar side chains, like glycine, valine, leucine, isoleucine or those possessing a ring, like proline, phenylalanine and/or tryptophan. The studies on the structure-activity analysis of peptides demonstrated the impact of N-terminal glycine, isoleucine, leucine, or valine on the ACE inhibitory activity. In turn, C-terminus of these peptides was composed of "ring containing amino acids" (ABACHI et al. 2019). Thus, the abundance of parent peptides in such residues affected the presence of ACE inhibitors. Moreover, loads of ACE inhibitors composed of the above-mentioned residues had a PeptideRanker Score > 0.5 (bold sequences). The PeptideRanker program estimates the likelihood of a peptide to be bioactive (with no indication of particular bioactivity). Its value ranges from 0.00 to 1.00, and the Score of a potentially active peptide should exceed 0.5 (MOONEY et al. 2012). According to the scientific reports, amino acids, like tryptophan or proline, were typical of peptides with DPP-IV inhibitory activity (RIVERO-PINO et al. 2020). It was also confirmed in the present study, where the majority of DPP-IV-inhibiting motifs having a PeptideRanker Score > 0.50 were abundant in such residues.

The fragmentomic analysis of the products of milk protein hydrolysis by bromelain and papain revealed that the antioxidant bioactivity was the third dominant bioactivity of the parent peptides. Antioxidant peptidic motifs were mostly rich in the amino acids possessing a ring or an apolar side chain (N-end) and proline or histidine, leucine, and valine (C-end). These observations were consistent with findings obtained by other authors who employed the chemometric analysis (i.e. multivariate regression) to find the "structure-activity" relationships of antioxidant peptides identified in the food-derived protein hydrolysates (UDENIGWE and ALUKO 2011).

The other motifs present in parent peptides acted mostly as antiamnestic, glucose absorption-simulating, antibacterial, antithrombotic, enzyme inhibiting agents etc. Their full list is found in Table 1 and Table 2. To the best of our knowledge, there are no literature works providing information on the structural nature of such peptides, which is probably due to several factors that impede the structure-function analysis of molecules.

These include, e.g., difficulties with the collection of an appropriate number of samples (understood as peptide sequences), variables to form a data matrix as well as problems with applying the appropriate measure of activity to run QSAR (i.e., quantitative structure-activity relationship) analysis. More details related to QSAR studies of peptides, including the pros and cons of methods used, were described by IWANIAK et al. (2015). Thus, the fragmentomic analysis of parent peptides may be useful for the brief finding of some regularities in motifs with specific activities assuming there is a plenty of peptides representing the specific activity. Moreover, calculation of PeptideRanker Scores might be one of the steps of the hybrid approach (see above), like the initial selection of relatively strong peptides (*in silico* part of the study) followed by their identification in the hydrolysate and determination of their bioactivity *in vitro*.

According to DALIRI et al. (2007), many scientific reports provide the data about peptides showing one biological effect, whereas relatively small amount of data refer to the multifunctional sequences. Thus, due to the multiple health benefits, peptides exhibiting more than one biological activity are in the focus of the scientific interests. It especially concerns the identification of such sequences in hydrolysates (DALIRI et al. 2007). Therefore, databases can offer a supportive tool to acquire the knowledge on multi-active peptides. Several functions were ascribed to the motifs encrypted in parent peptides theoretically produced from milk proteins hydrolyzed with papain and bromelain (see Table 1 and Table 2). A great majority of them had dual functions, like e.g. IW acting as ACE/DPP-IV inhibitor (parent source: IWCK released from α-lactalbumin, enzyme used: papain/bromelain). One peptide, PG (see below, bold font), showing 5 bioactivities, was encrypted in the following parent peptides: LNV**PG** and PF**PG** (source: *β*-casein hydrolyzed with papain, see Table 1) and RELEELNV**PG** (source: *β*-casein hydrolyzed with bromelain, see Table 2). PG was confirmed as ACE/DPP-IV inhibitor, stimulator of the action of stomach mucosa membrane as well as antiamnestic and antithrombotic peptide.

It needs to be highlighted that, although the bioinformatic analysis of multifunctional peptides is useful and easy, it has some limitations. They were discussed by IWANIAK and MOGUT (2020). Briefly, such an analysis is based on the so-called positive selection assuming that a peptide of interest matches the sequences present in the database used (IWANIAK and MOGUT 2020). At the time of data analysis, the BIOPEP-UWM database contained the information about 3,200 bioactive peptide sequences. Currently, it contains over 4,000 sequences. Thus, some authors postulate the regular update of the database (UDENIGWE 2014) to get more knowledge on the additional functions of peptides before running the *in vitro* part of

the experiment. Regular update of databases should be a golden standard to ensure the high quality of data when performing any type of computations, including those applied in our protocol.

Several parent peptides that were *in silico* released from milk proteins were known as bioactive themselves (Table 1 and Table 2), i.e.: LDIQK$^{0.11}$ (ACE inhibitor, source: *β*-lactoglobulin, enzyme applied: papain and bromelain), IPIQY$^{0.36}$ (DPP-IV inhibitor, source: *κ*-casein, enzyme applied: papain and bromelain), NQDK$^{0.07}$ (antithrombotic peptide, source: *κ*-casein, enzyme applied: papain and bromelain), TTMPLW$^{0.74}$ (ACE inhibitor/immunomodulator/opioid, source: α_{s1}-casein, enzyme applied: papain and bromelain), EQLTK$^{0.05}$ (antibacterial peptide, source: α-lactalbumin, enzyme applied: bromelain), and EMPFPK$^{0.77}$ (ACE inhibitor, source: *β*-casein, enzyme applied: bromelain). The superscripts mean the PeptideRanker Score of each peptide. All the above-mentioned peptides were identified experimentally in food sources. In the case of peptides acting as enzyme inhibitors, their bioactivity measured in experimental conditions was expressed as IC_{50} understood as the concentration of a molecule (i.e., peptide) corresponding to its half-maximal inhibition (Pripp and Ardö 2007). Comparison of theoretical vs. experimental bioactivity of these peptides (i.e., PeptideRanker Scores vs. IC_{50}) enabled various options of data interpretation: *a*) peptide with theoretically strong but experimentally weak activity; *b*) peptide with theoretically weak but experimentally strong activity; *c*) peptide with theoretically and experimentally strong activity, and *d*) peptide with theoretically and experimentally weak activity.

The first option was exemplified by the EMPFPK parent peptide (see Table 2). Its weak bioactivity was reported when studying the ACE inhibitory potential of the EMPFPK peptide (IC_{50} = 432.0 μg/mL) (Hayes et al. 2007), whereas its high potency was indicated by the PeptideRanker Score (0.77).

The LDIQK peptide (Table 1 and Table 2) is an example of a sequence with weak theoretical bioactivity but a strong experimental potency. According to the literature, LDIQK was an ACE inhibitor with IC_{50} = 27.6 μM (relatively potent) (Hernández-Ledesma et al. 2006), whereas its PeptideRanker Score (0.11) suggested its weak potential. Similar regularity was reported for the IPIQY sequence (DPP-IV inhibitor) with IC_{50} = 35.2 μM (Nongonierma et al. 2014) and 0.36 (PeptideRanker Score).

A strong theoretical and experimental potential was ascribed to the TTMPLW (Table 1 and Table 2) parent peptide acting as an ACE inhibitor. Its IC_{50} value was 16.0 μM (strong potential) (Fuglsang et al. 2003), which was also confirmed by a high PeptideRanker Score (0.74). This peptide was identified by Iwaniak et al. (2020a) in bovine milk protein concentrate hydrolyzed both *in silico* and *in vitro* by papain.

Finally, the parent peptide with a sequence NQDK exhibited weak potency according to the PeptideRanker Score (0.07) and literature data. According to literature findings, it inhibited the ADP-induced human platelet aggregation (IC_{50} = 400 μM) (FIAT et al. 1993). Weak theoretical and experimental bioactivity was also ascribed to the EQLTK sequence. Its PeptideRanker Score was 0.05 and, according to the literature, this peptide exhibited an antibacterial function. Its antibacterial potential was expressed as $\log N_0/N_1$ (N_0 – control number of colonies without antibacterial material; N_1 – the number of colonies containing antibacterial agent after an incubation period of 2 h), and the values calculated against different microbial strains showed its weak activity against Gram-negative bacteria compared to the other peptides (PELLEGRINI et al. 1999).

To recapitulate, according to FU et al. (2016), the PeptideRanker Score might be useful for the structure-activity analysis of peptides, but the "exact" prediction of bioactivity is rather impossible. The discrepancies between the theoretical and experimental bioactivities of peptides were also observed by FU et al. (2016) who assessed the potency of peptides derived from patatin (potato). Based on the literature search, it was found that FP had a weak ACE inhibitory potency (IC_{50} = 1215.7 μM), which was in opposition to the PeptideRanker Score (0.99) suggesting strong bioactivity. Another peptide (WG) had the same PeptideRanker Score as FG peptide, but there was no literature data about its potential measured *in vitro* (FU et al. 2016). It needs to be noted that the measure of the bioactivity of peptides might also be an important feature when comparing their predicted and experimental activities. No units are provided by PeptideRanker Scores, whereas experimental bioactivity is expressed in different units, which might affect the interpretation of results. Nevertheless, biological activity prediction may prove useful while selecting peptides for their synthesis in order to determine their effect *in vitro* (FU et al. 2016). Finally, our approach shows how to determine the potential of proteins as the sources of biopeptides. However, some discrepancies may appear when comparing the results of *in silico* and *in vitro* hydrolysis of proteins. Such phenomenon is quite common and possible factors affecting such discrpeancies were discussed by IWANIAK et al. (2020b). However, the analyses of large datasets involving bioinformatic-assisted methods enable to preselect the protein and protease candidates to produce peptides before their identification in the laboratory conditions.

Final remarks

The fragmentomic approach applied in this study showed the potential of milk protein-derived parent peptides to be bioactive. *β*-Casein was considered as the best source of biopeptides, which is the common fact. Despite this, the new parent peptide, i.e., PVQPFTESQSLTLTDVENLHLPPLLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK produced by the action of bromelain, showed the likelihood to act as a new strategic zone of *β*-casein due to the presence of plenty of motifs with various activities. Several parent peptides theoretically released from milk proteins possessed experimentally confirmed bioactivity. However, the analysis of their predicted and experimental potency showed some discrepancies. Despite the usefulness of the tools for peptide bioactivity prediction, critical thinking while planning the application of such data in future experiments would thus appear to be a worthwhile line of inquiry. It results from immense structural diversity of natural compounds and the complexity of structure-activity relationships. Nevertheless, the scientists highlight the suitability of bioinformatic-assisted analyses of large datasets to preselect the protein and protease candidates to produce peptide before discovering bioactive peptides in the laboratory conditions.

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References

Abachi S., Bazinet L., Beaulieu L. 2019. *Antihypertensive and angiotensin-i-converting enzyme (ACE)-inhibitory peptides from fish as potential cardioprotective compounds*. Mar. Drugs, 17(11): 613.

Aktayeva S., Akishev Zh., Khassenov B. 2018. *Proteolytic enzymes in cheese making*. Eurasia J. Food Biotechnol., 1, article ID: UDC 577.15.

Arlene A., Prima Kristijarti A., Ardelia I. 2015. *The Effects of the types of milk (cow, goat, soya) and enzymes (rennet, papain, bromelain) toward cheddar cheese production*. Makara J. Technol., 19(1): 31–37.

Berg J.M., Tymoczko J.L., Stryer L. 2002. *Biochemistry*. 5th edition. New York. W.H. Freeman. Chapter 3. *Protein structure and function*, https://www.ncbi.nlm.nih.gov/books/NBK21177/, access: 10.04.2021.

Bhandari D., Rafiq S., Gat Y., Gat P., Waghmare R., Kumar V. 2019. *A review on bioactive peptides. Physiological functions, bioavailability and safety*. Int. J. Pept. Res. Ther., 26: 139–150.

Capriotti A.L., Cavaliere C., Piovesana S., Samperi R., Laganà A. 2016. *Recent trends in the analysis of bioactive peptides in milk and dairy products*. Anal. Bioanal. Chem., 408(11): 2677–2685.

Carreiro A.L., Dhillon J., Gordon S., Higgins K.A., Jacobs A.G., McArthur B.M., Redan B.W., Rivera R.L., Schmidt L.R., Mattes R.D. 2016. The Macronutrients, Appetite and Energy Intake. An. Rev. Nutr., 17: 73–103.

CHANPUT W., NAKAI S., THEERAKULKAIT C. 2010. *Introduction of new computer softwares for classification and prediction purposes of bioactive peptides: case study in antioxidative tripeptides*. Int. J. Food Prop., 13: 947–959.

CHOOPINHAM S., JATURASITHA S., RAKARIYATHAM N., SUREE N., HATAICHANOKE N. 2015. *Antioxidant and antihypertensive activity of gelatin hydrolysate from Nile tilapia skin*. J. Food Sci. Technol., 52(5): 3134–3139.

DALIRI E.B., OH D.H., LEE B.H. 2017. *Bioactive peptides*. Foods, 6(5): 32.

DATE C.J. 2003. *An introduction to database systems, eight edition*. Boston, MA, Addison Wesley, USA.

FIAT A.M., MIGLIORE-SAMOUR D., JOLLÈS P., DROUET L., BAL DIT SOLLIER C., CAEN J. 1993. *Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities*. J. Dairy Sci., 76(1): 301–310.

FU Y., WU W., ZHU M., XIAO Z. 2016. *In silico assessment of the potential of the patatin as a precursor of bioactive peptides*. J. Food Biochem., 40: 366–370.

FUGLSANG A., NILSSON D., NYBORG N.C.B. 2003. *Characterization of New Milk-derived inhibitors of angiotensin converting enzyme in vitro and in vivo*. J. Enzyme Inhib. Med. Chem., 18(5): 407–412.

HAQUE E., CHAND R. 2008. *Antihypertensive and antimicrobial bioactive peptides from milk proteins*. Eur. Food Res. Technol., 227: 7–15.

HAYES M., STANTON C., SLATTERY H., O'SULLIVAN O., HILL C., FITZGERALD G.F., ROSS R.P. 2007. *Casein fermentate of Lactobacillus animalis DPC6134 contains a range of novel propeptide angiotensin-converting enzyme inhibitors*. Appl. Environ. Microbiol., 73(14): 4658–4667.

HERNÁNDEZ-LEDESMA B., LÓPEZ-EXPÓSITO I., RAMOS M., RECIO I. 2006. *Bioactive peptides from milk proteins*. In: *Immunochemistry in dairy research*. Ed. R. Pizzano. Kerala, India, Trivandrum, pp. 37–60.

IWANIAK A., DAREWICZ M., MOGUT D., MINKIEWICZ P. 2019. *Elucidation of the role* of *in silico methodologies in approaches to studying bioactive peptides derived from foods*. J. Funct. Foods, 61: 103486.

IWANIAK A., MINKIEWICZ P., DAREWICZ M., PROTASIEWICZ M., MOGUT D. 2015. *Chemometrics and cheminformatics in the analysis of biologically active peptides from food sources*. J. Funct. Foods, 16: 334–351.

IWANIAK A., MINKIEWICZ P., HRYNKIEWICZ M., BUCHOLSKA J., DAREWICZ M. 2020a. *Hybrid approach in the analysis of bovine milk protein hydrolysates as a source of peptides containing di- and tripeptide bitterness indicators*. Pol. J. Food Nutr. Sci., 70(2): 139–150.

IWANIAK A., MINKIEWICZ P., PLISZKA M., MOGUT D., DAREWICZ M. 2020b. *Characteristics of biopeptides released in silico from collagens using quantitative parameters*. Foods, 9(7): 965.

IWANIAK A., MOGUT D. 2020. *Metabolic syndrome-preventive peptides derived from milk proteins and their presence in cheeses*. A Review. Appl. Sci., 10: 2772.

MARCONI S., DURAZZO A., CAMILLI E., LISICIANI S., GABRIELLI P., AGUZZI A., GAMBELLI L., LUCARINI M., MARLETTA, L. 2018. *Food composition databases. Consideration about complex food matrices*. Foods, 7: 2.

MINKIEWICZ P., IWANIAK A., DAREWICZ M. 2019. *BIOPEP-UWM database of bioactive peptides: current opportunities*. Int. J. Mol. Sci., 20: 5978.

MOHANTY D.P., MOHAPATRA S., MISRA S., SAHU P.S. 2016. *Milk derived bioactive peptides and their impact on human health. A review*. Saudi J. Biol. Sci., 23(5): 577–583.

MOONEY C., HASLAM N.J., POLLASTRI G., SHIELDS D.C. 2012. *Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity*. PLoS ONE 7(10): e45012.

NONGONIERMA A.B., FITZGERALD R.J. 2014. *Susceptibility of milk protein-derived peptides to dipeptidyl peptidase IV (DPP-IV) hydrolysis*. Food Chem., 145(15): 845–852.

PELLEGRINI A., THOMAS U., BRAMAZ N., HUNZIKER P., VON FELLENBERG R. 1999. *Isolation and identification of three bactericidal domains in the bovine alpha-lactalbumin molecule*. Biochim. Biophys. Acta, 1426(3): 439–448.

Periwal V., Bassler S., Andrejev S., Gabrielli N., Typas A., Patil R. 2020. *Bioactivity assessment of natural compounds using machine learning models based on drug target similarity*. BioRxiv, doi: 10.1101/2020.11.06.371112.

Pripp A.H., Ardö S. 2007. *Modelling relationship between angiotensin-(I)-converting enzyme inhibition and the bitter taste of peptides*. Food Chem., 102: 880–888.

Rieder N., Gazzano-Santoro H., Schenerman M., Strause R., Fuchs C., Mire-Sluis A., McLeod L.D. 2010. *The roles of bioactivity assays in lot release and stability testing*. BioProcess Int., article ID: 53119054.

Rivero-Pino F., Espejo-Carpio F.J., Guadix E.M. 2020. *Antidiabetic food-derived peptides for functional feeding. Production, functionality and in vivo evidences*. Foods, 9(8): 983.

Rozin P., Fischler C., Imada S., Sarubin A., Wrzesniewski A. 1999. *Attitudes to food and the role of food in life in the U.S.A., Japan, Flemish Belgium and France. Possible implications for the diet–health debate*. Appetite, 33: 163–180.

Shu G., Huang J., Bao C., Meng J., Chen H., Cao J. 2018. *effect of different proteases on the degree of hydrolysis and angiotensin i-converting enzyme-inhibitory activity in goat and cow milk*. Biomolecules, 8(4): 101.

Sołowiej B., Dylewska A., Kowalczyk D., Sujka M., Tomczyńska-Mleko M., Mleko S. 2016. *The effect of pH and modified maize starches on texture, rheological properties and meltability of acid casein processed cheese analogues*. Eur. Food Res. Technol, 242: 1577–1585.

Udenigwe C.C. 2014. *Bioinformatic approaches, prospects and challenges of food bioactive peptide research*. Trends Food Sci. Technol., 36: 137–143.

Udenigwe C.C., Aluko R.E. 2011. *Chemometric analysis of the amino acid requirements of antioxidant food protein hydrolysates*. Int. J. Mol. Sci., 12(5): 3148–3161.

Zambrowicz A., Timmer M., Polanowski A., Lubec G., Trziszka T. 2013. *Manufacturing of peptides exhibiting biological activity*. Amino Acids, 44(2): 315–320.

Zamyatnin A.A. 2009. *Fragmentomics of natural peptide structures*. Biochemistry (Moscow), 74: 1575–1585.