PUBLISHED BY THE ACADEMY OF SCIENCES OF ALBANIA

AJNTS

ALBANIAN JOURNAL OF NATURAL AND TECHNICAL SCIENCES

2022 (2)
XXVII (55)

Introduction to the special edition

1ST INTERNATIONAL SCIENTIFIC CONFERENCE ON "BIOTECHNOLOGY AND GENETICS; -DEVELOPMENTS AND FUTURE CHALLENGES" (BGIC) 2021

The **1st International Scientific Conference "Biotechnology & Genetics -Developments and Future Challenges" was held from** 20- 21 May 2021 at the Albanian Academy of Sciences. This event was the first of its kind to be held in Tirana, Albania. By holding such a prestigious event it is hoped to find solution to the main questions about biotechnology and enhance this scientific area in the country as it represented a good opportunity for the researchers in the area to meet and share their knowledge in the field.

Main goals of BGCI-2021 were the:

- Evaluation of the developments of biotechnological and genetic applications in the country in order to promote the cooperation among local capacities;
- Promoting new collaboration among local and foreign institutions in the framework of regional, European and international Programs related to biotechnology and genetics;
- Establishment of new modern scientific research methods and infrastructures for interdisciplinary research and for collaboration among specialists of the fields;
- Beginning of a qualitative leap in applied biotechnology and genetics research in Albanian universities, scientific institutions and industry.

There were over 350 participants attending the event with over 110 scientific presentations in various fields of biotechnology. A symbol of the importance BGIC remains the participation of researchers from Italy, Israel, Egypt, Turkey, Republic of North Macedonia, Kosovo, etc. The backbone of the conference was the participation of young researchers.

At the end of the conference, a round table was organized where the conclusions of the conference were drawn as a means to address the activities in the future for the promotion of biotechnology and investments in this

scientific area of great interest. Recommendations were addressed to the decision-making institutions, institutions of higher education, scientific community, and the private sector to promote and increase cooperation among them, increase investment in scientific laboratories and alleviate many other problems in the field of biotechnology.

The topics covered but not limited to were:

Medicine Biotechnology

- Cytogenetic and molecular genetic testing in the diagnosis of genetic diseases
- Impact of genomics in the diagnosis and management of rare diseases
- Molecular genetic characterization of ß-thalassemia and sickle cell syndrome
- Molecules and Genes Regulations of Biological Human Pathways;
- Significance of genetic markers in autoimmune diseases and cancers;
- New perspectives in using bioengineered immune cells in the fight against cancer
- Biotechnologies Applied in Biomedical Vaccines.
- Importance of Ethical issues in Medical Biotechnology

Pharmaceutical Biotechnology

- Biopharmaceutical Products, Current Applications in Therapy and Market Analysis
- Formulation and Analysis of Biotech Products
- Biopharmaceutical Pharmacovigilance and Regulatory Affairs.
- Bioactive Natural Products as pharmaceuticals, nutraceuticals and cosmeceuticals;
- Genetic and Chemical Diversity in Medicinal and Aromatic Plants;

Populations Genetics

- Populations genetics of cultivated and spontaneous plants
- Biodiversity and genetic diversity of animal populations
- Genetics of human populations:
- Genetic relationships of Albanian populations evaluated by different markers
- Clinical and Medical populations genetics
- Physical activity, health nutrition and recreation in Albanian human populations

Plant Biotechnology

- Plant Genetic Improvement

- Plant Tissue Culture Innovations and Commercial Production
- Molecular Markers and Marker Assisted Selection in Crop Plants
- Plant Molecular Diagnostics
- Crop Protection Applications
- Plant Molecular Farming
- Biodiversity and *in vitro* Conservation
- Biosafety, Bioethics and IPR Issues in Plant Biotechnology.

Animal Biotechnology

- Animal Genetic resources conservation.
- Animal Tissue and Cell Culture (Growth and Propagation, Cryopreservation)
- Artificial Inseminations and *in vitro* Fertilization
- Biotechnology derived Therapeutics
- Veterinary Diagnostic System (Molecular and Serologic) on detection and control of animal infectious deceases.
- Biotechnological Methods of the Improvement of Health and Welfare of livestock animals and their products.
- Detection of mislabeling and species substitution in animal products by molecular methods.
- Food quality and safety for livestock and aquaculture.
- Utilization of organic and mineral wastes as well as gases eliminated from animals.

Food Biotechnology

- Food Processing (Culture starters, Enzymes, Immobilized enzymes and cells, etc.)
- New bioactive compounds, as ingredients in functional foods and packaging materials.
- Biomolecules as the markers for authenticity, traceability, geographical origin and development of food products.
- Genetic characterization of autochthonous food resources for forming cheese potential of milk and technological and nutritional quality of olive oil and grape.
- Food Safety (Biomarkers for toxic substances presence, contaminants, allergens, pesticides, microorganisms to the food complex systems, Food Preservation, etc.)

Environmental Biotechnology

- Molecular Ecology

- Contaminated land and bioremediation (Microbes and metabolism)
- Phytoremediation of Organic Pollutants
- Phytoremediation of Inorganics
- Constructed Wetlands
- Production of Biofuels and Plant Biomass

Valbona Sota

PHASEOLUS VULGARIS TREATED WITH PHYSICAL MUTAGENS AND ASSESSMENT OF CHLOROPHYLL MUTATIONS AND SYMBIOTIC BACTERIA

Arjana YLLI, Selvina ÇOLLAKU and Emanuela HAJDERAJ
Biotechnology Department, Faculty of Natural Science,
University of Tirana, Albania

ABSTRACT
The bean (*Phaseolus vulgaris*) represents a crop source for food and sustainable agriculture in the world. Unfortunately, climate change has a significant impact on their yield. Induced physical mutagenesis has a long-term remarkable potential of improving plant material with regard to their qualitative and quantitative production characteristics. The present paper investigates the seeds of *Phaseolus vulgaris,* a cultivar registered in the Albanian Genetic Bank. The seeds are treated with physical mutagens, gamma radiation Cs-137, in three different doses (50 Gy, 100 Gy and 150 Gy). The present investigation informs about the planting of these materials in three different environments; greenhouse, laboratories and experimental field. *Rhizobia* bacterium plays a significant role in provision of agricultural ecosystem services due to their ability to form symbiotic association with a wide range of leguminous plants that results in biological nitrogen fixation. The bacteria have been microscopically identified. Once identified, they were isolated from the root nodules of beans which were treated with physical mutagens, and young rhizobia cultures were grown in YEMA medium. Various mutations were reported throughout the experiment. In laboratory and greenhouse plants, some types of chlorophyll mutations appeared to be dependent upon the radiation dose. In addition, striata, maculate, chlorine and tigrine, types of chlorophyll mutations reported changes in the amount of chlorophyll. Compared to one year ago with M_1 generation, albviridis is the only chlorophyll mutation not occurring in the present investigation. Nodules are smaller in size due to mutagenesis. Stero-microscope images show nodules being green and brown in color due to the presence of chlorophyll in the cortical region of the nodule and the presence of leghemoglobin protein in the nodule, respectively.
Keywords: *Phaseolus vulgaris, Physical mutagens, Rhizobium*

1. INTRODUCTION

Ulukapi and Ozmen (2019) stated that common bean is an important crop for food and sustainable development in agriculture. *Phaseolus vulgaris* also

known as the bean belongs to the group of leguminous plants, and is widely found across the country. The bean is considered to be one of the most important products in the leguminous group (Ylli *et al*, 2019; Kodhelaj and Ylli 2021). *Phaseolus vulgaris* is very important for agricultural production due to its high genetic variability. Climate changes unavoidably project decrease in vegetables and legumes' yields (Heinemann *et al.*, 2017; Kodhelaj and Ylli 2021). Bean production decrease has led to many studies to power the elimination of performance losses in field. One of the problems identified is the abortion of flowers of the beans as legumes do not survive due to high temperatures (Ylli *et al*, 2013; Kodhelaj and Ylli 2017). As the decrease in bean yield results from a low percentage of fruit production from flowers when drought occurring during flowering and from embryos abortion during the pod-forming stage, continuous efforts have been made to adapt legumes to new environmental conditions. Induced mutations have been for a long time a means to address the new cultivars with improved features when compared to the parents. Induced mutagenesis technology has been recently recognized as a valuable additional tool to create improved cultivars in agriculture (FAO/IAEA, 2018). Induced mutation by using mutagen is appropriate for genetic variability (Kshirsagar *et al.*, 2014). The experience has shown that mutagenesis is one of the most important direction for the creation of new crop variety especially at leguminous. Physical mutagens are generally preferred by reason of being convenient, easily reproducibility, and user environment-friendly method (Çelik and Atak, 2017). Bacterial nitrogen fixation is the biological process by which atmospheric nitrogen is uptake by bacteroid located in plant root nodules and converted into ammonium through the enzymatic activity of nitrogenase. In practice, this biological process serves as a natural form of fertilization and its optimization has significant implications in sustainable agricultural programs (Resendis-Antonio *et al*, 2011). Rhizobia bacteria play a significant role in provision of the agricultural ecosystem services due to their ability to form symbiotic association with a wide range of leguminous plants that results in biological nitrogen fixation (Koskey *et al*, 2018). The infection of legume roots by rhizobia, leading to the formation of nitrogen-fixing nodules, is a clonal event and each individual bacterium that initiates an infection can grow rapidly (Frederix *et al*, 2014). Lateral gene transfer of specific symbiosis genes within rhizobia genera is an important mechanism allowing legumes to form symbioses with rhizobia adapted to particular soils. Rhizobia-legume symbiosis is a host-specific association and enhances the need to determine the strains and the diversity of rhizobia associated with specific type of legume for better exploitation of the benefits associated with the rhizobia biofertilizers (Batista 2015; Koskey *et al*, 2018). As strain-specific legume rhizobia symbioses can develop in particular habitats (Andrews and Andrews 2017), the present paper aims to identify the

presence of rhizobium bacteria in plants treated with mutagenesis and grown in different environments.

2. MATERIALS AND METHODS

The seeds of *Phaseolus vulgaris*, a cultivar registered in the Albanian Genetic Bank, are in the present investigation used. This cultivar is mainly planted in Fieri and Lushnja regions. The seeds of *Phaseolus vulgaris* are treated with physical mutagens, gamma radiation of radiation source Cs-137, in three doses of 50 Gy, 100 Gy and 150 Gy compared to the control at the Institute of Nuclear Physics, University of Tirana, Albania. Materials are planted in different environments; greenhouse, laboratories and experimental field in the outskirts of Tirana. *Rhizobium* was microscopically identified and quantified, and isolated from bean root nodules. Evaluation required plants with nodules for isolation purposes.

Rhizobium was isolated from bean root nodules treated with physical mutagens. Isolation of rhizobium was carried following the standard protocols as described in (Somasegaran and Hoben 1994). Healthy and undamaged root nodules of *Phaseolus Vulgaris* treated with physical mutagens from various environments; greenhouse, laboratories and experimental field were selected and used for the isolation of *rhizobia* bacteria. The nodules were washed and sterilized, and the 10 healthy and undamaged nodules were collected from each plant. The nodules were detached from the root by cutting 0.5 cm on each side of the node. Undamaged nodules were immersed for 5-10 seconds in 95% ethanol, and subsequently transferred to a 3% solution of sodium hypochlorite, for 4 minutes.

To make the nodule preparation, a loop of the nodule suspension is placed on a mannitol agar (YEMA) plate with yeast extract and then this material is incubated in the dark at 28^{o}C for 3-5 days. Maked wet mounts of the cultures were provided and examined under the phase contrast microscope, and the standard materials microbiological techniques described by (Somasegaran and Hoben, 1994) were employed. All the isolates were characterized by morphological parameters such as colony size, shape and color. Other tests that were carried out included gram staining where young pure isolates (5 days old) cultured on YEMA were smeared on clean microscope slides (Beck *et al*, 1993; Koskey *et al*, 2018).

3. RESULTS

There are numerous chlorophyll mutations reported which could be classified into 9 groups based on the Gustafsson's method. In the laboratory and greenhouse plants, some types of chlorophyll mutations appeared to be

related to the radiation dose. So, *Phaseolus Vulgaris* had unifoliate and trifoliate leaves, different in size. Seedlings grown from seeds irradiated with 50 Gy gamma dose and planted in Petri dishes were observed to grow faster. The Figure 1 depicts the plant with the chlorophyll mutation albviridis induced by 300 Gy gamma rays. Seed germination was reported in seeds irradiated with 150 Gy gamma dose. The striata mutation was characterized by pronounced longitudinal stripes, maculate stained throughout the leaf due to the destruction of the chlorophyll. The chlorine mutation was characterized by larger stains which had light color. The tigrine mutation was characterized by pigments destruction with the narrow transverse stripes which were yellow in color (Kodhelaj and Ylli 2017; Kodhelaj and Ylli 2021).

These types of mutations occur in all materials obtained after treatments. There were changes in chlorophyll pigment under natural conditions reported. Here, we could mention maculate, chlorine and viridis mutations. The latter is characterized by a light green. There are changes related to the morphology of bifoliate and tetra foliate leaves and the concentration of pigments causing chlorophyll mutations such as chlorine, viridis, maculate, xantha and albviridis if compared to one year ago with M1 generation (Figure 1). The latter is characterized by the green leaf and its most discolored tip. It is the only mutation that has not appeared in the present investigation (Gustafsson 1986). Chlorophyll mutations are the most frequent mutations and easily identifiable in the M2 generation.

Fig. 1: Different tipe of Chlorophyll mutation in *Phaseolus vulgaris* treated with physical mutagens in M1 generation.

Stress caused by high temperatures develops a deeper root system that absorbs more water, but the growth of the root system decreases the productivity. Here, either multiplication of roots hair for a more effective use of the same biomass or stimulation of a higher acid production in roots to keep the same production level or increasing it would be necessary.

Figures 2-4 show the nodes observed in the plants planted in Petri dishes for the materials irradiated with three different doses and their untreated control. In the Petri dishes the plant growth is more restricted, and the root system less developed. The Stereomiscroscope method was applied to measure the root 12 and 15 days after germination. The root system for bean plants treated with three doses of radiation are compared to the control, and the results show changes throughout their development process.

Fig. 2: Control plant growth in laboratories.

Fig. 3: Plant treated with 50GY doses.

Fig. 4: Plant treated with the dose 100 GY.

Fig. 5: Plant treated with the dose 150GY.

4. DISCUSSION

Leguminous plants have the capacity of fixing nitrogen in their roots. Nodules are smaller in size and amount than the control due to mutagenesis. The stereomicroscope images show green and brown nodules. The greenhouse and soil plants showed the presence of green nodules. These nodules are close to the earth's surface, and the green color proves the presence of chlorophyll in the cortical region of the nodule. The data obtained from the microscope show that the rhizobium bacteria had bacillus and rod-shaped form, gram negative and their number in plants irradiated with 50 Gy gamma doses and 100 Gy gamma doses was smaller than in control plants (Figure 6).

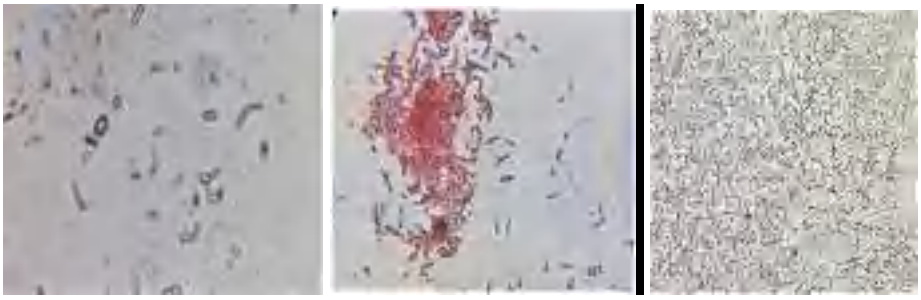


Fig.6: Rhizobium from plant treated with doses 50 Gy, 100 Gy and control under the microscope**.**

Mutation breeding can be used non only to induce mutations but also to promote genetic recombination to increase mutations' frequency (FAO/IAEA, 2018). Induced mutation is an important tool for producing genetic variation (Chusreeaeom and Khamsuk, 2019). Chlorophyll mutations appeared dependent upon radiation dose. The types of mutations observed during the experiment are macromutations in different degrees.This is most pronounced in the case of the xantha, viridis and alboviridis types and less pronounced in the case of the albina and tiger types (Gustafson 1938; Gustafsson 1986; FAO/IAEA, 2018).

The bean plants planted in the greenhouse showed undefined mutations accompanied by changes in the shape of the leaves, their wrinkles and color. Albine mutations report lethal mutation characterized by entirely white leaves of seedlings; seedlings survived for 10-12 days after germination. The bean plants undergoing the chlorine mutations have light green leaves. In addition, although most of the seedlings died within 20 days, few vigorous plants survived and were late in maturity. During the maculata mutation, seedlings showed either yellow or white dots on leaves, and these mutants survived till maturity producing few seeds. The xantha mutations displayed on the *Phaseolus Vulgaris* leaves gave the typical characteristics of light green color of leaves, most of the seedlings died within 20 days. In the leaves of our plants alboviridis mutations appear different colors at the leaf base and leaf tip. These mutations in the greenhouse were quite noticeable.

Chlorophyll mutations are frequently employed for the determination of mutagenic potency in inducing genetic variability as they are vital indicators in the assessment of induced genetic changes of mutagenized population (Raina and Khan. 2020). The green color appears due to the development of chlorophyll in the cortical region of the nodule, while the brown color proves the presence of the leghemoglobin protein in the nodule (Somasegaran and Hoben, 1994).

The root system is an important factor for plant productivity in leguminous plants. The length of the roots was measured 12 and 15 days after germination. The presence of a developed root system was observed in the three different environments where the mutagenized bean plants were planted. The plants planted in the ground are healthier and have rounded nodules as they grow under favorable conditions due to their natural environment. Gram staining results and growth on YEMA media, confirmed preliminary the standard morpho-cultural characteristics of *Rhizobium* species that nodulate with *P. vulgaris* (Koskey *et al*, 2018). Strain-specific legume rhizobia symbioses can develop in particular habitats (Andrews and Andrews 2017). The presence of the rhizobium bacterium in mutagen-treated plants also confirms that these plants have a great ability to fix the nitrogen.

REFERENCES

Andrews M, and Andrews ME. 2017. Specificity in legume-rhizobia symbiose. *The International Journal of Molecular Sciences*, **18 (705):** 1-3. DOI:10.3390/ijms18040705
Corpus ID: 9878768 2-35.

Batista L, Irisarri P, Rebuffo M, Cuitiño MJ, Sanjuán J, Monza J. 2015. Nodulation competitiveness as a requisite for improved rhizobial inoculants of *Trifoliu pratense*. *Biology and Fertility of Soils,* **51**: 11–20. doi: 10.1007/s00374-014-0946-3.

Beck DP, Materon LA, Afandi F. 1993. *Practical rhizobium-legume technology manual.* Aleppo: International Center for Agricultural Research in the Dry Areas (ICARDA). No.19 pp.389 pp.

Çelik Ö, Atak Ç. 2017. Applications of ionizing radiation in mutation breeding. *Research Gate,* 112-127. DOI:10.5772/66925.

Chusreeaeom K, Khamsuk O. 2019. Effects of gamma irradiation on lipid peroxidation, survival and growth of turmeric in vitro culture. *Journal of Physics: Conference Series (JPCS) 1285 012003,* 1-5.

FAO/IAEA. 2018. Manual on mutation breeding - Third edition. Food and Agriculture Organization of the United Nations. 5, 6, 49. ISBN 978-92-5-130526-3.

Frederix M, Edwards A, Swiderska A, Stanger A, Karunakaran R, Williams A, Abbruscato, P, Contreras MS, Poole, PS, Downie J. 2014. Mutation of *praR* in *Rhizobium leguminosarum* enhances root biofilms, improving nodulation competitiveness by increased expression of attachment proteins. *Molecular Microbiology*. **93(3):** 464–478.

Gustafson A. 1938. Studies on the Genetic basia of Chlorophyll Formation and the Mechanisms of Induced Mutating. *Online version,* 33-38.

Gustafsson, Å. 1986. Mutation and gene recombination principal tools in plant breeding Research and results in plant breeding. pp. 76-84.

Heinemann AB, Villegas JR, Stone LF, Didonet AD. 2017 Climate change determined drought stress profiles in rainfed common bean production systems in Brazil. *Agricultural and Forest Meteorology,* **246***:* 64-77.

Koskey G, Mburu SW, Kimiti JM, Ombori O, Maingi JM, Njeru EM. (2018). Genetic Characterization and Diversity of *Rhizobium* Isolated from Root Nodules of Mid-Altitude Climbing Bean (*Phaseolus vulgaris* L.) Varieties. https://doi.org/10.3389/fmicb.2018.00968.

Kodhelaj M, Ylli A. 2021. Induced mutagenesis in *Phaseolus vulgaris*. *International Journal of Ecosystems and Ecology Science* (IJEES) ISSN 2224-4980. **11 (2):** 349-354.

Kodhelaj M, Ylli A. 2021 Chlorophyll mutations in haricot plants treated with mutagens. *Buletini i shkencave të natyrës.* Botim i Fakultetit të Shkencave të Natyrës, Universiteti i Tiranës. Nr.30 Viti 2021. ISSN 2305 – 882X. P. 52-62.

Kodhelaj M, Ylli A. 2017. Influence of induced mutation in beans (*Phaseolus vulgaris*). Albanian j. agric. sci.; (Special edition). ISSN: 9789928146441 Page 395-398.

Kshirsagar JK, Dalvi VV, Bahave SG, Pethe UB, Mahadik SG. 2014. Induced Mutagenesis in Lablab Bean (Lablab pulpureus L. Sweet var. Typicus). *Research Journal of Agricultural Sciences*, **5(6):** 1215-1218.

Mahamune SE, Kothekar VS. 2012. Induced chemical and physical mutagenic studies in M1 generation of French bean (Phaseolus vulgaris L.). *Current Botany*, **3(3)**:17-21. Retrieved from https://updatepublishing.com/journal/index.php/cb/article/view/1404.

Oldasou Y, Rafi MY, Abdullah N, Hussin G, Ramli A, Rahim HA, Miah G, Usman M. 2015. Principle and application of plant mutagenesis in crop improvement: a review. https://doi.org/10.1080/13102818.2015.1087333.

Raina A, Khan S. 2020. Mutagenic effectiveness and efficiency of gamma rays and sodium azide in M_2 generation of Cowpea [*Vigna unguiculata* (L.) Walp.]. Researchgate 12-39. http://dx.doi.org/10.1101/2020.03.09.983486.

Resendis-Antonio O, Hernández M, Salazar E, Contreras S, Martínez- Batallar G, Mora Y, Encarnación S. 2011. Systems biology of bacterial nitrogen fixation: high-throughput technology and its

integrative description with constraint-based modeling. *BMC System Biology*, 1-8. doi: 10.1186/1752-0509-5-120.

Somasegaran P, and Hoben H J. 1994. Handbook for Rhizobia Methods in Legume - Rhizobium Technology. *Researchgate*, 7-73. DOI:10.1007/978-1-4613-8375-8.

Ulukapi K, Ozmen FS. 2018. Study of the effect of irradiation (^{60}Co) on M_1 plants of common bean (*Phaseolus vulgaris* L.) cultivars and determined of proper doses for mutation breeding. *Journal of Radiation Research and Applied Sciences*, 157-161. https://doi.org/10.1016/j.jrras.

Ylli A, Karcini M, Klemo M, Dodbiba A. 2013. Induced Mutation in Bean. *Aktet*. Reviste shkencore e Institutit te Alb-Shkencës **1(VI):** ISSN 2073-2244, 10-16.

Ylli A, Karcini M, Sula S. 2019. The measurement of chlorophyll pigments at *Phaseolus vulgaris* mutants line. *Aktet*. Reviste shkencore e Institutit te Alb-Shkencës. **2 (X):** ISSN 2073-2244 137-142.

POMOLOGICAL DIVERSITY OF LOCAL PLUM CULTIVARS

Frida ÇARKA and Raimonda SEVO
Institute of Plant Genetic Resources, Agricultural University of Tirana, Albania

ABSTRACT

There are tons of plum varieties typically categorized by European, Japanese, hybrid and American types—each with its own advantages. The present paper analyzes and assesses the pomological characteristics of five local plum cultivars (AGB 3232, AGB 3190, AGB 3142, AGB 3227, AGB 3133) at the Gene Bank Collection of Fruit Trees in Valias, Albania, via UPOV Code and plum descriptors. Tree habitat brunches, tree vigor, season of flowering and maturity, fruit size, fruit shape, fruit flesh color, flesh juiciness, sugar/acid ratio, length of fruit stalk, stone size and stone shape were statistically evaluated for the vegetation period 2018-2020. The results reported distinctive characteristics of the cultivars, regardless the similarities in various descriptors. In addition, a considerable polymorphism was found regarding their vegetative period and morphological features of fruit. Moreover, these local plum cultivars are pomologically unique. The present study is a means to address the identification and conservation of genetic resources of fruit species and the establishment of a valuable database with pomological data for scientists and growers.
Keywords: Albanian plum cultivars; biodiversity; pomological approach

1. INTRODUCTION

Plum is one of the most widespread fruit tree species in Albania. The 2019 Albania plum crop is 40 928 tons. Total 2019 bearing acreage is estimated at 2626, raking second after apple (INSTAT, 2020). There is a long history in the realm of growing plum trees in Albania, and plum crop is important for the market and industrial sector. In addition, the relatively high content of phenols and antioxidants make plums of nutritional benefit for the people (Kim, *et al.,* 2003; Rupasinghe *et al.,* 2006).

The diversity of plum cultivars in Albania was investigated during exploration missions from 2009 to 2014, allowing the registration of 107 local plum cultivars in the database. There are 62 accessions of plum cultivars

conserved at the National Collection of the Albanian Gene Bank, Valias, Albania. They are collected from different areas, and their diversity is expressed by the pomological characteristics that distinguish them. In addition, they represent local genotypes of great interest. The characterization and evaluation of the pomological characteristics of the plum accessions of the National Collection is one of the objectives of Albanian Gene Bank as a first step towards their further identification, removal of possible duplicates and recommendation to the farmers **(**Hârţa *et al.*, 2016; Mićić *et al.*, 2019). Consequently, an investigation was carried out to characterize and evaluate these five local plum accessions: AGB 3232, AGB 3190, AGB 3142, AGB 3227 and AGB 3133.

2. MATERIALS AND METHODS

The present investigation involves AGB 3232, AGB 3190, AGB 3142, AGB 3227, AGB 3133, the five plum accessions planted in 2011 at National Collection of Albanian Gene Bank, Valias, Albania. They were *in situ* investigated from 2009 to 2010. Table 1 reports the collection sites. The plants selected were healthy and in full production. The UPOV Code, plum descriptors and guidelines set by International Board of Plant Genetic Resources were employed during 2018-2020 for the characterization and evaluation of these accessions (Plum Descriptors, 1984). The following phenologic characteristics were determined: the time of beginning of flowering was considered when at least 5% of the flowers bloomed; when at least 80% of the flowers bloomed was recorded as full flowering, and the end of flowering was determined when 90% of the flowers bloomed and corollas began to fall off. The harvest date was determined when the fruits were colored and were soft to be eaten (Funt 1998).

During the vegetative period, the tree vigor and tree habitat brunches were determined. The tree vigor was considered as the overall abundance of vegetative growth (UPOV) (International Bord for Plant Genetic Resources, 1984).

At full maturity when fruit has typical taste, color and firmness the samples of 25 fruits were collected from each accession to be pomologically investigated for the following parameters: fruit weight, fruit shape, skin color, ground color, flesh color, flesh firmness, stone weight (Plum Descriptors, 1984).

A digital scale was used for the weight of each fruit (g) and their stone, and the manual caliber for the size of fruits (mm).

The sweetness of the fruit was measured in juice obtained using a home blender by a refractometer expressed as degrees Brix. The total acidity of the fruit was measured by titration with 0.1 N NaOH.

The overall data were analyzed by one-way analysis of variance (ANOVA). Differences were considered statistically significant at the level of $p < 0.05$.

3. RESULTS

The phenological characteristics of five local plum cultivars conserved in the Albanian Gene Bank were determined based on field observations and the data analyzed showed that these cultivars bloom from 05 March to 24 March. The AGB 3227 has the earliest time of flowering, while AGB 3232, AGB 3190, AGB 3142, AGB 3133 bloom a few days later—and intermediate time for the beginning of flowering. Considered with medium maturity period, the cultivars AGB 3190 and AGB 3142 ripen during July 20-30, while AGB 3232 and AGB 3227 ripen late (15-30 August). The cultivar AGB 3133 is the latest cultivar which fruits are harvested (15-25 September) (Table1). The tree vigor is considered intermediate to AGB 3190, AGB 3142, AGB 3227 and strong to AGB 3232, AGB 3133, while tree habitat branches is spreading to AGB 3190, AGB 3142, AGB 3133 and upright to AGB 3232, AGB 3227.

Table 1 The phenological characteristics of five local plum cultivars

Accession Code	Accession Name (local plum cultivars)	Origin	Time of beginning of flowering	Time of beginning of harvesting
AGB 3232	E kuqja e Elbasanit	Gjinar	5	7
AGB 3190	Çifte e Elbasanit	Karkavec	5	5
AGB 3142	Violet e Gjinarit	Gjinar	5	5
AGB 3227	E verdha e Tiranës	Tiranë	3	7
AGB 3133	E verdha e Pashtreshit	Pashtresh	5	9

Time beginning of flowering: 3=early; 5=intermediate;
Time beginning of harvesting: 5=mid-season; 7=late; 9=extremely late.

All the data showed that there have been similarities in various descriptors, but most of the characteristics are distinctive. The duration of vegetative period and some of morphological features of fruit could be distinguished the most. The analyses of the average fruit weight and fruit size, considered as very important characteristics for commercial market, (Mićić *et al.*, 2019) showed that fruit weight ranged from 16.48± 0.254 g (AGB 3232) to 45.32± 0.276 g (AGB 3190). The size of fruit is considered extremely small to AGB 3232 and AGB 3142, very small to AGB 3227 and AGB 3133 and small to AGB 3190. The skin color is more diverse. It differs from yellow light to dark as ground color and the over color of the skin is different; pink, red and violet. Flesh firmness is soft to AGB 3133 and AGB 3232 while medium to AGB

3190 and AGB 3142. AGB 3133 has the firmest flesh. Stone weight ranged from 0.74 ±0.02to O.92 ±0.06. Regarding the sugar/acid content and eating quality the following cultivars: AGB 3232, AGB 3190 and AGB 3142 (Table 2) were determined on higher level when compared with the others.

Table 2 The pomological characteristics of five local plum cultivars

Accession number	Fruit weight (g)	Fruit size	Fruit shape	Fruit ground color	Fruit over color	Flesh firmness	Stone weight (g)	Eating quality
AGB 3232	26.54±0.320	1	2	3	2	3	0.85±0.03	7
AGB 3190	45.32.±0.276	3	2	4	3	5	0.74±0.02	7
AGB 3142	16.48±0.254	1	3	5	4	5	0.83±0.03	7
AGB 3227	23.95±0.234	2	2	3	1	3	0.92±0.06	5
AGB 3133	19.45.±0.346	2	3	3	2	7	0.76±0.02	5

fruit size: 1=extremely small; 2=very small; 3=small; fruit shape: 2=rounded; 3=elliptic; fruit ground color: 3= light yellow; 4=yellow; 5=dark yellow; fruit over color: 1=pink; 2=red; 3=red violet; 4= violet; flesh firmness: 3=soft; 5= medium; 7= firm; eating quality:5= fair; 7=good

5. CONCLUSIONS

The characterization and evaluation of pomological characteristics based on UPOV Code and plum descriptors approved by the International Board for Plant Genetic Resources of the local plum cultivars: AGB 3232, AGB 3190, AGB 3142, AGB 3227, AGB 3133 conserved at National Collection, Albanian Gene Bank revealed that these local plum cultivars are unique.

The fruits of these cultivars are extremely small in size and unattractive to the market, but AGB 3232, AGB 3190 and AGB 3142 appear to be of good quality. AGB 3133 has firm flesh. All of them have different and attractive color skin. These local plum cultivars are of local importance and work for genetic improvement must further.

The characterization and evaluation of their pomological characteristics is not only unavoidable for their identification and conservation of fruit tree genetic resources, but also a means to address the establishment of a database with pomological data that could be considered as a valuable source for scientists and growers.

REFERENCES:

Funt RC. 1998. *Plums: A guide to selection and use*. Ohio State University Extension Fact Sheet.

Hârţa Monica, Sisea CR, Pop R, Szabo K, Zănescu M, Clapa D, Domokos D, Mihai Botu, Pamfil D. 2016. The Current Status of Germplum Database: a Tool for Characterization of Plum Genetic Resources in Romania.

Bulletin UASVM Horticulture 73(2) Print ISSN 1843-5254, Electronic ISSN 1843-5394 DOI:10.15835/buasvmcn-hort:12324.

INSTAT. 2020. Vjetari rajonal statistikor.

International Bord for Plant Genetic Resources, Plum Descriptors. 1984.
/https://www.bioversityinternational.org/fileadmin/_migrated/uploads/tx_news/Plum_descriptors_1

Kim DO, Jeong SW, Lee CY. 2003. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. *Food Chemistry*, **81:** 321–326. [CrossRef].

Mićić N, Cvetković M, Đurić G, Vučković D. 2019. Pomological characteristics of plum cultivars introduced under the agro-ecological conditions of the Banja Luka region. *Acta Horticulturae*. 1260, 145-152 DOI:10.17660/ActaHortic.2019.1260.23
https://doi.org/10.17660/ActaHortic.2019.1260.23.

Rupasinghe HPV, Jayasanka S, Lay W. 2006. Variation in total phenolic contents and antioxidant capacity among European plum genotypes. Scientia Horticulturae, **108 (3):** 243–246.

PHYTOMINING AND AGROMINING CANDIDATE PLANTS OF HYPERACCUMULTIVE FLORA OF KOSOVO

Muharrem SALIHAJ
International Business College, Mitrovica, Kosovo
Aida BANI
Department of Agro-Environmental, Faculty of Agronomy and Environment, Agricultural University of Tirana, Albania

---

ABSTRACT
Phytomining technology employs hyperaccumulator plants to take up metal in harvestable plant biomass. Harvesting, drying and incineration of the biomass generates a high-grade bio-ore. We propose that "agromining" (a variant of phytomining) could provide local communities with an alternative type of agriculture on degraded lands; farming not for food crops, but for metals such as nickel (Ni). This type of operation is most efficient using perennial species that regenerate aboveground biomass rapidly after harvesting. This is a biennial investigation that involves the hyperaccumulative flora of Kosovo. As the present investigation aims to identify plant species for phytoremediation and phytomining purposes, *Odontarrhena muralis (synonym Alyssum murale)* and *Noccaea ochrolecum* which belong to Brassicaceae family were identified, because they are able to uptake more than 1000 mg kg^{-1} of Ni from the corresponding soil and accumulate that in their tissues.
Samples were collected from ten sampling sites across Kosovo. The results showed Ni levels varying from moderately high 1586 mg kg^{-1} to high (up to 7564 mg kg^{-1}) in all the *Odontarrhena muralis* samples, and Zn extracted in tissues of *Noccaea ochrolecum* resulted 798.7 and 5888 mg kg^{-1}. It has been proved that *Odontarrhena muralis* from serpentine soils of Kosova could be a candidate for agromining and phytomining.
Keywords: agromining, phytomining, Ni hyperaccumulation, ultramafic, Kosovo

1. INTRODUCTION

Phytomining is the phytoextraction and recovery of metals for commercial gain (Chaney *et al*., 2007; Tang *et al*., 2012). Ni hyperaccumulation has been

defined as the accumulation of at least 1,000 mg kg^{-1} Ni in the dry biomass of plants grown on a natural substrate (Brooks *et al*., 1977).

However, for a potential use in phytomining, we need to focus on "hypernickelophorous" species that can accumulate more than 10,000 mg kg^{-1} (Chaney *et al*., 2007; Bani *et al*., 2014).

Ultramafic outcrops in Europe cover more than 10,000 km^2 and soils derived from this bedrock are generally characterized by low fertility (low total N and available K and P contents) and productivity, making them unattractive for agriculture (Bani *et al.*, 2015a,b; Bani *et al*., 2019). In Albania, ultramafic outcrops cover 11% of the surface and the Mg-rich agricultural soil have been estimated to cover about 20907.4 ha of the about 313300 ha of total area of ultramafic substrates available in the country (Lekaj *et al*., 2019).

Serpentine soils cover 4.48 % of the territory of Kosovo from which 1.31 % (142.8 km^2) are soils developed on serpentinite and 3.16 % (344.32 km^2) are soils on harzburgite (peridotite).

The Ibri Valley in the North of Kosovo represents the largest ultramafic complex. Other ultramafic complexes are the Golesh Massif in Central Kosovo, and the southwestern Kosovo.

Several plant species that we know elsewhere that are Ni-hyperaccumulators (Bani *et al*., 2009; Bani *et al*., 2010; Bani *et al*., 2014) or that are suspected to be hyperaccumulators (i.e. Bornmuellera dieckii) could also be found in Kosovo (Stevanovic´ *et al*., 2003; Salihaj *et al*., 2016; Salihaj *et al*., 2018) including *Odontarrhena muralis and Noccaea ochroleuca(Boiss and Heldr) (*Meyer F.K.. 1973). Salihaj *et al*., (2018) said that the ultramafic hyperaccumulators grown in Kosovo are able to accumulate extremely high concentrations of Ni, and sometimes Co, in their aerial biomass. It has been showed that we can apply phytomining, cultivating *O. chalcidica (syn. Alyssum murale)* plants that are able to accumulate trace metals from metal-rich soils and transport them to the shoots (>1%), which can then be harvested as a bio-ore to recover highly valuable metals such as Ni (Li *et al*., 2003; Bani *et al*., 2007; 2015a, b; van der Ent *et al*., 2015; Bani *et al*., 2019). It was proven to be an efficient opportunity in (Chaney *et al*., 2007; Bani *et al*., 2015a; b).

The ultramafic soils of Kosovo are considered suitable for the cultivation of plants that could be used for phytomining and agromining purposes. It is worth mentioning that most of these areas are state property, and here the access is easier and more convenient.

The present investigation aims at identifying the hyperaccumulator plants in serpentine habitats, and the scanning for Ni hyperaccumulation along with their potential to be used for phytomining and agromining based on soil characteristics.

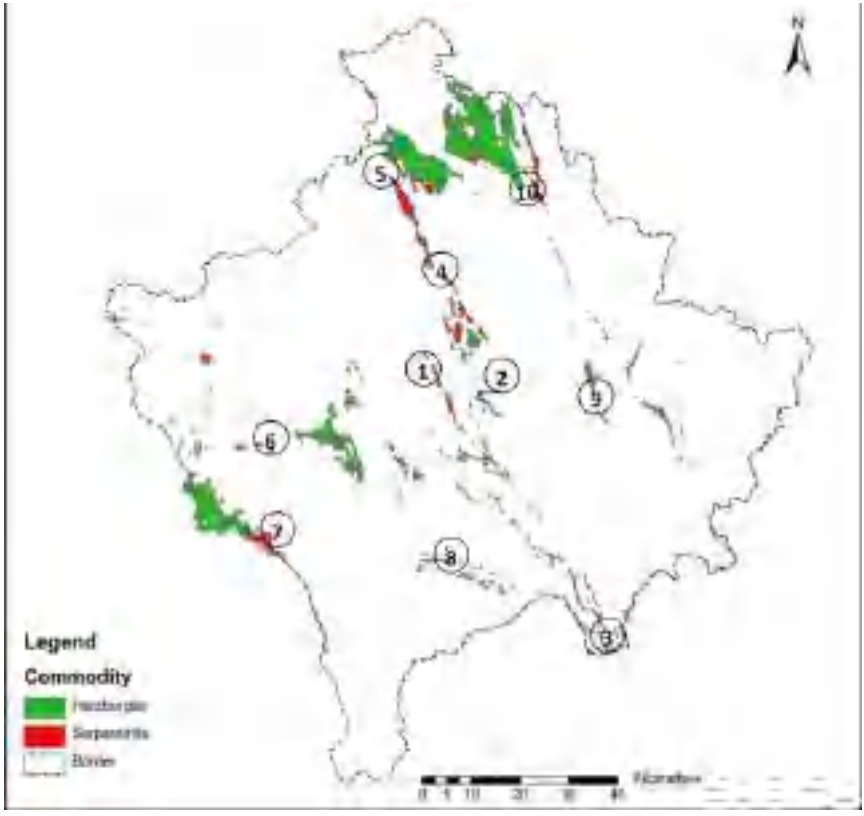


Fig. 1. The map of ultramafic soils of Kosovo.

2. MATERIALS AND METHODS

Sampling was carried out in ten sampling sites across Kosovo on June 2014. These areas are the most representative in terms of phytommining and agromining candidate plants. Twelve representative soil profiles from 10 different sites across the country were dug, and 27 horizon samples were collected. Regarding serpentine flora, at total of 162 plant species present in the ultramafic sites were collected. *Odontarrhena muralis* (syn. *Alyssum murale* Waldst. and Kit) and *Noccaea ochroleuca* (Boiss and Heldr.) Meyer F.K. 1973 (syn.*Thlaspi ochroleucum*) were the only potential candidate plant species for phytomining and agromining. In addition, the entire flora here located was identified, collected, air-dried, sieved to less than 2 mm and analysed for heavy metals concentrations and the calcium and magnesium

content. Soil samples were collected from the rhizosphere area of the plants. The soil samples were air-dried ground and sieved to 2 mm. Once air-dried ground and sieved, the samples were transported in polyethylene bags to the laboratory to analyse the total Ca, Mg, Ni, and Zn, and the results showed that each site exhibited a high concentration of one or more heavy metals.

All soil and plant's samples were air-dried ground and sieved to 2 mm or less. Then they were mineralized with a microwave digester. Conditions for mineralization were 6 ml HCl, 2 ml HNO3, and 3 ml H2O2, per 0.5-g soil. Soils were air-dried and sieved to mm. Total major (N, P, K, Ca and Mg) and some of the trace elements (Ni, Zn,) were determined in mineralization solution by atomic absorption spectrophotometry. The Mechlich 3 method was applied for the extraction of Ni, Ca, and Mg bioavailability in different soil samples.

3. RESULTS AND DISCUSSIONS

Soil characteristics

All the sampling sites were characterized by high levels of heavy metals exhibiting typical properties of an ultramafic environment.

Total Ni availability varied from 872 and 3298 mg kg^{-1}, whereas bioavailable Ni is found at the range of 57.2 mg kg^{-1} and 125 mg kg^{-1}, respectively. There are some correlations between the bioavailable Ni in soil with Ni level found in *Odontarrhena muralis* such as sample site Golesh #2 where bioavailable Ni in soil (**table 1.**) corresponds with hyperaccumulated Ni in this plant (**table 2.**) Regarding the Ca content in serpentine soil, results showed that the bioavailable Ca concentration is very low compared to Mg, which results in a negative Ca/Mg ratio in most sampling sites. Regarding the N, P and K content, most of the soil samples are characterized by low level of these nutrients, which is a general characteristic of ultramafic soils (Whittaker 1954; Brooks 1987). Fertilization of native *A. murale* with NPK promoted shoot biomass yields without affecting Ni concentration in shoot, resulting in increased Ni removal (Bani *et al.*, 2007). In fertilized plots the biomass yields progressively improved from 2.6 to 6.0 t ha^{-1}, and Ni removal increased from 22.6 to 69 kg ha^{-1} (Bani *et al.*, 2007; 2009).

The low content of available phosphorus in serpentine soils might be connected to the high affinity of soluble phosphates to serpentine (Brooks *et al.*, 1987).

Potassium content is in Table 3 reported. As it could be noted the potassium content is very low. Moreover, the content of bioavailable macro elements such as Ca, K is also very low. It varies from 1028 to 3480, and from 52.2 to 471.7 for Ca and K, respectively. This is typical of ultramafic soils.

Levels of Mg and Fe proved that serpentine soils are lithological weathered products which consist predominantly of ferromagnesian silicate minerals.

Ni content in hyperaccumulative plants

Odontarrhena muralis reported the highest Ni concentration. This plant species is dominant in all the serpentine sites. The Ni uptake varied from 1585-7564 mgkg-1, which is lower than Ni concentration found in the same species grown in the serpentine soils of Albania (19100 mgkg-1). Here, the edaphic characteristics (topographic characteristics) of these sampling sites might be the source of low levels of Ni concentration rates. As the sites are very steep, nutrients specifically nitrogen leach from runoff waters. Consequently, Ni uptake by hyperaccumulator plants has significantly reduced.

On the other hand, Ni concentration found in *Noccaea* grown Kosovo varies between 799 mg kg^{-1} and 5888 mg kg^{-1}, which is higher than Ni concentration found in *Noccaea* grown in Albania (1360 mg kg^{-1}), or in Bulgaria (3400 mg kg^{-1}).

Basically Ca/Mg ratio to Nickel hyperaccumulator plants is greater than one (>1), which is contradictory to serpentine associated soils. This ratio is between 2 and 21 for *Odontarrhena muralis*, while for *Noccaea* is between 2 and 10 (**Table 2**.). Positive Ca/Mg ratio has also been observed for the *O. chalcidica* grown in serpentine soils of Albania.

Table 1 Zn, Ni, Ca and Mg concentrations in soil and Ca/Mg Ratio

	Total trace elements			Exchangeable elements		
Sample point	Zn	Ni mgkg^{-1}	**Ni**	**Ca** mgkg^{-1}	**Mg**	**Ca/Mg Ratio**
Site 1 Kishnareke P#1	31.8±0.9	2248±104.4	110±12	3480±74	4239±121	0.82
Site 1 Kishnareke P#2	35.6±2.7	2275±85.2	101±6.6	1082±40	3912±98	0.28
Site 2 Golesh P#1	31.8±2.6	872±18.2	115±5.4	1150±52	3784±120	0.30
Site 2 Golesh P#2	42.3±2.7	1347±23	125±3.4	1869±42	3331±116	0.56
Site 3 Rezhance	51.4±1.4	3298±13.0	68±6.4	1028±41	3645±68	0.28
Site 4 Vejshtine	32.7±2.7	1543±123	57.2±3.2	3061±68	1289±48	2.38
Site 5 Çaber	39±5.3	2235±47	40.8±3.5	1164±52	1548±78	0.75
Site 6 Radoniq H-A	31.7±1.7	2234±51.6	101±13	1711±62	1004±32	1.70
Site 7 Q. Prushit H-A	38.9±4.1	2272±85.2	90.7±5.8	2289±94	1782±44	1.28
Site 8 Mushtisht H-A	34.8±2.9	2428±84.4	68.7±8.2	1235±75	2680±115	0.46
Site 9 Badovc H-A	56.4±2.1	1049±98.3	67.4±7.6	2323±78	1928±65	1.21
Site 10 Kaqandoll H-A	33.7±1.7	1837±66.5	62.9±6.3	1300±110	1315±42	0.99

High calcium content in these hyperaccumulator plants is due to the unusual ability of these plants to accumulate high Ca concentration in their tissues, even from the soils with low Ca/Mg ratio which is typical property of serpentines soils. Consequently, these plants can lower Ca deficiency stress for themselves.

Table 2. Hyperaccumulators candidates for phytomining and agromining

Nr	**Species**		**Zn**	**Ni**	**Ca**	**Mg**	
		Sample Site		mgkg^{-1}			Ca/Mg Ratio
1	*Odontarrhena muralis (1)*	1	87.8	7010	34700	1640	21
2	*Odontarrhena muralis (2)*	1	58.9	7280	16490	2730	6
3	*Odontarrhena muralis (1)*	2	73.4	7564	23720	1860	13
4	*Odontarrhena muralis (2)*	2	59.2	6871	11580	1820	6
5	*Noccaea ochroleuca (1)*	2	425	4303	12840	1400	9
6	*Noccaea ochroleuca(2)*	2	512	5888	8770	900	10
7	*Odontarrhena muralis*	3	35.1	3066	13055	1138	11
8	*Odontarrhena muralis*	4	143	4039	5136	2343	2
9	*Odontarrhena muralis*	5	30.8	1586	20313	6252	3
10	*Odontarrhena muralis*	6	70.2	2980	15625	2343	7
11	*Noccaea ochroleuca*	6	593	799	14844	1772	8
12	*Odontarrhena muralis*	7	19.8	2742	5131	524	10
13	*Odontarrhena muralis*	8	34.8	7117	10493	2658	4
14	*Odontarrhena muralis*	9	22.1	2446	2905	1165	2
15	*Noccaea ochroleuca*	9	177	5862	6548	7859	1
16	*Odontarrhena muralis*	10	73.6	2764	16861	3609	5

Table 3. Macronutrients concentrations in soil

Site		**mg kg^{-1} DM**			**% DM**	**% DM**
	N	**P**	**K**	**Ca**	**Mg**	**Fe**
Vejshtine HA	440	105	104.7	3709	17.90	4.87
Vejshtine HB	320	91	88.6	5422	18.76	5.06
Çaber HA	510	120	471.7	1837	17.77	5.15
Çaber HB	470	89	140.9	728	19.20	4.50

Çaber HC	670	108	52.2	1072	19.29	3.65
Radoniq HA	1020	370	382.6	1845	16.00	3.93
Radoniq HB	980	215	149.5	629	19.37	2.43

4. CONCLUSIONS

The serpentine soils samples collected from the 10 different sites showed high Ni, Zn, Mg and Fe concentration. The high concentration of available Mg and Fe, and low to moderately high available Ca concentration make the soils be more or less of typical ultramafic content.

Presence of hyperaccumulator plants is so diverse. *Odontarrhena muralis* and *Noccaea* are dominant species, but *Noccaea* could be occasionally found in serpentine areas. Results report about a close relationship between the Ni amount in soil and Ni uptake in plants. The highest concentration of Ni in *Odontarrhena muralis* is exactly where the bioavailable Ni is at its spike. The ratio between the concentration of bioavailable Ni in the hyperaccumulative plant and the corresponding soil is 60:1.

These plants species can be used for phytomining and agromining purposes, when applying some additional measures to improve the edaphic conditions and fertilizers such as NPK and moderate amounts of Ca to soil not only to support the growth of plants, but also to decrease the soil pH, hence increasing the metal bioavailability (Bani *et. al*., 2008)

REFERENCES

Bani A, Echvarria G. **2019**. Can organic amendments replace chemical fertilizers in nickel agromining cropping systems in Albania? *International Journal of Phytoremediation*,**21(1):** 43-51. doi: 10.1080/15226514.2018.1523871. Epub 2019 Jan 16. PMID: 30648409.

Bani A, Echvarria G. Zhang X, Simonnot M. 2015. The effect of plant density in nickel phytomining field experiments with *Alyssum murale* in Albania

Bani A, Echvarria, G, Sulçe S, Morel L 2014. Improving the Agronomy of Alyssum Murale for extensive phytomining: A five Year Field Study. *International Journal of Phytoremidiation* Volume 17, 2015 Pages 117-127.

Bani A, Echvarria, G, Sulçe S, Mullaj A. 2007 In-situ phytoextraction of Ni by a native population of Alyssum murale on an ultramafic site (Albania)

Bani A, Echvarria, G, Sulçe S, Mullaj A. 2009. Nickel Hyperaccumulation by Brassicaceae in Serpentine Soils of Albania and Northwestern Greece.

Bani A, Echvarria, Sulçe S, Pavlova D. 2010. Nickel hyperaccumulation by the species of Alyssum and Thlaspi (Brassicaceae) from the ultramafic soils of the Balkans.

Brooks R.R, Lee J. Reeves, R. D, Jaffer T. 1977. Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. *Exploration* **7:** 49-57.

Brooks, R.R. 1987. Serpentine and its vegetation. A Multidisciplinary Approach. Dioscorides Press, Portland.

Chaney R.L. Angle J.S, Broadhurst C.L, Peters C.A, Tapero, R.V, Sparks, D, L 2007. Improved understanding of hyperaccumulation yields commercial phytoextraction and phytomining technologies. Journal of Environmental Quality 36: pages 1429-1443.

Lekaj E, Teqja Z, Bani A. 2019. The dynamics of land cover changes and the impact of climate change on ultramafic areas of Albania.

Li Y-M, Chaney RL, Brewer E, Roseberg R, Angle JS, Baker AJM, Pavlova D, Bani A, Xhaferi B, Vila D, Vila K. 2018. Effect of Nickel on Seed Germination of Alyssum Species with potential for phytomining in Albania.

Meyer FK 1973. Conspectus der "Thlaspi"-Arten Europas, Afrikas und Vorderasiens. Feddes Repert 84: 449-470.

Salihaj M, Bani A. 2018. Chemical properties of serpentine soils from Kosovo.

Salihaj M, Bani A. 2016. Heavy Metals Uptake by Hyperaccumulating Flora in the Serpentine Soils of Kosovo.

Stevanovic V, Tan K, Iatro G. 2003. Distribution of the endemic Balkan flora on serpentine I. - Obligate serpentine endemics.

Tang Y-T, Deng T-H-B, Wu Q-H, Wang S-Z, Qiu R-L, Wei Z-B. **2012**. Designing cropping systems for metal-contaminated sites: A review. *Pedosphere*, **22**: 470-488.

Van der Ent, A.; Erskine, P.; Sumail, S. 2015 Ecology of nickelhyperaccumulator plants from ultramafic soils in Sabah (Malaysia). Chemoecology.

Whitaker R.H. 1954. The ecology of serpentine soils. *Ecology* **35(2):** 258-288.

ASSESSMENT OF PHYSICO-CHEMICAL PROPERTIES OF WINE PRODUCED TRADITIONALLY IN THE SOUTHEAST ALBANIA

Amilda BALLATA and Jonida CANAJ
Department of Industrial Chemistry, Faculty Natural Sciences,
University of Tirana, Albania

ABSTRACT
Variety, composition and treatment of grapes, along with wine production technology, fermentation process, wine must treatments, sulfidation, etc.,—all impacting the physico-chemical characteristics of wine. Consequently, different wines have different characteristics. The present paper assesses the physico-chemical parameters of traditionally produced wine. Samples were collected from the final packed product provided by the canteen in the southeast Albania and compared with the national standards. For commercial reasons, the name of canteen is not mentioned. The densitometry method was applied for the wine density. The Gibertini distiller expressed in v / v was used for the alcohol content. The method of determining the specific mass (direct method) expressed to gr / l was applied for the total extract. The blue bromothymol expressed in gr / l ac tartaric was used for the total titrated acidity, free sulfur dioxide and the total sulfur dioxide. Laboratory investigation was carried out at the Customs Laboratory in Tirana, Albania. Results reported that all the physico-chemical parameters were within the permissible limits and, could be considered of good quality.
***Keywords*:** total sulfur dioxide SO_2, red wine, white wine, alcoholic degree

1. INTRODUCTION

Wine is an alcoholic beverage obtained by the complete or partial alcoholic fermentation of fresh grapes or wine must. Wine consists of a large number of organic or inorganic compounds, some of which are present in the wine must, while some others decrease or increase during fermentation. As the physico-chemical characteristics of wine depend on many external and internal factors such as variety, composition, treatment of grapes, wine production technology, fermentation process, wine must treatments, sulfidation, etc., different wines appear to have different characteristics (Grainger and Tattersall 2005; Burns and Osborne 2013). The samples were collected from white and red wines produced in the traditional way for

personal use from grape varieties in the hilly area, southeast Albania (Muscat, Tokay and Tempranillo, etc.). Thus, our study focused on the evaluation of the physic-chemical parameters to see if these parameters comply with the standard used in the Albania Republic State and if these wines can be used. Based on the study of these characteristics we can obtain a database of physic-chemical characteristics and information about the method of production, applied techniques, packaging and storage conditions. Ethyl alcohol is the ingredient in the largest amount, its content is directly related to the amount of sugar must, from which it is related and the fermenting power of yeast (Burns and Osborne 2013). It is a very good solvent for aromatic substances and contributes to the aroma of wine. Ethyl alcohol has an important role in the preservation of wine from pathogenic microorganisms. Tartaric acid is resistant to bacterial activity, but some lactic acid bacteria can react with tartaric acid, to form acetic acid and lactic acid. In this case, the wine loses its fixed acidity. Sulphite components have antiseptic, selective, and acidifying properties, when preventing the microorganisms that cause the breakdown of malic and tartaric acid from development. In addition, they have clarifying properties when allowing the rapid clearing of cider, antioxidant properties, protects wine must and wine from oxidation, solvent properties help in the digestion of substances, found in the skin of grains (Standartet Shtetërore 1982). Wine acidity is one of the important qualitative indicators. Wine characteristics depend on grapes composition (The free encyclopedia of wine and winemaking).

2. MATERIAL AND METHODS

Description of the sample

Representing the final product, the samples were collected from white and red wine bottles— 4 bottles per each, respectively. White and red wine were synchronously produced in the southeast hilly area in Albania.

Methods

The present paper investigates the physico-chemical characteristics of wine produced for domestic use, and the results are compared to the national standards. Laboratory investigation was carried out at the Customs laboratory, Tirana. the remainder of this subchapter describes the physic-chemical parameters investigated and the methods applied per each parameter. Wine samples were taken to laboratory using a chemical glass 500 ml. The Anton Paar DM 500 densitometry was used for the wine density at 20^0C after immersing the probe in the glass. Determination of total sulfur dioxide (SO_2) is carried out in a100 ml flask where 25 ml NaOH 1 N and 50 ml of wine are added. The balloon is allowed to rest for 15 minutes to release the anhydride

that is bound. Once is released, the anhydride is treated with 10 ml sulfuric acid (H_2SO_4) and 1 gram of starch. Once treated with H_2SO_4 and 1 gram of starch, it is placed in an electric blender to titrate with 0.02N Iodine solution. The free sulfuric anhydride is determined as following: 50 ml of wine sample is added to a chemical glass 250 ml and treated with 3 ml of H_2SO_4 (1: 4) and 1 gram of starch. Once treated, it gets titrated with Iodine N / 50 solution. Ethyl alcohol is determined based on the distillation wine method in the D.E.E Gibertini apparatus. 100 ml of wine sample is measured in the tarred flask of the apparatus which is placed in the bath at 20^0C for about 30 minutes. Once placed for 30 min. it is transferred to the distillation apparatus flask (after making sure that the discharge tap is completely closed). The tarred balloon is washed several times with distilled water (about 40 ml in total). Once washed, 5-10 drops of anti-foam and 5-10 drops of 2M lime solution are added in the distillation balloon. 2 ml of distilled water are added to the same flask which is placed in the same side where the distillate is taken. If 80% of the distillate is obtained, distillation process is automatically stopped. A quantity of distilled water not up to the mark is here subsequently added. The balloon is placed back in the bath at 20^0C for about 30 minutes. It is then brought finally it to the mark with distilled water and stirred prior to the measurement with the Anton Paar DMA 500M densitometer. The total acidity determined based on the neutralization of the sample with 1N sodium hydroxide solution in the presence of blue indicator thymol bromide. To this is added 10 ml of wine, 50 ml of distilled water and 0.5 ml of blue thymol bromide to an electric blender. It is then titrated with 0,1N sodium hydroxide until clear blue (with a single dot). The determination of the total extract is based on the determination of the specific mass of residue after distillation, carried to the initial volume with distilled water. 100 ml of wine is taken in a flask which is placed in the water bath until 20 ^{0}C is reached for about 30 minutes. The flask content is placed in a distillation flask which is filled with distilled water up to 1/3 of the flask. The balloon is montaged in the manual distillation apparatus which is switched on to allow the water enter in order to obtain 80-90 ml distillates. The distillation residue obtained after cooling is carefully poured into a 100 ml tarred flask, the distillation flask is rinsed several times with water and thrown into the same tarred flask, tossed with water at a temperature of 20^0C, mixed to homogenize and set specific mass of liquid in DMA densitometer 500 M Ant on Paar.

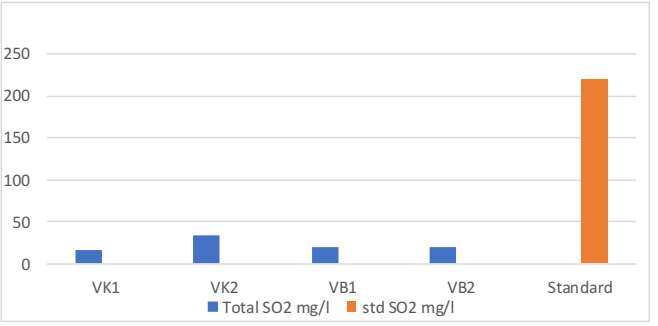
3. RESULTS

The Tables 1-4 provide the information about the physico-chemical parameters of wine, while the Graphs compare these data to the National

Standards (Standartet Shtetërore-1982). As it could be noted the parameters are within permissible data.

Table 1. Total sulfur dioxide mg/l results of red and white wine compared to standard.

Sample	Total SO_2 mg/l	Total SO_2 mg/l Standard
VK1	15.36	
VK2	33.2	
VB1	19.96	
VB2	20.03	
		Not more than 220 mg/l

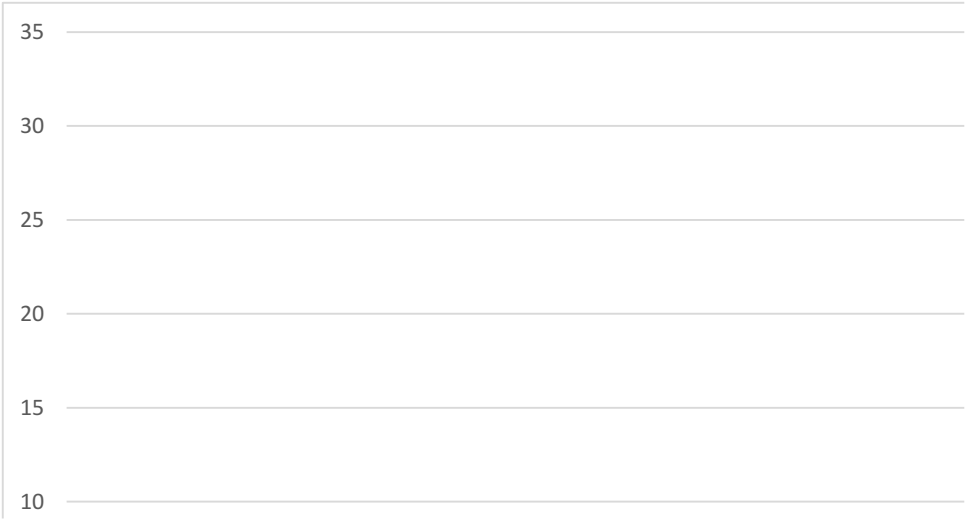


Graph.1. Total sulfur dioxide mg/l results of red and white wine compared to standard.

The Graph. 1 plots the values of total sulfur dioxide (SO_2) of the red and white wine varying 15.36-33.2 mg / l and 19.96-20.03 mg / l, respectively. The total values of sulfur dioxide (SO_2) for the analyzed wine samples vary from 15.36 to 20.03 mg / l, i.e., within the permissible values (not more than 220 mg / l).

Table 2. Free sulfur dioxide (SO_2) mg/l results of red and white wine compared to standard.

Sample	Free SO_2 mg/l	Free SO_2 mg/l Standard
VK1	5.76	
VK2	6.4	
VB1	7.89	
VB2	7.96	
		Not more than 30

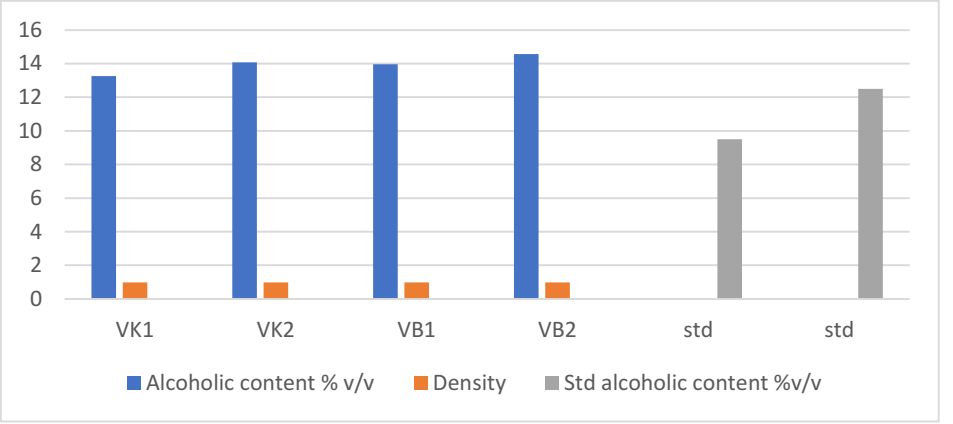


Graph. 2. Free sulfur dioxide mg/l results of red and white wine compared to standard.

The Graph. 2 plots the values of free sulfur dioxide (SO_2) varying from 5.76-6.4 mg /l and 7.89-7.96 mg /l for the red and white wine, respectively, i.e., within permissible limits.

Table 3. Alcoholic content %v/v and density results of red and white wine.

Sample	Alcoholic content % v/v	Density
VK1	13.26	0.9979950
VK2	14.08	0.980012
VB1	13.964	0.975220
VB2	14.562	0.977511

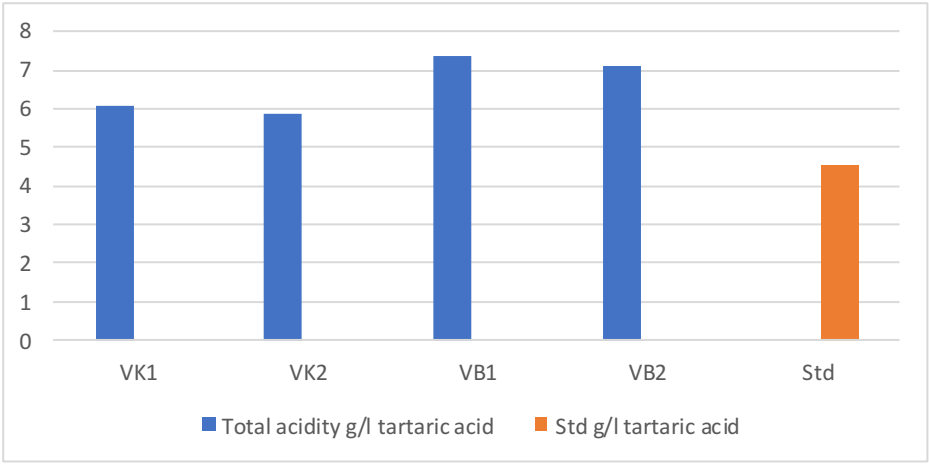


Graph. 3. Alcoholic content %v/v and density results of red and white wine.

In general, for all samples the results of the alcohol content are within the standard 9.5-12.5% v / v. Alcoholic content values in red wine vary from 13.26-14.08% v / v, in white wine vary from 13.964-14.562% v / v. Density is an indicator of fermentation performance. Density in red wine varies from 0.97990-0.980012. In white wine it varies from 0.975220-0.977511.

Table 4. Total acidity g/l results of red and white wine compared to standard.

Sample	Total acidity g/l tartaric acid	Total acidity g/l tartaric acid Standard
VK1	6.075	
VK2	5.853	
VB1	7.364	
VB2	7.100	
		Not less than 4.5

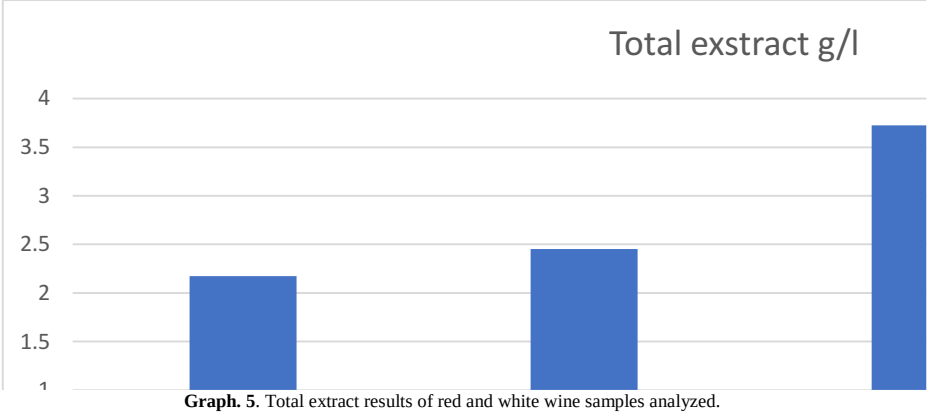


Graphic 4. Total acidity g/l results of red and white wine compared to standard.

Results report that the level of total acidity is within permissible limits. In red wine it ranges from 5.853-6.075 g / l tartaric acid. In white wine it varies from 7.100 to 7.364 g / l tartaric acid.

Table 5. Total extract results of red and white wine samples analyzed compared to standard.

Sample	Total extract g/l
VK1	2.173
VK2	2.452
VB1	3.725
VB2	2.998



Graph. 5. Total extract results of red and white wine samples analyzed.
The extract values appear to be 2.173-2.452 g/l and 2.998- 3.725 for the red and white wine, respectively g/l.

4. CONCLUSIONS

The following conclusions could be drawn:

The density varies from 0.975220-0.980012 showing a whole fermentation process.

Alcoholic content for the analyzed wine samples ranges from 13.26-14.562% v / v, which is within permissible limits as based on the national standards.

The total acidity values range from 5.853- 7.100 g /l tartaric acid —within the national standard values (not less than 4.5g /l) —proving their unmodified content and being undiluted with water.

The values of free sulfur gas vary from 5.76 to 7.96 mg / l, which are within the standard values (not more than 30 mg / l).

The total SO_2 for the analyzed wine samples results to be within permissible limits, preventing wine from oxidation and bacterial diseases (spoilage by bacteria). The values of total sulfur dioxide vary from 15.36-20.03mg /l (not more than 220mg /l).

The general extract is a specific indicator of grape cultivation and shows the content of organic matter of grapes and the wine produced from contain. The content of the extract is also related to the agrotechnics used for grape cultivation and the type of pruning that significantly affects the structure of the wine obtained. The total extract ranges from 2.173 to 3.725 g /l, proving that both red and white wine are naturally produced.

REFERENCES:

Burns TR, Osborne JP. 2013. Impact of malolactic fermentation on the color and color stability of Pinot noir and Merlot Wine. *American Journal of Enology and Viticulture*, **64(3):** 370-377.

Gayon RP, Dubourdie D, Doneche B, Lonvaud A. 2006. Handbook of Enology. **1**, The Microbiology of Vine and Vinifications 2-nd Edition, France.

Grainger K, Tattersall H. 2005. Wine production. 9781405113656.

Standartet Shtetërore-1982.

The free encyclopedia of wine. **2005**. 978-1405305174.

CONTROL OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS INFECTION IN A DAIRY FARM IN ALBANIA

Liljana LUFO, Kastriot KORRO
Faculty of Veterinary Medicine, Agriculture University of Tirana
Gjena DURA
Laboratory BIO-V, Bio-diagnostic Veterinary and Food Laboratory
Gani MOKA
Faculty of Veterinary Medicine, Agriculture University of Tirana

---

ABSTRACT

Paratuberculosis is a chronic and contagious granulomatous enteritis. In cattle it is characterized by persistent diarrhea, and progressive weight loss (Whittington and Sergeant 2001). The *Mycobacterium avium subsp. paratuberculosis* (MAP) is the etiologic agent and, is excreted in large numbers in feces of infected animals and less in colostrum and milk. Fecal shedding begins before the appearance of clinical signs, and subclinical animals are important sources of transmission. Infected animals can appear healthy for months to years. The present paper reports the successful decrease from 2018-2021 of the seroprevalence and infection rates in a herd with MAP infection. A total of 1613 sera and 47 stool samples underwent RT PCR and microscopic investigation. Stool and blood samples were collected from animals of different age groups. The Real Time PCR employing TaqMan® MAP Reagents & Xeno™ DNA Controls and DNA amplification involving Applied Biosystems 7500 Real-Time PCR System was used to test the bacterial shedding of stool samples. The Ziehl-Neelsen method was applied for the direct detection of MAP in stained faecal smears. Individual serum samples tested via cELISA were used to detect antibodies against MAP (https://www.idexx.co.uk/en-gb/livestock/livestock-tests/ruminant-tests/idexx-paratuberculosis-verification-ab-test/). High percentage in positive results (6.8% seroprevalence, and 77% - 88% agent detection) show the possibility of a long-term infection persistence in this heard. Detailed analysis was necessary for the evaluation of positivity. In addition, management measures for the disease control were considered. Applying different laboratory methods is an added value to early detection of infected animals (Pinedo *et al*. 2008). Testing strategy accompanied by appropriate management measures in the herd show impressive decrease in the prevalence of infection.
Keywords: MAP, seroprevalence, Real Time PCR, cELISA, microscopy

1. INTRODUCTION

Animal production in Albania is considered one of the most important branches of the economy, because it's the main source of income for the rural population. Cattle are raised almost everywhere, even in remote mountainous areas. The official data of 2020 report that the population of cattle and calves is approximately 370 thousand, 300 thousand of which are dairy cows. In total, there are 122 thousand cow–calf herds which makes up almost 1/3 of herds in the country. Herds with over 100 cows are few, just several dozen. There are many and complex reasons behind the challenges the cattle breeding sector is facing. One of the most sensitive aspects in cattle breeding is the control and monitoring of diseases, especially those that cause economic damage to the herd, paratuberculosis or Johne's Disease is among of them.

Paratuberculosis is a chronic, and contagious granulomatous enteritis. In cattle it is characterized by persistent diarrhea, and progressive weight loss (Whittington and Sergeant 2001). Mycobacterium avium subsp. paratuberculosis (MAP) is the agent of John's disease (JD) in cattle. M. Avium subsp. paratuberculosis belongs to the species M. avium, subspecies: M. Avium subsp. Avium (synonym, M. avium, M. Avium subsp. Paratuberculosis (synonym, M. paratuberculosis) (Imirzalioglu *et al*, 2011). MAP lives in intestinal cells and lymph nodes, and it's excreted in large numbers in feces of infected animals and less in colostrum and milk (Collins 2021). Calves become infected usually within first month of age, but also during fetal life. Fecal shedding begins months even years before the appearance of clinical signs. The main source of transmission is subclinical animals that can appear healthy for months to years.

The present investigation aims to serologically, microscopically, and molecularly diagnose the MAP in dairy cows. The diagnosis tests were chosen based on the costs and number of samples. Direct diagnosis is based on the detection of MAP in feces using Real Time PCR (Stevenson 2010), and microscopic examination via Ziehl - Neelsen (ZN) stain method. For the detection of antibodies against MAP in individual serum, ELISA test is generally used. It is best used to determine the infection prevalence in a herd, and to detect infection in the later stages of infection, but also it's a rapid and low-cost method (Collins *et al*., 2006).

2. MATERIALS AND METHODS

2.1 Sample selection

Fecal and blood samples were collected from animals of different age groups of one of the biggest herds in Albania. These samples are used in regular basis to detect *Mycobacterium avium subsp paratuberculosis*. Fecal culture is considered more sensitive and specific, and pooling of fecal samples of individual animals may reduce the high costs of fecal culture (Van Schaik 2007).

Initially, the criteria used to determine the heard health status, was testing all animal. Intending strategy was to remove infected cows along with limited measures to protect vulnerable calves. In the first year blood samples were taken from all adult dairy cows and heifers. In third were included in sampling calves less than 6 months off age.

2.2 Samples preparation

Blood samples were collected in plastic tubes, without anticoagulants. The serum was separated in the laboratory and stored in congealment till testing time. From suspected cows or those with clinical signs of disease, fecal samples were collected. Fecal samples were collected in 200 ml plastic cups and transported to the laboratory under cooling condition. The fresh stool sample is stored for at least 1-2 hours at room temperature. Once brought to laboratory, the samples underwent microscopic examination and stored at -18°C for further examination.

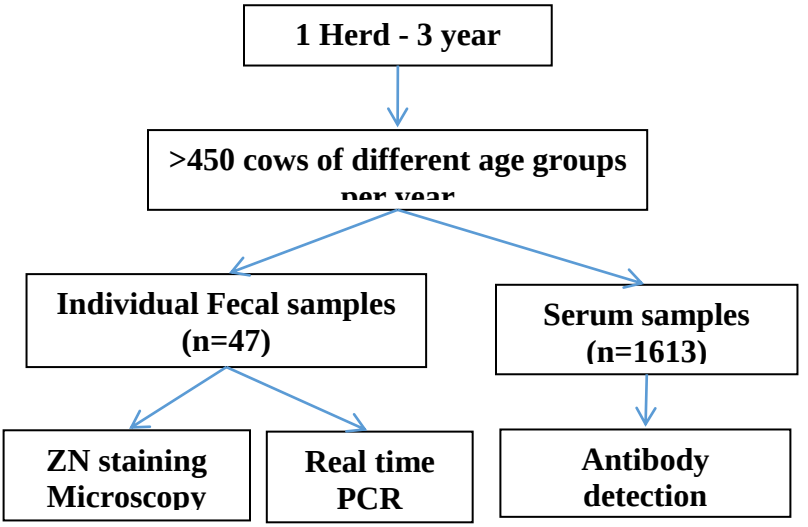


Fig. 1: Overview of study design.

2.3 METHODS

Microscopic investigation employing the Ziehl-Neelsen method determines the presence of mycobacteria directly in stained fecal smears. The method is based on phenol allowing fuchsine solution to penetrate inside bacterial cells. Once stained with fuchsine solution, some mycobacterium remained red even after the application of the acid-alcoholic solution, which means that this bacteria is acid-alcohol resistant. Other bacteria lose their red color and turn blue from the application of methylene blue solution.

Detection of MAP using RT PCR was carried out on individual fecal sample collected from suspected animals (Imirzalioglu *et al*., 2011). The total DNA is extracted using PSP Spin Stool DNA kit. Once extraction occurs, the master mix process involving the TaqMan® MAP Reagents & TaqMan® MAP and Xeno™ DNA Controls and DNA amplification using the Applied Biosystems 7500 Real-Time PCR System subsequently follows. The amount of specific target sequences present in a sample was calculated by measuring *ct* values and using standard curves generated with a series of known quantities of target sequences.

For the detection of antibodies directed against MAP in individual serum and plasma samples an ELISA test was used (Hirst *et al*., 2002). IDEXX paratuberculosis verification. microplates are coated in alternance with control antigen (-Ag) and with MAP antigen (+Ag). Once pre-incubated (30 – 120 min) with *M. phlei* extract to bind unspecific antibodies, the samples are transferred and incubated in the wells of coated microplates. Once the

unbound material is washed, the anti-ruminates antibody enzyme conjugate is added, which binds to any Ag-Ab immune-complex. The unbound conjugate is subsequently washed away and a substrate/chromogen solution is added. In the presence of enzyme, substrate reacts with the chromogen to generate a blue color. Upon addition of stop solution, a yellow color is generated. This color development is directly related to the amount of antibodies against MAP present in the test sample.

3. RESULTS

1613 cows blood samples were submitted to tests. 47 out of 1613 cows' blood samples underwent real time PCR and ZN staining for microscopy. ELISA test provided 6.8% positive results (31/450) in the first year. 7 out of 9 stool samples showed pigmentation via microscopic examination. All fecal samples regardless the microscopic data underwent real time PCR, and results showed 8 positive cases. In the second and third year number of positive animals decreased noticeably. The change in seroprevalence and direct detection of MAP is in the Table 1 reported.

Table 1. Positivity % of MAP in, using 3 different methods

	ELISA		MICROSCOPY		RT PCR	
Year	**Sample no./+**	**%Positive**	**Sample no.**	**Positive**	**Sample no.**	**Positive**
2018	450/31	6.8%	9/7	77.7%	9/8	88.8%
2019	468/29	6.1%	26/5	19.2%	26/7	26.9%
2020	695/7	1%	12/3	25%	12/4	33.3%

Regardless the method applied and the high number of animals of all age categories included, the total percentage of positivity is quite low in the third. Table 2 reports the positive relationship between agent and antibody detection**.**

Table 2. Comparing positivity % of MAP between methods

	ELISA		MICROSCOPY		RT PCR	
Year	**Sample no./+**	**%Positive**	**Positive**	**% in herd**	**Positive**	**% in herd**
2018	450/31	6.8%	7	1.5%	8	1.7%
2019	468/29	6.1%	5	1.0%	7	1.4%
2020	695/7	1%	3	0%	4	0.5%

4. DISCUSSION

MAP appears in a range of forms of disease with high prevalence. Detection of infected animals within a herd relies on use of different laboratory tests. A combination of laboratory tests to animals in different stages of the disease and would provide appropriate information about the prevalence of the disease in the herd. In addition, hygienic conditions and breeding in stables are necessary. The time factor and tests repetition are the greatest allies of MAP detection because of the disease progress and effectiveness of most of the tests in the later stages of the disease.

In the farm prevalence of infection decreased because the infectious pressure was reduced by culling of heavily shedding animals and the limited measures to prevent the calves from exposure to Mycobacterium avium subsp. paratuberculosis. Cows that resulted positive when tested with ELISA were separated from other animals and placed in a different compartment. All management and milking activities were separately carried out.

Other management measures for a higher protection of young animals through separation from infected cows and their colostrum would have reduced the risk of neonatal infection. Calves born from positive mothers were fed pasteurized colostrum and milk, or fed by other healthy cows in the herd. This was considered the most effective measure in reducing the risk of infection in newborn calves.

Shedding cows within a herd will be a source for the contamination of environment with MAP until they are removed from the herd, contributing to the presentation of new infections, if no control program is established. Quantitative use of ELISA to identify animals for selective culling or isolation in herd is a cost-effective strategy for disease control; lower ELISA values are associated with lower probabilities of infection and lower rates of fecal shedding.

Despite the importance of the cattle production, MAP in Albanian herds remains relatively unexamined, and very limited epidemiological information and data especially on molecular characterization of MAP are available. The present investigation is the first of its kind in Albania, and additional studies must further. Application of molecular methods allow for the identification of various MAP isolates present in our country, in addition to their genetic description and differentiation.

REFERENCES

Collins MT, Gardner IA, Garry FB, Roussel AJ, Wells SJ. 2006. Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States. *Journal of the American Veterinary Medical Association*, **229(12):** 1912–1919.

Collins MT. 2021. Paratuberculosis in Ruminants (Johne's Disease). Last full review/revision Feb 2021.

Hirst HL, Garry FB, Salman MD. 2002. Assessment of test results when using a commercial enzyme-linked immunosorbent assay for diagnosis of paratuberculosis in repeated samples collected from adult dairy cattle. *Journal of the American Veterinary Medical Association*, **220 (11):** 1685–1689.

IDEXX Paratuberculosis Verification Ab Test Manual. https://www.idexx.co.uk/en-gb/livestock/livestock-tests/ruminant-tests/idexx-paratuberculosis-verification-ab-test/

Imirzalioglu C, Dahmen H, Hain T, Billion A, Kuenne C, Chakraborty T, Domann E. 2011. Highly specific and quick detection of Mycobacterium avium subsp. paratuberculosis in feces and gut tissue of cattle and humans by multiple real-time PCR assays. *Journal of Clinical Microbiology*, **49(5):** 1843-52. doi: 10.1128/JCM.01492-10. Epub 2011 Mar 23. PMID: 21430100; PMCID: PMC3122678.

Pinedo PJ, Rae DO, Williams JE, Donovan G, Melendez AP, Buergelt CD. 2008. Association among results of serum ELISA, faecal culture and nested PCR on milk, blood and faeces for the detection of paratuberculosis in dairy cows. *Transboundary and Emerging Diseases*, **55(2):** 125–133.

Stevenson K. 2010. Diagnosis of Johne's disease: current limitations and perspectives. *Cattle Practice*, **18 (2):** 104–109.

Van Schaik G, Pradenas M, Mella A, Kruze J. 2007. Diagnostic validity and costs of pooled fecal samples and individual blood or fecal samples to determine the cow- and herd-status for *Mycobacterium avium* subsp. *Paratuberculosis*. *Preventive Veterinary Medicine*, **82(1-2)**: 159–165.

Whittington RJ, Sergeant E. 2001. Progress towards understanding the spread, detection and control of *Mycobacterium avium* subsp *paratuberculosis* in animal populations. *Australian Veterinary Journal*, **79(4):** 267–278.

BIOMARKERS AND GENETIC FACTORS RELATED TO PHYSICAL ACTIVITY AND ITS HEALTH BENEFITS

DHURATA BOZO and SUELA XHUFI
Department of Physical Activity and Health, Institute for Sport Research, University of Sports, Tirana, Albania

---

ABSTRACT

The benefits of physical activity (PA) on health and fitness are well known. Regular physical activity (PA) is associated with a reduced risk for many chronic medical conditions, including depression, cardiovascular disease; type 2 diabetes, obesity, and cancer. Recent studies show that heritability plays a significant role on the health benefits and the reduced risk for both prevention and treatment of chronic medical conditions. The purpose of this minireview is to summarize the existing literature on the role of biomarkers in general and genetic determinants of PA, on biological response and other biological factors on physical activity and its beneficiary effects for human health.Use of biochemical and physiological biomarkers, in conjunction with genetic factors are important and useful indicators to assess the human health status in general and health effects of physical activity and sports, the biological response to physical activity and in the context of the prevention and treatment of diseases arising from sedentary life related or not to biological and physical aging. The use of bio-genetic markers presents limitations related to their sensitivity, the lack of reference values for groups engaged in physical activity and sports, and others. In this context, the exploration of the genetic basis and hereditary factors, in collaboration with the above, takes on special importance for the purposes of this paper. The Human Gene Map for Performance and Health related Fitness Phenotypes considers a number of genes associated with exercise performance and response to exercise training. These genes interact with physical activity and/or exercise to influence multiple physical and physiological traits, biochemical parameters, and hemodynamics. The functions of these genes are consistent with the fitness phenotypes of physical activity and provide insight on how physiologic processes might drive the capacity for physical activity and the relative associations with performance phenotypes and their potentials to be used as markers for talent identification and trainability in sport. However, Physical Activity, biological response and its health benefits and sport performance is a complex multifactorial phenomenon governed by several intrinsic factors such as genetic polymorphism, psychomotor skills, physical fitness that are greatly influenced by a variety of

extrinsic factors such as diet, exercise andtraining, behavioral, social and cultural factors as well.
Keywords: biomarkers, Physical Activity, HGMPH, sport genes, polymorphisms

1. INTRODUCTION

Biomarkers are small molecules widely used for assessing the health status of individuals engaged in physical and recreational activity and for athletes in the context of tracking and monitoring physical and athletic performance. Traditionally, biomarkers started to be used in the field of sports, to assess, monitor and track athletic and training performance and to identify the conditions of training overload of athletes (Lee *et al*., 2017).

Biochemical and genetic markers are used today to assess the physical fitness, the cardiovascular capacity, muscle strength or power, oxidative stress, muscle fatigue, response to exercise, etc. Recently, there has been a growing interest in biomarkers used for assessing health-related aspects, both in individuals who engage in sports activity and those engaged in physical activity for health or recreation (Lightfoot 2013; Palacios *et al*., 2015).

The biological values of biomarkers depend on the health status of individuals, the status and level of physical activity and exercise load, type of sport, intensity and duration of exercise, in addition to other factors such as age group, gender, and special physiological, pathological or nutritional conditions. International studies provide recommendations and biomarkers lists to be used for tracking and monitoring changes in individuals engaged in physical activity and various exercise programs and for athletes of specific types of sports (Lee *et al*., 2017). Biochemical and physiological biomarkers present also some limitations related to their specificity, need to be used in combination with others, lack of reference values in active people and athletes, and others (Gielen *et* al., 2014).

The aforementioned limitations have called the need to explore more about genetic and molecular markers, which are the basis for the production and synthesis of the biomarker molecules themselves. However, it should be noted that the weight of genetic determinants is not absolute, as the fitness phenotype, the biological response to PA and its health benefits, as well as sport performance is a complex multifactorial trait determined by the combination and coordination between intrinsic (genetic polymorphism, psychomotor skills, physical fitness/health) and extrinsic factors (diet, exercise/ training behavior, social/educational/cultural factors) and others (Zhang *et al*., 2018).

Over the last 20 years the genetics of Physical Activity and sports has developed significantly and the obtained results/discoveries are impressive. It has become possible today to discover and identify the location and role of

hundreds of specific genes that directly or indirectly encode the traits related to fitness, exercise and sport performance (Lightfoot *et al*., 2018).

The genetic basis of the response to physical activity for health, recreation and sports is the most explored field especially in the last 10 years. There have been identified and defined genes that affect muscle strength, resistance, 'speed' , the bodily changes in response to exercise, those responsible for glucose and fat metabolism in response to exercise, for energy balance and obesity, including those dealing with interaction with exstrinsic factors. These discoveries enable today the design of special exercise programs for groups, individuals within the various goals of physical activity, to the design of specialized personalized programs and the identification and selection of talents in various sports. These studies and discoveries have also paved the way for the genetic modification techniques and methods that constitute the basis of genetic doping, a critical and much debated issue in the field of sports, directly related to the health of physical exercisers (Dias, 2011; Lee *et al*., 2017).

Given the importance of these markers and the limited knowledge about them and their use, the present paper reviews the current data regarding the types of biomarkers, their importance with a special focus on the genetic markers used to evaluate and asses the health-related fitness phenotypes and the impact of physical activity on human health.

2. MATERIALS AND METHODS

This publication is a summary mini-review on issues and scientific aspects related to the current state of knowledge on biomarkers used in the context of the impact that health activity has on its broad conception of human health, of individuals engaged in physical and recreational activities as well as to guarantee the health of athletes of various sports. The literature references of this mini-review have been selected among those reported during the last 10 years from reliable and reputable sources and publications in scientific journals.

3. RESULTS AND DISCUSSION

Biomarkers for Physical Activity and Health

Biomarkers are useful tools for assessing and monitoring health, training status and performance.

Traditionally, biomarkers have been of interest in sports in order to measure performance, progress in training and for identifying overtraining, to identify the degree of physical fitness, chronic stress, overtraining, cardiovascular risk, oxidative stress and inflammation. In sport, biomarkers

are key parameters to assess the impact of exercise on different systems, tissues and organs. During the last years, growing interest is set on biomarkers aiming at evaluating health-related aspects which can be modulated by regular physical activity and sport. Today we can estimate parameters for assessing the degree of fitness, muscle damage, hydration/dehydration, inflammation, oxidative damage, fatigue, overtraining, etc, which facilitate the evaluation of the response of the human body at the different levels of physical activity or training being carried out. Biomarkers can also be used to measure the impact of training on the long term or the acute effect of exercise (Papa *et al*., 2015).

Depending on the purpose of their use and application, they can be classified and grouped into markers that assess and monitor muscle condition, inflammation in general and muscle in particular (including cardiac), muscle strength and strength, metabolic changes such as consequence of exercise, the level of parameters such as glycemia or cholesterolemia as an effect of exercise. In the field of applications in the field of sports they are classified into biomarkers that assess muscle energy status, lactate production rate, hydration status, inflammatory effects of exercise, recovery after exercise, inflammatory and other injuries caused by excessive or poor exercise, etc. (Van der Mee *et al*., 2018).

The value or concentration of a biomarker depends on many factors, as the training status of the subject, the degree of fatigue and the type and duration of exercise, apart from age and gender, among others. Other useful biomarkers are body composition (specifically muscle mass, fat mass, weight), physical fitness (cardiovascular capacity, strength, agility, flexibility), heart rate and blood pressure. Depending on the aim, one or several biomarkers should be measured. It may differ if it is for research purpose or for the follow up of training (Lee *et al*., 2017).

A single measurement of a biomarker does not enable a complete and accurate assessment of an individual's health status. Even when specific molecular and biochemical markers have high sensitivity, their combined use does not always yield accurate and well-referenced data. This is further complicated by the fact that individuals exhibit an extremely wide inter-individual absolute and relative variance even under normal conditions, the more it becomes difficult to interpret and compare in the conditions of PA and sports. Moreover, reference values for biomarker values or concentrations specifically to physically active people and athletes are lacking, which may lead to incorrect interpretation of the results (Palacios *et al*., 2015).

A research about the hematological biochemical parameters of a target groups of athletes of different sports has been carried out at the Institute for Sport Research, University of Sports, Tirana, Albania, and the results reported that the reference values used as a basis for evaluation or comparison are different and variable. In addition, it is known that individuals engaged in

regular physical activity and athletes may have biomarker values / concentrations which would be considered in the range as pathological in untrained people, as observed in the hematological and biochemical parameters of routine in laboratory analysis. Therefore, it is important to adjust the reference values as much as possible and to regularly check each item to set its reference rate. In order to avoid this limitation of biomarkers as much as possible, in addition to continuing studies in this field, our research team is currently elaborating a set of reference values for some biochemical and hematological indicators specially adapted for the assessment of health status and the athletic performance of athletes of various sports (Bozo and Lleshi 2012; Della Valle, 2013).

Another several-year study in female volleyball players, showed abnormal variations in a number (7 out of 13) of critical biochemical-hematological parameters, most of them related to red blood cells, haemoglobin, iron status, etc. These call for special attention to anemia problems within the teams and medical care as a matter to be seriously addressed by the coaches, as one of the main female triade health issue in sports (Della Valle and Haas 2013; Bozo and Lleshi, 2014).

Genetic determinants related to physical fitness and health

Although it is aleady known that there is a considerable genetic component, PA is such a complex phenotype quantification and clear understanding still remains a challenge for future studies (Karvinen *et al*., 2015).

Current understanding of the genetic architecture contributing to PA is limited, especially compared with other phenotypes like height, and genetic diseases like obesity and diabetes. As the ultimate level of PA is an interaction between the genes and the environment, it is more correct to state that genetics influences a predisposition to engage in activity, which is then expressed or not in relation to environmental factors (Aaltonen *et al*., 2013).

Family studies show that genetic factors contribute to variation in PA with heritability estimates ranging from 9% to 57%. A number of family studies suggest that it is difficult to disentangle the effects of genetics from shared environmental effects. Twin studies may address better this issue and reported that heritability of PA is between 43% to 52% in adolescents, 30% in young adulthood and no uniparental heritability was found (Bouchard, 2011; De Vilhena et al., 2012).

Genome-wide linkage studies (GWAS) use association-based candidate-gene methods to provide additional insights into the genetic architecture underlying human PA (Kim et al., 2011; Dias, 2011).

GWAS enables also a more precise location of the potentially causal genes involved in the PA phenotype (Church *et al*., 2011; Stenholm *et al*, 2014; Pedersen and Saltin 2015).

Thanks to these modern techniques and methods, hundreds of candidate genes have been identified as directly or indirectly related to Physical Activity and its health impact and for the treatment of health conditions, physical fitness and sport performance (De Vilhena *et al*., 2012; Gielen *et al*., 2014; De Geus *et al*., 2014;).

Physical Performance and Health-related Fitness Genes

The human gene map for performance and health-related fitness phenotypes (HGMPHFP) contributed significantly to the identification and gene mapping of physical performance and health-related fitness phenotypes. A total of 239 genes and markers with evidence of association or linkage with a performance or a fitness phenotype are positioned on the map of autosomes (214), X chromosome (7) and mitochondrial DNA (18). The map is continuously growing in number and complexity (De Geus *et al.*, 2014; Diego *et al*., 2015; Lighfoot, 2018; Zhang and Speekman, 2018;).

Physical performance and health-related fitness phenotypes genes are classified based on different criteria and depending on the study or the purpose of use use.

Endurance / resistance genes.

These group of genes encode for molecules that define or influence human structures and functions, increase endurance and muscular endurance and in turn promote the increase of muscle mass. They affect contraction power, blood flow, aerobic fitness, insulin sensitivity, response to hypoxia and other traits, related directly or indirectly to physical activity/sports and health benefits (Lee *et al*., 2017; Dias 2011).

ACE gene is responsible for the production of the Angiotensin-Converting Enzyme, which catalyzes the conversion of angiotensin I to its active form, II. ACE has multiple effects as a vasoconstrictor, regulator of salt and water homeostasis, of inflammatory reactions, erythropoiesis, tissue oxygenation, and skeletal muscle efficiency. Of the two alleles (I and D), the D allele produces a more active enzyme, associated with physical and sports traits common in power sports athletes: I allele associates to anabolic response in bursting power sports. These results makes ACE an important gene to track PA and health benefits outcome (Gielen *et al*., 2014; Bruneau *et al*., 2017; Lightfoot *et al*., 2018).

PPAR-delta (Peroxisome-proliferator Activator Receptor**) gene** regulates the expression of several other genes that promote the further proliferation of slow muscle fibers, increasing the slow and fast muscle fibers, thus affecting

speed and endurance and fat breakdown activity in adipose tissue (De Vilhena *et al*., 2012).

PPARGC1A (peroxisome proliferators-activated receptor g coactivator 1a**) gene** produces an PPAR activator enzyme that controls the glyco-lipid metabolism and conversion of fats into sugars for immediate energy use, thus promoting the increase of muscle fibers and the mitochondrial biogenesis. Two alleles (A and G) are known to correlate with opposite features of muscle performance, speed or resistance (Lee *et al*., 2017).

Genes that affect muscle performance

This category includes candidate genes that influence muscle structure, strength and speed and other properties related to physical and athletic performance.

MSTN (myostatin) gene codes for myostatin, a protein which acts as a negative regulator of skeletal muscle growth, whose inactivation promotes abnormal increase of muscle mass. MSTN is a candidate gene for the treatment of atrophy and muscular dystrophy and subject to doping in sports. Myostatin inhibitors are used as doping agents and listed among the limited inhibitors by the World Anti-Doping Agency (WADA) (Bouchard 2011).

ACTN3 gene: produces Alpha-Actinin3, the binding protein of actin fibers in fast fibers of scheletal muscle, where its presence in high amounts causes a fast explosive powerful muscle contraction. Of the two R and X alleles of ACTN3, R (rapid) determines the increased amount of actin-3 in fast muscle fibers. Variants containing the R allele are very common (about 85%) in the population of Jamaica while in other population it doesn't exceed 14-20% (Lee et al., 2017; Gielen et al., 2014).

CK-MM gene is responsible for the synthesis of Creatine-kinase (CK), which catalyses the conversion of creatine into phosphocreatine (PCr), expressed by various tissue cells, In tissues that consume ATP rapidly, i.e. skeletal muscle, PCr serves as an energy reservoir for the rapid regeneration of ATP. The CK-MM isoenzyme is found in the skeletal and cardiac muscle and increases in cases of muscle damage/injury (Kleiner *et al*.; 2013; Koch *et al*., 2014).

Currently, the Institute of Sports Research, at the University of Sports of Tirana is investigating on a set of biochemical and genetic (ACE, ACTN3, MSTN and CK-MM) in a group of Albanian athletes.

Genes that affect cardiac and respiratory functions

NRF (nuclear respiratory factors) **genes** affect cardiac and respiratory functions, studied in the context of the genetic and biochemical basis of increased cardiac / respiratory capacity, and the ability to respond to long-term adaptation to exercise (Bruneau, *et al*., 2017).

The three types of NRF genes (NRF1, NRF2 and NFE2L2) play a special role in adaptation to exercise. The various genotypes (GG, AA, GA) affect cellular energy economy, increase mitochondrial density in muscles, activate of oxidative phosphorylation (aerobic) and translation of biochemical signals into physiological adaptive responses and increasing endurance and resistance to PA/exercise (Church *et al*., 2011).

Endotelin1 gene produces a protein expressed in vascular endothelium, acts as a vasoconstrictor regulating (increasing) blood pressure and is higly tempered by activity level, cardiac fitness and adaptation. The G allele is associated with increased cardiorespiratory fitness, while T allele with increased hypertension and pulse pressure response to training (Zhang and Speakman, 2019).

The Nitric oxide synthase gene (NOS) produces endothelial nitric oxide (NO) acting as a vasodilator and increasing the blood flow to the skeletal muscle. The NOS3 variant might be a candidate for the adaptive cardiac capacity in elite endurance athletes (Gielen *et al*., 2014).

- ***Training response genes***

Under this group are considered a number of genes that direct and control important enzymes in the metabolism of nutrients and energy, responsible for the metabolism of glucose and fats in response to exercise, etc. Instead than single genes, is the combination, integration and interaction of multiple genes and extrinsic factors that affect performance, determine the individual variation of the response to exercise, make an individual/athlete fit and respond better by increasing physical and athletic performance while maintaining a healthy state (Church *et al*., 2011; Stenholm *et al*., 2014).

This group includes also 127 genes considered as candidate genes associated with obesity and the impact of exercise. These include genes that respond to different traits and functions, that affect energy balance, the body weight, metabolism according to diet, predisposing to weight/obesity, and others (De Vilhena *et al*., 2012; De Geus *et al*., 2014).

REFERENCES

Aaltonen S, Ortega-Alonso A, Kujala UM, Kaprio J. 2013. Genetic and environmental influences on longitudinal changes in leisure-time physical activity from adolescence to young adulthood. *Twin Res Hum Genet. 16(2):535*

Bouchard C. 2011. Overcoming barriers to progress in exercise genomics. *Exerc Sport Sci Rev. 39(4):212–217*

Bozo Dh, Lleshi E. 2014. Anaemia and iron deficiency in female volleyball players; comparison with a reference group. Journal of Human Sport and Exercise. ISSN 1988-5202. Vol. 9(1) (special issue).

Bruneau M Jr, Angelopoulos TJ, Gordon P, Moyna N, Visich P, Zoeller R, Seip R, Bilbie S, Thompson P, Devaney J, GordishDressman H, Hoffman E, Pescatello LS. 2017. The angiotensin-converting enzyme insertion/deletion polymorphism rs4340 associates with habitual physical activity among European American adults. *Mol Genet Genomic Med. 5(5):524–530*.

Church TS, Thomas DM, Tudor-Locke C, et al. 2011. Trends over 5 decades in U.S. occupation–related physical activity and their associations with obesity. *PLoS One. 6(5):e19657*

De Geus EJ, Bartels M, Kaprio J, Lightfoot JT, Thomis M. 2014. Genetics of regular exercise and sedentary behaviors. *Twin Res Hum Genet. 17(4):262–271*

De Geus EJ, De Moor MH.2008. A genetic perspective on the association between exercise and mental health. *Ment Health Phys Act. 1(2):53–61*.

De Vilhena e Santos DM, Katzmarzyk PT, Seabra AFT, Maia JAR. 2012. Genetics of Physical Activity and Physical Inactivity in Humans. *Behaviour Genetics. Springer*. DOI 10.1007/s10519-012-9534-1

DellaValle, DM. 2013. Iron supplementation for female athletes: Effects on iron status and performance outcomes. *Curr Sports Med Rep 12: 234–239*

Dias RG. 2011. Genética, performance física humana e doping genético: o senso comum versus a realidade científica. *Rev Bras Med Esporte.*Vol.17 no.1 São Paulo. ISSN 1517-8692.

Diego VP, De Chaves RN, Blangero J, et al. 2015. Sex-specific genetic effects in physical activity: results from a quantitative genetic analysis. *BMC Med Genet. 16:58*

Gielen M, Westerterp-Plantenga MS, Bouwman FG, et al. 2014. Heritability and genetic etiology of habitual physical activity: a twin study with objective measures. *Genes Nutr. 9(4):415.*

Gielen M, Westerterp-Plantenga MS, Bouwman FG, Joosen AM, Vlietinck R, Derom C, Zeegers MP, Mariman EC, Westerterp KR. 2014. Heritability and genetic etiology of habitual physical activity: a twin study with objective measures. *Genes Nutr;9(4):4*

Karvinen S, Waller K, Silvennoinen M, Koch LG, Britton SL, Kaprio J, Kainulainen H, Kujala UM. 2015. Physical activity in adulthood: genes and mortality. *Sci Rep. 5(1):18259*.

Kim J, Oh S, Min H, Kim Y, Park T. 2011. Practical issues in genomewide association studies for physical activity. *Ann N Y Acad Sci;1229(1):38–44.*

Kleiner, G, Marcuzzi, A, Zanin, V, Monasta, L, and Zauli, G. 2013. Cytokine levels in the serum of healthy subjects. *Mediators Inflamm 2013: 434010.*

Koch AJ, Pereira R, and Machado, M. 2014. The creatine kinase response to resistance exercise. *J Musculoskelet Neuronal Interact 14: 68–77*

Lee EC, Fragala MS, Kavouras SA, Queen RM, Pryor JL, and Casa DJ. 2017. Biomarkers in sports and exercise: tracking health, performance, and recovery in athletes. *J Strength Cond Res 31 (10): 2920–2937*

Lightfoot JT, De Geus EJC, Booth FW, Bray MS, M. DEN Hoed, Kaprio J, S. A. Kelly, D. Pomp, M. C. Saul, M. A. Thomis, T. Garland JR, and C. Bouchard. 2018. *Biological/Genetic Regulation of Physical Activity Level: Consensus from GenBioPAC. Med. Sci. Sports Exerc., Vol. 50, No. 4, pp. 863–873*

Lightfoot JT. 2013. Why control activity? Evolutionary selection pressures affecting the development of physical activity genetic and biological regulation. *BioMed Res Int; 2013:821678*

Palacios G, Pedrero-Chamizo1 R, Palacios N, Maroto-Sánchez1 B, Aznar S, and González-Gross M. 2015. Biomarkers of physical activity and health. *CIBERobn (Fisiopatología de la Obesidad y la Nutrición CB12/03/30038).*

Papa L, Ramia MM, Edwards D, Johnson BD, and Slobounov SM. 2015. Systematic review of clinical studies examining biomarkers of brain injury in athletes after sports-related concussion. *J Neurotrauma 32: 661–673.*

Pedersen BK, Saltin B. 2015. Exercise as medicine - evidence for prescribing exercise as therapy in 26 different chronic diseases. *Scand J Med Sci Sports;25(Suppl 3):1–72*.

Stenholm S, Mehta NK, S, Elo IT, Heliövaara M, Koskinen Aromaa A. 2014. Obesity and muscle strength as long-term determinants of all-cause mortality--a 33-year follow-up of the Mini-Finland Health Examination Survey. *Int J Obes (Lond) ;38(8):1126-32*

Van Der Mee DJ, Fedko IO, Hottenga JJ, Ehli EA, Van Der Zee MD, Ligthart L, Van Beijsterveldt TCEM, Davies GE, Bartels M, Landers JG, De Geus EJC. 2018. Dopaminergic genetic variants and voluntary externally paced exercise behavior. *Med Sci Sports Exerc. 50(4):700–708*.

Vink JM, Boomsma DI, Medland SE, et al. 2011. Variance components models for physical activity with age as modifier: a comparative twin study in seven countries. *Twin Res Hum Genet. 14(1): 25–34.*

Zhang X and R. Speakman, J .2019. Genetic Factors Associated With Human Physical Activity: Are Your Genes Too Tight To Prevent You Exercising*? ORCiD numbers: 0000-0002-2457-1823*

AN INTEGRATIVE TAXONOMIC STUDY OF MOLLUSCS FROM BELSHI KARSTIC LAKES USING MOLECULAR GENETIC ANALYSIS

Xhoia MUJALI
Department of Biology, Faculty of Natural Sciences, University of Tirana, Albania
Elisabeth HARING, Helmut SATTMANN, Michael DUDA
Natural History Museum Vienna, Austria
Sajmir BEQIRAJ
Department of Biology, Faculty of Natural Sciences, University of Tirana, Albania

---

ABSTRACT
High species diversity and the large number of endemic species make the mollusc fauna of the Balkan Peninsula one of the most important hotspots of the Holarctic region. Belshi lakes, situated in central Albania, represent a typical and interesting area of karstic lakes. As their mollusc fauna has been randomly investigated, information remains very limited. Although molecular genetic methods, especially DNA barcoding techniques have significantly become important in biodiversity research, they have been rarely applied in studies of aquatic fauna of Albania, and even lesser in molluscan studies. This paper represents a survey of the malacofauna of the Belshi lakes and first steps into DNA barcoding analysis of selected molluscs collected there. Molluscs samples were collected from 34 lakes in the spring and autumn of 2018. The DNA barcoding analyses included sequencing of a short fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene and comparisons with a library of DNA barcodes of known species (Barcode of Life Data Base BOLD or GenBank). Standard DNA extraction and PCR of the COI marker sequence were carried out via standard barcoding primers and Sanger sequencing of the PCR products. DNA analysis confirmed the presence of six molluscs species, two of which did not match well with sequences available for comparison. The species thought to be *Gyraulus albus* was quite distantly related to that taxon. Also, the species that was initially identified as *Dreissena polymorpha* turned out to be very closely related to the *Dreissena carinata* clade. The results here reported show also the importance of integrative taxonomy, which along with morphological and genetical analysis help to better understand the relationships among freshwater mollsucs in the Balkans and their taxonomic issues.
***Keywords*:** barcoding, freshwater malacofauna, Balkan Peninsula, Albania

1. INTRODUCTION

Molluscs, like many other groups of macroinvertebrates have an essential role in the functional processes of aquatic ecosystems such as marine, freshwater and brackish. They have a key position within food networks, being at the same time decomposers and / or filters even for higher trophic levels (Griffiths, 1991).

Integrated taxonomy is the study of taxonomy, genetics, origin, phylogenetic relationships, biogeography, development, behaviour, ecology etc. of species, combining (integrating) morphological and anatomical characteristics with those of molecular genetics (Araujo, 2007).

In Albania, DNA barcoding techniques have been scarcely used, mainly for some species of medicinal plants and domesticated animal species in agriculture. The application of these techniques is almost absent in studies of wild fauna, aquatic fauna and especially molluscs in Albania.

Belshi lakes are of karstic origins and located in Central Albania. They represent the group with the largest number of lakes (88 lakes in total) in the hydrogeographical network of Albania.

Currently, there is no information about the malacofauna of Belshi Lakes. The summary lists of continental molluscs of Albania, referred to Dhora and Welter-Schultes (1999), Fehér and Eröss (2009) and Dhora (2010) do not mention mollusks from Belshi lakes.

The present paper aims to: i) identify the mollusks' species based on the integrated taxonomy, meaning the combination of morphological and anatomical data with those of molecular genetics to increase the accuracy and confidence in species identification and, ii) to compare the mollusks' species from Belshi lakes with the same species from the Balkan region and larger via molecular genetic analysis.

This investigation is the first attempt to obtain information about the malacofauna of these lakes and to use molecular genetic analyses and integrative taxonomy for a better understanding of relationships among freshwater mollsucs in the region.

2. MATERIAL AND METHODS

Sampled lakes and sampling periods

Mollusk sampling was carried out in 34 lakes in Belshi. The selection of lakes has generally been random, but also taking into account the possibility of easy access.

Fig. 1. Map of Belshi area, showing the sampled lakes.

Table 1. List of sampled lakes and their respective geographical coordinates

	Lake	COORDINATES
1	Liqeni i Katundit	40°57'54.1"N 19°58'23.3"E
2	Liqen pa emër	40°57'45.1"N 19°58'37.2"E
3	Liqeni i Miloshit	40°57'49.9"N 19°58'45.3"E
4	Liqeni i Bicit	40°57'39.0"N 19°58'42.1"E
5	Liqeni i Ulzës	40°58'07.0"N 19°58'12.0"E
6	Liqeni Kashaj	40°57'55.3"N 19°58'01.4"E
7	Liqen pa emer	40°58'03.2"N 19°57'56.1"E
8	Liqeni i Komnecit	40°58'00.4"N 19°57'24.3"E
9	Liqeni i Godës	40°58'37.1"N 19°57'47.9"E
10	Liqeni i Kashtës	40°59'05.7"N 19°56'52.0"E

11	Liqeni i Thatë	40°59'15.5"N 19°56'20.7"E
12	Liqen pa emër	40°59'00.1"N 19°55'43.4"E
13	Liqen pa emër	40°58'58.3"N 19°55'39.7"E
14	Liqeni i Strehës	40°58'41.1"N 19°55'38.0"E
15	Liqen pa emër	40°58'44.2"N 19°55'23.3"E
16	Liqeni i Belshit	40°58'42.4"N 19°53'32.1"E
17	Liqen pa emër	40°58'57.1"N 19°54'15.7"E
18	Liqeni i Gjatë	40°59'04.2"N 19°54'25.5"E
19	Liqen pa emër	40°59'16.6"N 19°54'27.6"E
20	Liqeni i Trojsit	40°59'18.6"N 19°53'50.4"E
21	Liqen pa emër	40°59'02.5"N 19°53'48.6"E
22	Liqeni i Dorbnit	40°57'06.3"N 19°52'29.2"E
23	Liqeni i Merhojës	40°56'36.6"N 19°52'25.1"E
24	Liqeni i Cestijes	40°55'09.1"N 19°52'01.7"E
25	Liqeni i Seferanit	40°57'18.8"N 19°53'55.0"E
26	Liqeni i Civiles	40°57'21.6"N 19°54'28.8"E
27	Liqen pa emër	40°57'33.5"N 19°55'30.9"E
28	Liqeni i Paçit	40°57'33.1"N 19°55'38.3"E
29	Liqeni i Këlvorës	40°57'16.2"N 19°55'33.9"E
30	Liqen pa emër	40°53'34.9"N 19°53'48.9"E
31	Gjoli i Turbullt	40°53'49.7"N 19°53'29.8"E
32	Liqeni Gropa e Selamit	40°53'40.8"N 19°53'09.1"E
33	Liqen pa emër	40°53'47.3"N 19°52'16.2"E
34	Liqeni i Gropës së Madhe	40°53'47.9"N 19°52'01.5"E

Sampling was carried out in spring (April - May) 2018 and autumn (September - October) 2018. In the lakes of the Mediterranean climatic zone (which includes the lakes of Belshi, and all the other lakes in Albania), these periods generally coincide with the highest values of dissolved oxygen in the water and their better environmental condition (in spring), and the decrease of the values of dissolved oxygen and increase of eutrophication and environmental stress (in late summer and early autumn) as stated in (Wetzel 2001).

Mollusks' sampling

The molluscs sampling is based on (Hájek *et al*., 2006; Horsak *et al*., 2015). Each lake was sampled at three equidistant points that were selected based on the diversity of micro-habitats in the lake, type of substrate, the presence or absence of macrovegetation, and the presence of stones, wood, or artificial materials on substrate. Sampling of aquatic mollusks was done in transects at 1 m distance from the shore, along a 10 m linear stripe on lake substrata. Sampling time interval was 20 minutes at each point. Samples on soft bottom and macrovegetation were taken with a hand net of 0.5 mm mesh size and a diameter of 20

cm. Hand picking with forceps was applied on hard substrata and woods. The samples were stored in plastic bottles with 90% alcohol and transported to the laboratory.

Taxonomic identification and molecular genetic analyses of mollusks

Taxonomic identification of mollusks was initially done at the Laboratory of Environmental Biology, Faculty of Natural Sciences, University of Tirana, Albania. Later on, the samples were transferred for molecular genetic analysis at the Laboratory of Molecular Systematics of the Museum of Natural History, Vienna, Austria. Small tissue fragments were taken from each animal's body for DNA extraction and they were allowed to dry from the remaining ethanol. Tissues were treated for extraction using the Qiagen 2011 method (QIAamp®DNA Mini Kit). After extraction, the next step in DNA sequencing was the PCR. The results were processed through applications for processing DNA sequences such as: BioEdit, referred to Hall T. A (1999), GenBank (https://www.ncbi.nlm.nih.gov/), Molecular Evolutionary Genetics Analysis (MEGA) (https://www.megasoftware.net) and The Barcode of Life Database (BOLD) (http://www.barcodinglife.org).

3. RESULTS AND DISCUSSIONS

There are eight mollusks species found in Belshi lakes as listed below:

GASTROPODA
Radix auricularia (Linnaeus, 1758)
Ampullaceana balthica (Linnaeus, 1758)
Galba truncatula (O. F. Müller, 1774)
Gyraulus chinensis (Dunker, 1848)
Physella acuta (Draparnaud, 1805)
Valvata piscinalis (O. F. Müller, 1774)

BIVALVIA
Musculium lacustre (O. F. Müller, 1774)
Dreissena carinata (Dunker, 1853)

Six mollusks' taxa underwent molecular genetic analyses. Two species, namely *Ampullaceana balthica* and *Valvata piscinalis*, did not undergo such analysis as they were found only as empty shells. Consequently, molecular genetic analyses appeared impossible.

Genus *Radix*

Based on data from the Laboratory of Environmental Biology in Tirana and the Department of Invertebrate Zoology, Museum of Natural History, Vienna, Austria, it was initially thought that from the genus Radix in our samples, in addition to *(Radix) Ampullaceana balthica* (Linnaeus, 1758), we had two other species: *Radix auricularia* (Linnaeus, 1758) and *Radix balthica* (Linnaeus, 1758). DNA analysis reported that both supposed taxa belong to the species *Radix auricularia* (Linnaeus, 1758). This is could be clearly noted in the phylogenetic tree (Figure 3), where the sequences of individuals found in Belshi lakes stand in the same lineage with *Radix auricularia* sequences found in Greece, Montenegro, Croatia, and Germany. These sequences appear to be far from *Radix balthica* species (Linnaeus, 1758).

Genus *Physella*

Although in the present in phylogenetic tree (Figure 3) and the existing databases, there are few species of *Physella acuta* from our region (the Balkans). Its position in the lineage of the species *Physella acuta* (Draparnaud, 1805) from samples originating from many countries like Greece, Italy and Austria is very much clear.

Genus *Galba*

Regarding this genus, there are no species from the Balkans in the presented phylogenetic tree and in the existing databases. Regarding the species *Galba truncatula* (O. F. Müller, 1774), there were considering similarities between the sequence of the species found in Belshi lakes and those from Germany and Spain, but also from countries far from our region such as Russia and Peru (Figure 3).

Genus *Gyraulus*

The individuals of genus *Gyraulus* were initially identified as *Gyraulus albus* (O.F. Muller, 1774), supported by existing reports of this species in Albania (Dhora and Welter-Schultes 1999; Fehér and

Erös 2009), but DNA sequences of it turned out to be similar by over 99.2% to those of *Gyraulus chinensis* (Dunker, 1848), (Figure 2), which is clearly distinguishable even in the phylogenetic tree (Figure 3). As aforementioned said, the species *Gyraulus albus*, stands far from the *Gyraulus chinensis* lineage and DNA sequences from our samples.

Fig. 2. Estimated similarity of DNA sequences between different *Gyraulus* sampled in the Belshi lakes and different *Gyraulus* according to the Barcode of Life Database (BOLD).

Genus *Musculium*

Regarding the genus *Musculium*, the species found in the Belshi lakes has been identified from the beginning as *Musculium lacustre* (O. F. Muller, 1774). Comparison of its DNA sequences has been made with the species found in Germany and Georgia. Although with few sequences to compare, from the analyzes performed there was no doubt about the position of the sequence of the species found in Belshi lakes as *Musculium lacustre* (Figure 3).

Genus *Dreissena*

Through identification keys, the species of this genus from Belshi lakes was initially identified as *Dreissena blanci*, referring to existing reports of this species in Albania (Dhora and Welter-Schultes, 1999; Dhora and Beqiraj, 2001; Dhora 2002; 2004; Beqiraj *et al*., 2006; Beqiraj *et al*., 2012; Beqiraj 2013; Peçulaj 2014).

Based on DNA analysis, the phylogenetic tree showed that this species found in Belshi lakes, is not only part of the clade *Dreissena carinata* (= *D. blanci, D. presbensis, D. stankovici*) from the *Dreissena* sequence analysed in other Balkan countries such as the Republic of

North Macedonia, Greece, Croatia, Montenegro, Hungary, and Mediterranean countries such as Italy but also in more distant countries such as Germany and the USA. Here, clarifying the name and the synonymy of this species would be necessary. There has been a recent revision with regard. Until a few years ago, the taxa *Dreissena stankovici* (Lvova and Starobogatov, 1982) and *Dreissena blanci* (Westerlund 1890) were considered as different species and as such they are still found in the database of Fauna Europaea (https://fauna-eu.org/). Following the recent revision, these two taxa, together with the *Dreissena blanci var. presbensis* (Kobelt 1915) are not accepted as species and have been replaced by *Dreissena carinata* (Dunker, 1853), as currently found in the MolluscaBase (https://www.molluscabase.org/) and WoRMS databases (http: //www.marinespecies .org).

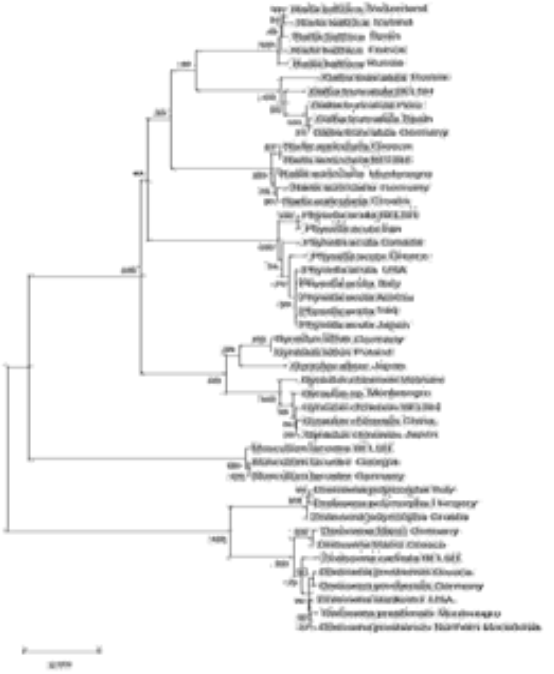


Fig. 3: Phylogenetic tree of live mollusk species found in Belshi lakes, compared to sequences of similar species from the region and larger.

DNA analyses carried out according to the methods of molecular genetics for the species *Gyraulus chinensis* and *Dreissena carinata*, and the revision of their taxonomy on a global scale, make the taxonomic review of the species from the genera *Gyraulus* and *Dreissena* previously reported in Albania from different habitats necessary.

REFERENCES:

Araujo R. 2007. Fauna Europaea: Mollusca, Bivalvia. version 1.3. http://www.faunaeur.org.

Beqiraj S, Dhora D, Dervishi L, Kromidha G. 2006. Joint Strategic Action Plan for Shkodra/Skadar Lake. GEF & MEFWA: 120 p.

Beqiraj S. 2013. Assessment of macrozoobenthos in Buna River, Albania. Project report. 24 p.

Beqiraj S, Dervishi L, Kromidha G. 2012. Strategic Action Plan for Shkodra Lake Protected Area. GEF & MoE: 112 p.

BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**:95-98.

Dhora DH, Welter-Schultes FW. 1999. Mollusc cenoses from different environments in Albania. - Schriften zur Malakozoologie. Cismar/Ostholstein:**13:** 61-66.

Dhora Dh. 2010. Regjistër i specieve të faunës së Shqipërisë. "Camaj – Pipa". Shkodër: 207 p.

Dhora, Dh, Beqiraj S. 2001. Biodiversity of Buna River. APAWA, Albania/REC, Hungary. 28 pp.

Dhora Dh. 2004. Mbi Molusqet e Shqipërisë. "Camaj – Pipa". Shkodër: 194 p.

Fehér Z, Erőss Z. 2009. Contribution to the Mollusca fauna of Albania. Results of the field trips of the Hungarian Natural History Museum between 1992 and 2007. Schriften zur Malakozoologie. Cismar/Ostholstein: **25**: 3-21.

Fehér Z, Erőss Z. 2009. Checklist of the Albanian molluscs fauna. Schriften zur Malakozoologie. Cismar/Ostholstein: 25: 22-38.

Griffiths H. 1991. Application of stable isotope technology in physiological ecology. *Functional Ecology*, **(5):** 254 – 269.

Hájek M, Horsák M, Hájková P, Dítě D. 2006. Habitat diversity of central European fens in relation to environmental gradients and an effort to standardise fen terminology in ecological studies. *Perspectives in Plant Ecology Evolution and Systematics*, **8(2):** 97-114.

Horsák M, Rádková V, Syrovátka V, Bojková, J, Křoupalová V, Schenková J, Zajacová J. 2015. Drivers of aquatic macroinvertebrate

richness in spring fens in relation to habitat specialization and dispersal mode. *Journal of Biogeography*, **(42) 11:** 2112 – 2121.

Peçulaj A. 2014. Studim i makrozoobentosit të Lumit Buna. Punim Diplome. Fakulteti i Shkencave të Natyrës, Universiteti i Tiranës: 64 p.

Wetzel R. 2001. Limnology - Lake and river ecosystem. Academic Press: 9 – 125; 665 – 725.

https://www.molluscabase.org. MolluscaBase.

https://fauna-eu.org. Fauna Europaea.

http://www.marinespecies.org. World Register of Marine Species (WoRMS).

http://www.fishbase.org. FishBase.

https://www.ncbi.nlm.nih.gov. GenBank.

https://www.megasoftware.net. Molecular Evolutionary Genetics Analysis (MEGA)

http://www.barcodinglife.org.The Barcode of Life Database (BOLD).

PRESENCE OF SOMATIC CELLS AND HETEROTROPHIC BACTERIA IN MILK, INDICATOR FOR ASSESSING ANIMAL HEALTH AND MILK QUALITY

Klementina PUTO
Department of Biotechnology, Faculty of Natural Sciences, University of Tirana, Albania
Ermelinda NEXHIPI
Institute of Food and Veterinary Safety, Tirana, Albania
Linda LUARASI and Anida BASHURI
Department of Biotechnology, Faculty of Natural Sciences, University of Tirana, Albania

---

ABSTRACT

The safety/ quality of milk and dairy products, which is delineated by a series of challenges, has been one of the major societal concerns. It unavoidably requires continuous investments**.** The increase of investments in small farms, livestock complexes and new diary processing plants in 2019 led to an increase by 0.49% of milk production when compared to 2018, unavoidably affecting nutritional values and its quality. Low bacterial counts and low somatic cell counts are the key indicators of milk quality, and as their numbers increase, there is a higher risk for contamination of milk and cheese with pathogens. Their increased number indicates the presence of pathogenic microorganisms and negatively affects the quality and technological performance of milk. The paper identifies the amount of bacterial microflora and the number of somatic cells (SCC) present in milk for the period January - June 2020. A total of 50 milk samples collected from the milk provided by the farms in the Tirana region were analyzed for the presence of SCC and heterotrophic microorganisms in milk, and the results showed high levels of SCC (38%) and heterotrophic microorganisms (45%). The European Community Directives 92/46 and 92/47 (European Economic Community, 1992) define regulations that established hygienic standards for raw milk collection and transport that focus on issues, such as temperature, sanitation, and microbiological standards, enabling the production of raw milk of the highest possible quality. Cow milk shall conform to the following standards: a standard plate count at 30°C of <100,000 cfu/mL and somatic cell counts of ≤400,000 $mL^{-1}$of milk. For the heterotrophic microorganisms the permissible levels are < 10^5. Dairy producers are responsible for the safety of their products and

must guarantee food safety through the implementation of Hazard Analysis Critical Control Point (HACCP).
Keywords: heterotrophic bacteria, milk, number of somatic cells (SCC), public health

1. INTRODUCTION

Milk is a nutrient-rich liquid food produced mammals. It is the primary source of nutrition for young mammals (including breastfed human infants) before they are able to digest solid food. Early-lactation milk, which is called colostrum, contains antibodies that strengthen the immune system, and thus reduces the risk of many diseases. Milk contains many other nutrients, including protein and lactose. It must provide the necessary nutritional values and meet the requirements on food quality. From milking to its final use, milk is subjected to various physical, enzymatic and thermal treatments, which ensure its preservation.

Producers are responsible for the safety of their products and must guarantee the food safety of dairy products, for which the dairy industries are subject to the Hazard Analysis Critical Control Point (HACCP). This allows for the quality of the final products through a control of the entire product management chain. The quality and safety of raw milk is essential for the quality and safety of milk and dairy products. The quality and safety of milk is related to the contamination of milk with microorganisms, chemical residues and other contaminants.

Livestock is a very important agro-food sector in Albania. About half of the farmers are engaged in livestock, including the dairy sector. Dairy products occupy an important part in the consumer basket of Albanian households. Milk production is dominated mainly by cow's milk (more than 4/5).

Albania faces serious problems in the national food safety control system in terms of legislation, infrastructure, institutional capacity, control and law enforcement, which affect the real and perceived safety risks for consumers.

Imami *et al*. (2011) and Verçuni *et al*. (2016) stated that food safety a major concern for Albanian consumers.

Food safety and consumers' health is at the heart of Albanian government's policies (https://sane27.com/wp-content/uploads/Law-no.9863-of-28.01.2008.pdf.). The law sets out the government's requirements to be met by food chain operators to ensure food safety and quality for humans and animals, to some extent in line with EU provisions.

The International Dairy Federation Report (1980) clearly states the importance of monitoring the key indicators that ensure a high-quality product.

Dairy products are generally destined for the domestic market, so the increase in production is mainly driven by the increase in domestic demand.

But most farmers do not have enough knowledge about the microbiological content and quality of raw milk. Although most farmers state that they have a register of farm animals, they do not know which institution is responsible for their control (Zhllima *et al*. 2015).

Dairy farms with a small number of cows usually milk the cows by hand. Only farms whose main activity is milk production (normally with more than six cows) have started to buy simple milking machines. When a farm has more than 50 cows, it usually invests in a milking parlor, milk storage tanks and cooling systems (Verçuni *et al*. 2016).

The data here reported are based on the information obtained from the interviews. Hygienic conditions and the quality of raw milk remain of great concern. Milk is mostly provided by small farms; more than 59.2% of which have one cow and considerable problems with dairy standards. From all the farms that have up to 20 cows, with a total of 94,481 cows, individual farms produced 28.8% of the amount of milk (INSTAT 2017 - data published by the agricultural census).

The organization system (fragmentation) is the source of bad sanitary conditions which unavoidably affects the quality and quantity of milk production. Milking doesn't occur in milking barns, and containers used for milking, storing and transporting milk are substandard. In addition to the organization system, lack of knowledge, information and skills on dairy hygiene, breast health, milking techniques, storage and cooling, affects the quality standards.

Given the aforementioned situation, analyzing somatic cell count (SCC) as a basic indicator of cow health and milk quality is of great concern. Leukocytes are somatic mammary cells and depend on the intensity of cellular immune defense. Some of the cells derive from the mammary pathways. Mastitis, one of the most common diseases of dairy cows, might cause significant economic losses to dairy farmers. The number of somatic cells in milk has been accepted as the world standard for the diagnosis of mastitis (Dairy Federation Report 148A, 1995).

Regulation no 853/2004 of the European Parliament and of the Council lays down specific hygiene rules for food of animal origin. The increased number of SCC shows the presence of pathogenic microorganisms which unavoidably affect both quality and technology of milk production.

SCC is defined as the number of cells per ml of milk. In general terms:

- A cow of 100,000 SCC or less indicates an uninfected cow, where there is no significant production loss due to subclinical mastitis.

- A SCC threshold of 200,000 would determine if a cow is infected with mastitis. With a SCC greater than 200,000 they are more likely to become infected in at least 25%.
- Cows infected with significant pathogens have an SCC of 300,000 or higher.
- Milk with a value of 400,000 or more is estimated to be unfit for human consumption.

SCCs vary, however, due to many factors, including seasonal and management effects. Dairy farmers are financially rewarded if they have low SCCs and are penalized for high ones.

Table 1. Impact of somatic cells on the reduction of milk production, according to the standards of National Mastitis Council, US (NMC Standard)

Number of somatic cells	Coefficient	Losses in %
200,000	1	0
200,000-500,000	0.94	6
500,000-1,000,000	0.82	18
1,000,000-1,500,000	0.71	29

Good-quality raw milk is required to make good-quality dairy products. Once raw milk is defective, it cannot be improved during processing, and defects often become more pronounced. Therefore, it is important that raw milk be produced and handled from farm to plant under conditions that do not reduce its quality or, consequently, the quality of the product. Many factors can influence the quality of raw milk.

The present study overviews freshness, quality, safety and naturalness of milk by analyzing the quality and quantity of bacterial microflora based on public health safety standards. In addition, it reports about the effect of somatic cell count (SCC) which affects milk yield and composition (National Mastitis Council).

2. MATERIALS AND METHODS

Basically, somatic cell count (SCC) indicates milk quality. Lower the SCC, higher the milk quality and vice versa. The present study reports about the presence of somatic cells and heterotrophic bacteria in milk as a means to address animal health and milk quality and the impact on human health as based on the horizontal method ISO 4833: 2003 at 30^{o}C. The investigation was carried out at the Institute of Food Safety and Veterinary (IFSV).

A total of 50 milk samples were collected from milk obtained from the farms in the Tirana region and analyzed for mesophilic loads and the total number of somatic cells to investigate the safety of the milk.

The horizontal method was applied as defined by the ISO 4833-1: 2013 for the counting of microorganisms that are able to grow and form colonies in a solid environment once aerobic incubation at 30°C was applied to determine the total microbial number. The method is also applicable to the products that require a reliable count when a low detection limit is specified (below 10^2 / ml for liquid samples).

Plate Count Agar (PCA) was employed to determine the total number of live, aerobic bacteria in a sample. The number of bacteria is expressed as colony forming units per ml (CFU / ml) in liquid samples. Here, the pouring plate technique would be recommended. The samples were diluted and the appropriate dilutions added to the Petri dishes. Sterile melted agar is added to these dishes. Once the sterile melted agar is added to the dishes, the dishes were incubated at 20 or 30°C for three days. Subsequently, the number of colonies is calculated on the plate with 25-250 colonies, which is considered to give the most accurate result.

The following formula was employed for the calculation:

$$N = \frac{\Sigma C}{V \times [n_1 + (n_1 + 0.1n_2)] \times d}$$

where:

- ∑C is the sum of the colonies numbered in all dishes obtained from two successive dilutions at least where one of them contains at least 15 colonies
- V is the volume of inoculate, in milliliters, cast on each plate
- n_1 is the number of dishes taken in the first dilution
- n_2 is the number of dishes in the second dilution
- d is the dilution factor corresponding to the first dilution obtained [d = 1 in cases (liquid products) when the test sample is inoculated directly].

Microbiological samples of milk were classified based on the quality criteria applied. The criteria of <100000 col/ml for quality premium rate and > 100000 col/ml for milk unsuitable for processing at the industrial level are set in accordance with the requirements of Regulation no. 853/2004.

Microscopic technique involving the ISO 13366-1: 2008 was used for the somatic cell count. Laboratory milk samples are stored at 4°C and analyzed within 6 hours after the arrival at the laboratory.

Initially, the sample is heated in the bath for a short time at 40°C, left at room temperature and then diluted with PSB (Phosphate buffer solution)

which were recorded. 0.1 ml of the prepared samples was taken and all the spaces of the marked surface of the blade were carefully filled. The inoculated blades are completely dried and then immersed in the Newman Stain Solution dye where they are left for 15 minutes. Once rinsed, the count was performed under a microscope (Fig. 1). The following formula was used for the interpretation of the results:

$$C = fwx\left[\frac{N_t}{N_b} \times \frac{1}{d}\right]$$

where:

- C - number of somatic cells
- f - displacement factor
- wx - track width in mm
- Nt- total number of numbered cell nuclei
- Nb- total number of transits
- d- dilution factor

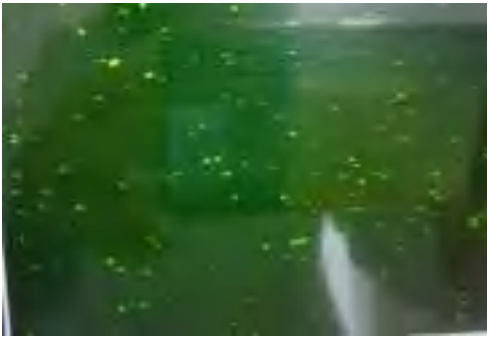


Fig. 1: View of somatic cells

3. RESULTS AND DISCUSSIONS

Monitoring the total number of bacteria at 30°C is necessary for the hygienic and sanitary quality of milk. The current EU regulation requires MAB calculations to be below 100,000 CFU/mL (Regulation EC 853/2004), while EU standards by food business operators normally set a stricter limit for the production of high-quality milk (30,000 CFU/mL).

The number of somatic cells in milk has been accepted as the world standard for the diagnosis of mastitis (International IDF Standard 148A, 1995). SCC is a key indicator of milk quality.

We note that the 50 samples analyzed for the presence of heterotrophic bacteria and the number of somatic cells were from fresh milk of individual cows (Figure 2 and 3).

The mesophilic bacteria were analyzed as based on the Regulation 853/2004 for product safety as it defines the quality criteria of bacterial colonies per ml of milk, and information about the load of mesophilic bacteria for all the samples analyzed are in the Table 2 reported. Exceeding values could be clearly noted.

Table 2. Number of mesophilic bacteria load colonies in milk and number of somatic cells in milk

Number of samples	Number of mesophilic bacteria, rate 100 000	Somatic cells, rate 400000
1	210 000	2 209 000
2	19 000	1 702 000
3	25 000	449 000
4	800 000	222 000
5	60 000	56 000
6	150 000	13 000
7	65 000	134 000
8	600 000	74 000
9	17 000	254 000
10	78 000	17 000
11	120 000	300 000
12	100 000	43 000
13	18 000	43 000
14	3 500	220 000
15	52 000	434 000
16	10 000	66 000
17	270 000	76 000
18	16 000	90 000
19	350 000	730 000
20	4 600 000	357 000
21	35 000	15 000
22	250 000	1 712 000

23	2 200 000	2 100 000
24	27 000	2 200
25	5 500	436 000
26	4 800	430 000
27	4 000	310 000
28	26 000	684 000
29	4 700	332 000
30	17 000	315 000
31	98 000	414 000
32	74 000	358 000
33	123 000	158 000
34	5 300	198 000
35	7 200	279 000
36	10 000	498 000
37	45 000	520 000
38	38 000	230 000
39	65 000	100 000
40	100 000	174 000
41	55 000	50 000
42	33 000	1 000 000
43	99 000	453 000
44	230 000	330 000
45	24 000	231 000
46	32 000	110 000
47	7 500	98 000
48	24 000	76 000
49	6 500	65 100
50	8 400	

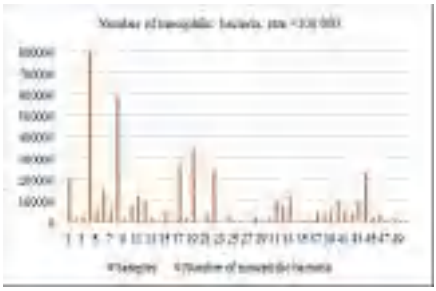


Fig. 2: Number of mesophilic bacteria

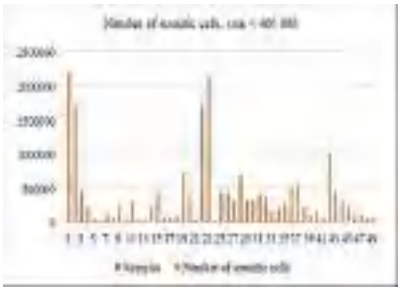


Fig. 3: Number of somatic cells

Regulation no. 853/2004 defines <100000 col/ml as a quality premium rate, and >100000 col/ml for industrially unsuitable milk (Table 3).

Table 3. Bacteriological standards of fresh milk

CFU/ml	ASSESSMENT
Not more than 100,000 col/ml	Acceptable
Over 100 000 col/ml	Not acceptable

About 72% of the analyzed samples meet the microbiological criteria and are safe for consumption. The Figure 4 depicts the test results for all the milk samples.

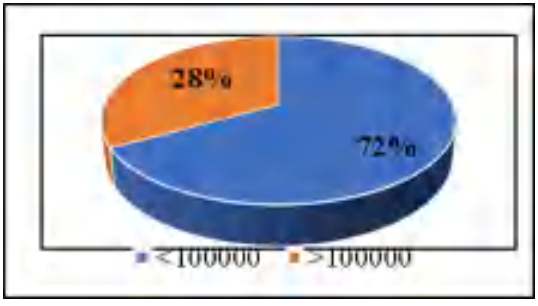


Fig. 4: % e mesophilic bacteria according the norms (rate < 100 000 col/ml)

Results on somatic cell count

A somatic cell count (SCC) is a cell count of somatic cells in a fluid specimen, usually milk. In dairying, the SCC is an indicator of the quality of milk—specifically, its low likeliness to contain harmful bacteria, and thus its high food safety.

An increase in SCC causes a decrease in milk yield and affects milk composition, which leads to reduced production of dairy products.

SCCs vary due to many factors, including seasonal effects and management. Milk with an SCC of more than 400,000 is deemed unfit for human consumption by the European Union. A particularly low SCC is sometimes considered a sign of a weak immune response, but in general terms this does not necessarily have to be true; it may happen that there is simply a low level of current infection. The immune response is best measured by how quickly the immune system responds to the challenges of the disease; not how many white blood cells are present before infection occurs.

High SCC is not only associated with udder health and milk losses, but also negatively affects the longevity (Sewalem *et al*., 2006) and fertility (Rekik *et al*.,2008) of dairy cows.

A low SCC means increased income from more milk, increased quality premiums, and decreased mastitis costs.

Table 4. Number of colonies with somatic cells according to certain norms

	Number of samples	**< 400000 col/ml**	**> 400000 col/ml**
Milk	50	35	15

As it could be noted from the Table 4, 35 samples out of 50 resulted in a number of somatic cells ranging from 13000 colonies/ml to 400000 colonies/ml—70% of the analyzed samples meeting the microbiological criteria. The reminder did not meet the criteria. The values are quite variable, showing in some cases considerable exceedance of the permissible values. The milk provided by individual farmers is of poor quality and dangerous for human consumption. Milk becomes a source of infections if overloaded with bacterial microflora and the number of somatic cells.

4. CONCLUSION

The following conclusions could be drawn: i) milk produced by the individual farmers is of poor quality; not only dangerous for human consumption, but also a source of milk-borne infections, ii) 72% of the cases resulted with heterotrophic bacteria load less than 100000 col / ml, iii) 70% of the cases resulted with somatic cell counts less than 400000 col/ml, iv) 28% of the cases resulted in a load of heterotrophic bacteria greater than 100000 col/ml and, v) 30% of the cases resulted with somatic cells count greater than 400000 col/ml.

5. RECOMMENDATIONS

The present paper highlights the importance and demand of estimating somatic cell count (SCC) and its effects on milk quality and human health. Dairy producers are responsible for the safety of their products and must guarantee food safety through the Hazard Analysis Critical Control Point (HACCP).

REFERENCES:

Committee on Infectious Diseases and Committee on Nutrition of the American Academy of Pediatrics. 2014. Consumption of raw or unpasteurized milk and milk products by pregnant women and children. *Pediatrics* **133**:175-179.

Imami D, Chan-Halbrendt C, Zhang Q, Zhllima E. 2011. Conjoint analysis of consumer preferences for lamb meat in central and southwest urban Albania. *International Food and Agribusiness Management Review*, **14(3).**

INSTAT. 2017. Baza e të dhënave www.instat.gov.al

International Dairy Federation Report (IDFR): 1980. Factors influencing the bacteriological quality of raw milk, International Dairy Federation, Document 120, Brussels.

International IDF Standard 148A: 1995. Milk enumeration of somatic cells. International Dairy Federation, Brussels, Belgium. International IDF Standard 141B: 1996. (1996). Whole milk: Determination of milk fat, protein and lactose content—guide for the operation of mid-infrared instruments.

ISO 13366-1:2008(en) Milk — Enumeration of somatic cells

ISO 4833-1:2013. Microbiology of the food chain — Horizontal method for the enumeration of microorganisms — Part 1: Colony count at 30°C by the pour plate technique.

Law, *On food*, no. 98638, Albania, dated 28.01.2008.

Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin.

Rekik B, Ajili N, Belhani H, Ben Gara A, Rouissi H. 2008. Effect of somatic cell count on milk and protein yields and female fertility in Tunisian Holstein dairy cows. *Livestock Science*, **116**:309–17.

Sewalem A, Miglior F, Kistemaker GJ, Van Doormaal BJ. 2006. Analysis of the relationship between somatic cell score and functional longevity in Canadian dairy cattle. *Journal of Dairy Science* (JDS); **89**:3609–14.

Verçuni A, Zhllima E, Imami, D, Bijo B, Hamiti X, Bicoku, Y. 2016. Analysis of consumer awareness and perceptions about food safety in Tirana, Albania. *Albanian Journal of Agricultural Sciences*, **15(1): 19**.

Zhllima E, Imami D, Canavari M. 2015. Consumer perceptions of food safety risk: Evidence from a segmentation study in Albania. *Journal of Integrative Agriculture*, **14(6):** 1142-1152.

THE USE DIFFERENT CHEMICALS AND UV RADIATION FOR THE REDUCTION OF MICROORGANISM IN THE EGGSHELL AND THEIR COMPARISON

Nora RRAHIMI HASANI and Gentian HASANI
Department of Microbiology, Faculty of Natural Sciences, University of Prishtina, Kosovo

ABSTRACT

The present paper evaluates the effect of different chemicals on microorganisms in the eggshells and eggs' quality. 120 chicken eggs were randomly collected from a poultry farm in Kosovo and divided for treatment purposes into the following gropus: Group 1 undergoing the 13.33 g/m^3 formaldehyde fumigation, Group 2 undergoing the ozone fumigation 5-10 ppm, Group 3 undergoing the 6.36 mW/cm^2 light UV-C radiation, Group 4 undergoing the spraying with 1.56% hydrogen peroxide, Group 5 undergoing the spraying with water (wet control) and Group 6 not undergoing any disinfection procedure (dry control). Samples were divided into 6 groups of 10 eggs per each procedure; before and after the disinfection process to evaluate the presence of *Salmonella, Enterobacteriaceae* and total bacteria in the eggshell. Results reported that only the eggs undergoing formaldehyde and UV treatments showed a significant reduction in the total number of aerobic mesophilic bacteria in the eggshell, when compared to those of the dry control group. The treatments did not affect neither the thickness of the eggshell nor its resistance. The UV treatment effectively reduces microbial load in the eggshells without affecting their quality.
***Keywords*:** ozone, hydrogen peroxide, bacterial count, UV light, chicken eggs

1. INTRODUCTION

Approximately 1.140 billion eggs are annually produced and, 700 billion/year are consumed worldwide (Mantouanelli *et al*., 2001; Rehault-Godbert 2019). They are less expensive and a source of dietary protein (Mantouanelli *et al*., 2001). In addition, they are considered functional food due to their high nutritional content (Mantouanelli *et al*., 2001; Bradley and King, 2016). However, eggs might be a source of foodborne illness caused by *Salmonella* spp. In general, there are two possible routes of transmission: i) horizontal, from penetration through the eggshell from the colonized intestine or from feces contaminated during or after issues eggs, and ii) vertical, from

direct contamination of egg yolk, albumin, eggshell membranes or eggshell before ovulation (Mantouanelli *et al*., 2001). *Salmonella* is the second leading cause of foodborne illness in poultry production, after *Campylobacter*, (Efsa 2009) with a mortality rate of less than 1%. About 2 million diseases/year occur due to *Salmonella*-contaminated eggs in the United States, while in Europe, the range is 7,400 cases/year (Hald 2013).

The calcareous skin surrounding the egg is porous and permeable to bacteria (Solomon, 2010). The cuticle is a film protein covering the egg shell that provides a natural barrier to help prevent internal bacterial contamination, however, defects in the shell or thinning of the cuticle can lead to invasion of the egg shell by bacteria on the surface. *Salmonella* can easily penetrate the egg cuticle and contaminate the internal contents.

In eggshells, the total number of aerobic mesophilic bacteria can reach 3.75 to 7.07 $\log_{10}$ colony forming units (CFU) per egg. Therefore, reducing the microbial load of the eggshell through disinfection procedures would improve the quality of the egg to be incubated and reduce the incidence of bacterial infections in newborn embryos and chickens.

Currently, *Salmonella* is the most prolific pathogenic bacterium associated with egg contamination (Cox 2007). Vertical transmission of *Salmonella* from chickens to eggs can occur from contamination of egg components in the reproductive tract or from contact with fecal material (Cox 2007).

Emphasis is placed on HACCP (Hazard analysis and critical control points)-based programs for identifying and preventing potential microbiological hazards that may arise from raw material, processing stages, product, and food plants (Mantouanelli *et al*., 2001; Giaccone *et al*., 2002).

Currently, formaldehyde fumigation is one of the most widely used disinfectants for hatching eggs. However, this substance has been shown to have side effects on the embryo, as reported in a systematic review about formaldehyde fumigation (Cadirci 2009). Due to its ability to disperse into eggs through their pores, this gas can alkylate cellular components, such as the purine and pyrimidine bases present in embryonic DNA and RNA, causing embryonic death, mainly in the early stages. of embryonic development (Cadirci 2009). In addition, the use of formaldehyde has been known to adversely affect the health of farm workers and attractors, encouraging researchers to seek alternative methods of disinfection (Berrang *et al*., 2016).

In this context, research is needed to provide effective methods for egg hygiene, taking into account the high probability of shell contamination after laying, as well as poultry health, poultry waste quality and proper egg collection procedures (Wells *et al*., 2010). Use of alternative shell disinfection with procedures, such as spraying with peroxide-based disinfectants, (Cox *et al*., 2007; Wells *et al*., 2011) ozone gas fumigation, (Braun *et al*., 2011) and

the use of UV-C light radiation, (Wells *et al*., 2011) has been effective in reducing the number of bacteria in egg shells. Moreover, since these methods do not emit toxic waste after their use, (Braun *et al*., 2011) they can also be characterized as environmentally friendly.

In 1982, ozone was generally recognized as safe (GRAS-Government Receipt Accounting System) by the Food and Drug Administration (FDA), and in 2001 the direct use of ozone was recognized in food products including fish, red meat, and chicken and used it in the food industry (Mielcke and Ried 2004). Ozone, which is a strong oxidizer, is effective against Gram-positive and Gram-negative bacteria, yeasts, fungi and viruses. Since ozone leaves no material in food products, it does not make a difference in the taste and color of the product (Okayama *et al*., 2002).

Ultraviolet (UV) light is the electromagnetic radiation found in the electromagnetic spectrum between X-rays and visible light and includes wavelengths between 200 and 400 nm. UV light is subcategorized into UV-A (400-315 nm), UV-B (315-280 nm) and UV-C (280-100 nm) based on the respective wavelength range (Iso 2007).

The main mechanism of microbial inactivation by UV radiation is the dimerization of DNA bases. The formation of these dimers within bacterial DNA prevents DNA duplication, eventually leading to a reduction in the bacterial population. These bases have a maximum UV absorption velocity at a wavelength of 260 nm, which corresponds to the bactericidal peak effectiveness of UV radiation which varies between 260 and 270 nm. Pyrimidine bases are 10 times more reactive than purine bases at a wavelength of 254 nm, the predominant wavelength of which is irradiated by UV-killing micro-lamps.

Thymine pyrimidine requires the least amount of energy to form a dimer, consequently, the complex thymine dimer is the predominant photoproduct of UV254 radiation. UV-C includes the wavelength of 254 nm within its range 200-290 nm and is therefore often referred to as UV germicide.

UV radiation does not expose eggs to toxic chemicals or by-products and is safe for the environment (Coufal *et al*., 2003). Except it is a low-heat process, UV cannot reach the developing embryo or cause DNA damage as UV does not penetrate the eggshell (De Reu *et al*., 2006).

Hydrogen peroxide (H_2O_2) is a very strong oxidizer that forms free radicals exerting a destructive effect on cell membranes. As a result, it has found wide application as a biocidal (Linley *et al*., 2012). When O_3 is exposed to UV the net reaction results in the formation of hydrogen peroxide (H_2O_2) and any hydroxyl radicals formed when O_3 reacts with UV are unable to escape this solvent cage. Although the advanced oxidation process (AOP) O_3/UV is an effective disinfectant, the bactericidal properties are the result of

the production of hydrogen peroxide instead of the hydroxyl radicals formed by the initial O_3 molecule.

The net photolysis of H_2O_2 yields two hydroxyl radicals, per quantum of absorbed radiation, which can continue to form peroxyl radicals leading to secondary oxidation reactions (Wells *et al*., 2011). The H_2O_2/UV photolytic reaction is one of the most widely used AOPs, and has been demonstrated to effectively inactivate vegetative bacteria, bacterial spores and viruses (Ikai *et al*., 2010). After treatment of eggs, H_2O_2 evaporates easily without leaving chemical waste and poses minimal safety issues for workers or embryo development (Keita *et al*., 2016). The bactericidal effects of H_2O_2 increase after UV photolysis (Ikai *et al*., 2010).

Other benefits of this system include the commercial availability of H_2O_2, its endless water solubility, and lower health risk than O_3 for workers. These benefits together with its effectiveness as a cleaner make the H_2O_2/UV AOP system an attractive method of eggs disinfection.

The present investigation aims to compare the results obtained from the use different chemicals and UV radiation for the reduction of microorganism in the eggshell of the eggs collected from a farm in Kosovo, to supply the highest quality and safe products to the customer.

2. MATERIALS AND METHODS

In the present investigation the 120 eggs have been randomly collected from a poultry farm in Kosovo and underwent two procedural phases: i) eggs collection and disinfection in the farm and, ii) microbiological investigation at the Laboratory of microbiology, the Faculty of Medicine, Prishtina, Kosovo. Eggs were collected from the nests using disposable latex gloves to avoid any sort of contamination from the collector's hands. Once collected, the eggs were placed in the litter and packed in previously disinfected plastic boxes. and distributed in disinfection treatments. The 120 eggs randomly selected underwent four different disinfection procedures (ozone fumigation, formaldehyde fumigation, type C ultraviolet irradiation and hydrogen peroxide spraying) and two control procedures (water spraying and no disinfection procedure) with subsequent microbiological investigation of the eggshell. The forthcoming paragraphs provide information about the disinfection procedures and control treatments.

For the egg procedure without disinfection, the eggs were kept in the same room where the other treatments were performed. Thermometer and thermohygrometer were used to measure room temperature and humidity, respectively, which varied from 27.5 to 30.4^{o}C and from 49 to 55%.

The water spraying process consisted of the collected eggs only sprayed with water. Water spraying was carried out to investigate how egg wetting affected the variables studied.

The water temperature went up to 26ºC in all disinfection processes, and was measured in the same way as in other spraying procedures. The spraying time varied from 8 to 11 min.

For ozone fumigation, eggs were disinfected with ozone gas at a concentration of 5-10 ppm for 20 min, as recommended by the ozone supply company. Each collected egg was placed in an exposed plastic box, inside a $3m^3$ fumigation room for disinfection purposes. The relative humidity of the air was adjusted to 70% inside the room, as suggested in (Braun 2011), and was measured through a thermo hygrometer (Boeco Germany). After fumigation, the product was consumed and the eggs were sent for bacterial count.

For formaldehyde fumigation, a concentration of 13.33 g m^{-3} formaldehyde was used to disinfect eggs as recommended in (Cadirci 2009). The egg box used for this procedure was plastic and was placed in the fumigation chamber which was the same room used to disinfect the eggs with ozone. Therefore, 1 hour (fumigation time plus the interval for the total waste disposal of the previously applied product). The relative humidity of the air inside the room was also adjusted to 70%, as recommended in (Cadirci 2009), and measured as during ozone fumigation process.

For the C ultraviolet radiation, disposable latex gloves were used and the eggs placed one by one in aluminum trays with a capacity of 10 eggs and then placed inside an enclosed room where the UV-C lamp -30W at a distance of 80 cm and 254 nm- provided an average light intensity of 6.36 mW cm^{-2}, as adapted by (Gottselig *et al.*, 2016). Egg compartments, designed to prevent eggs from touching each other and, consequently, to allow greater exposure to UV-C light, were placed in the center of the room to allow radiation from the bulb located over the entire length of the tray. In order to obtain significant reductions in the number of eggshell microbes, the disinfection time was 60s as in (Chavez 2002).

The temperature inside the room which ranged from 28.6 to 31.3°C throughout the disinfection period was measured by a probe equipped with a digital thermometer.

For hydrogen peroxide spraying, a solution of 1.56% hydrogen peroxide with 650 ppm active product was used to disinfect the eggs, as recommended by the manufacturer. Once collected and separated, 10 ml of disinfectant solution was sprayed on 10 eggs at the same time using a hand sprayer. A thermometer was employed to measure the temperature of the solution which ranged from 26 to 29ºC. For a complete spraying, the boxes of the eggs were placed on a horizontal surface and 5 ml of solution was dispersed on one side

of the eggs, and 5 ml were dispersed on the other side. An average of 1 mL of hydrogen peroxide was sprayed over each egg. Disinfection procedure lasted 9 to 12 min.

After each collection, shortly before and 1 hour after disinfection, 10 eggs undergoing each treatment were randomly selected to count the microbes in the eggshell. The eggs, collected with disposable handles, were placed in groups in autoclaved bags, which were properly identified according to each treatment and then refrigerated at 4ºC. The samples were transported to the laboratory for microbiological investigation purposes carried out 24 hours after cooling. Each bag was opened, and the eggs were transferred to another autoclaved bag, to which 250 ml of buffer phosphate (PBS) solution was added. The eggs were massaged for 5 min to remove bacterial cells from their eggshell surfaces. Then, a 1.0 ml sample of PBS was taken from each bag, and plaque was placed on the agar, in order to obtain the count of *Salmonella*, *Enterobacteriaceae* and total other bacteria.

The plates were incubated at 37°C for 24 to 48 hours and, subsequently, bacterial colonies were counted and recorded. The microbial count was expressed as $\log_{10}$ CFU 1.0 mL^{-1} batch of eggs.

3. RESULTS AND DISCUSSION

The present paper evaluates the effectiveness of the chemicals used to disinfect the chicken eggs. *Salmonella* contamination is considered an important hygienic issue, especially on small-scale farms that are not controlled by an authorized agency or when a Risk Analysis Critical Control Plan (HACCP 2001) is unavailable.

First, the bacterial count was carried out on the undisinfected eggs, and the results are in the Figure 1 depicted. Total number of aerobic mesophilic bacteria resulted to be of 3.8 and 4.56 $\log_{10}$ CFU/eggs, of which 1.37$\log_{10}$ CFU/eggs were *Enterobacteriaceae*. *Salmonella* resulted to be of 2.55 log10 CFU/eggs. These eggs were then treated with chemicals to investigate impact on *Salmonella enterica*, *Enterobacteriaceae* and total aerobic mesophilic bacteria from the egg shell.

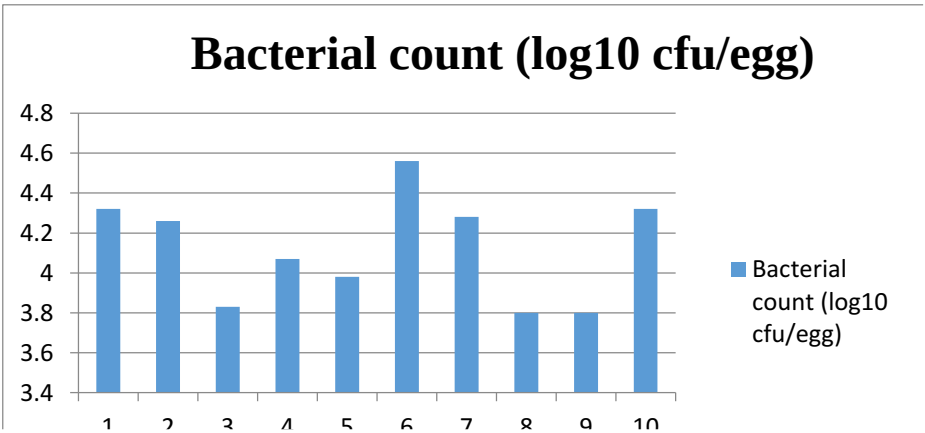


Fig. 1: Count of total bacteria in samples taken at a farm in Kosovo.

Among the various disinfection procedures, only eggs subjected to formaldehyde and UV treatments showed a significant reduction in the total mesophilic aerobic bacteria count (where here we are focused on eliminating *Enterobacteriaceae*) and *Salmonella* to relative to the control group (Figure 1). In this case, formaldehyde-treated and UV-treated eggs presented the lowest count of total aerobic mesophilic bacteria (*Enterobacteriacea*) and *Salmonela*.

Eggshell contamination with total mesophilic aerophilic bacteria, prior to each disinfection procedure, ranged from 3.8 and 4.56 $\log_{10}$ CFU/egg, where from them, 1.37$\log_{10}$ CFU/egg were *Enterobacteriacea*, confirming the results reported in (Coufal 2003) for eggs collected from nests. However, values between 4.0 and 7.0 $\log_{10}$ CFU for sterile eggs were also reported in (Wells et al., 2011; Zeweil *et al*., 2014) proving that values in the initial contamination of the eggshell vary greatly between studies. Despite the microbial challenge that eggs underwent prior to disinfection, formaldehyde and UV treatments were effective in reducing egg shell contamination by total mesophilic aerobic bacteria at 1.56 $\log_{10}$ CFU/egg (1.01 $\log_{10}$ CFU/egg *Enterobacteriacea*), and 1.52 $\log_{10}$ CFU/egg total mesophilic aerobic bacteria (1.02 $\log_{10}$ CFU/egg *Enterobacteriacea*). After treatment with formaldehyde a slight decrease of *Salmonella* was observed at 1.28 $\log_{10}$ CFU/egg, while after UV treatment we had a decrease to 1.26 $\log_{10}$ CFU/egg.

Compared to formaldehyde fumigation, achieving the same pattern of microbial reduction with UV light was impossible, because as Coufal *et al*., (2003) said this might be due to the difficulty of UV light reaching the entire surface of the egg, making it impossible for bacteria to be exposed to radiation and, therefore, causing a greater reduction in the number of eggshell

microbes. Other possible explanations might be the insufficient light intensity and exposure time to meet or exceed the reduced values due to formaldehyde smoking.

Although low, the total number of mesophilic aerobic bacteria for egg groups disinfected with ozone was 2.65 (1.08 $\log_{10}$ CFU/egg *Enterobacteriaceae*) and with hydrogen peroxide 2.55 (1.06 $\log_{10}$ CFU/egg *Enterobacteriaceae*). After ozone treatment, a slight decrease of *Salmonella* was observed at 2.11 $\log_{10}$ CFU/egg, while after treatment with hydrogen peroxide, we had a decrease to 1.98 $\log_{10}$ CFU/egg (Table 1).

It was proved that disinfectants were ineffective in reducing the number of microbes in the egg shells probably due to the long disinfection time which is industrially unfit.

Table 1. Total aerobic count of aerobic mesophilic, *Enterobacteriaceae* and *Salmonella* before and after disinfection of the chicken eggshells of a farm in Kosovo, using ozone, formaldehyde, UV-C light, hydrogen peroxide, water spraying and control dry.

Treatment	*Salmonella enterica* ($\log_{10}$ cfu/egg)		*Enterobacteriaceae* ($\log_{10}$ cfu/egg)		*Total aerobic mesofile bacteria* ($\log_{10}$ cfu/egg)	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
Dry control (without disinfection)	2.55	2.55	1.37	1.37	4.12	4.12
Spraying with water	2.55	2.55	1.37	1.37	4.12	4.12
Ozone	2.55	2.11	1.37	1.08	4.12	2.65
Formaldehyde	2.55	1.28	1.37	1.01	4.12	1.56
UV-C light	2.55	1.26	1.37	1.02	4.12	1.52
Hydrogen peroxide	2.55	1.98	1.37	1.06	4.12	2.55

Even when the manufacturer's recommendations were followed, noticing the microbial content reduction in the egg shells was irrelevant. The presence of *Salmonella* and total aerophilic mesophilic bacteria reduce Cox *et al*., (2007) and Wells *et al*., (2011) reported that the presence of *Salmonella* and total aerophilic mesophilic bacteria reduce diminishes when concentration of these products increase.

In the present investigation, very low counts of *Enterobacteriaceae* were found in all the groups, even before disinfection, as reported in (Musgrove). The rapid penetration of this group of bacteria through the pores of the eggs after laying and the lack of colonization of *Enterobacteriaceae* in the eggshell might be the source of the presence at low levels of these microorganisms.

4. CONCLUSIONS

The following conclusions could be drawn: i) only UV light irradiation and formaldehyde fumigation proved to be effective in reducing the bacterial count of *Salmonella*, *Enterobacteriaceae* and *mesophilic* bacteria in the eggshells. Formaldehyde after being detected long time ago that it has side effects on the embryo and adverse effects in the health of farm workers, alternative methods of disinfection are required, so formaldehyde is not preferable, ii) UV light could be recommended as an effective alternative procedure for large-scale disinfection of eggs, iii) other disinfectants such as ozone and hydrogen peroxide degrade longer times and higher concentration than that used in the work, leading to high reductions in Salmonella, Enterobacteriacea and total aerobic mesophilic bacteria.

REFERENCES:

Berrang ME, Cox NA, Frank JE, Buhr RJ, Bailey JS. 2000. Hatching egg sanitization for prevention or reduction of human enteropathogens: a review. *The Journal of Applied Poultry Research*, **9**: 279-284,. DOI: 10.1093/ japr/9.2.279.

Bradley F, King A. 2016. Pages 8154 in Egg Basics for the Consumer: Packaging, Storage, and Nutritional Information. *UCANR Publications*, Novato, CA.

Braun PG, Fernandez N, Fuhrmann H. 2011. Investigations on the effect of ozone as a disinfectant of egg surfaces. Ozone: science & engineering. *The journal of the International Ozone Association*, **33** p.374-378. DOI: 10.1080/01919512.2011.589359.

Cadirci S. **2009**. Disinfection of hatching eggs by formaldehyde fumigation – a review. *Archiv für Geflügelkunde*, **73**: 116- 123.

Chavez C, Knape KD, Coufal CD, Carey JB. 2002. Reduction of eggshell aerobic plate counts by ultraviolet irradiation. *Poultry Science*, **81**: 1132-1135,. DOI: 10.1093/ps/81.8.1132.

Coufal CD, Chavez C, Knape KD, Carey JB. 2003. Evaluation of a method of ultraviolet light sanitation of broiler hatching eggs. *Poultry Science*, **82**:754-759.

Cox NA, Cason JA, Berrang ME. 2000. Salmonella penetration of egg shells and proliferation in broiler hatching eggs: A review. *Poultry Science*, **79**:1571-1574.

Cox NA, Richardson LJ, Buhr RJ, Musgrove MT, Berrang ME, Bright W. 2007. Bactericidal effect of several chemicals on hatching eggs

inoculated with *Salmonella* serovar Typhimurium. *The Journal of Applied Poultry Research,* v.16, p.623-627,. DOI: 10.3382/japr.2007-00039.

De Reu, K., K. Grijspeerdt, L. Herman, M. Heyndrickx, M. Uyttendaele, J. Debevere, F.F. Putirulan, and N. M. Bolder. 2006. The effect of a commercial UV disinfection system on the bacterial load of shell eggs. *Lett. Appl. Microbiol*. 42:144-148.

Efsa. 2009. Scientific Opinion of the Panel on Biological Hazards on a request from the European Commission on Special measures to reduce the risk for consumers through Salmonella in table eggs - e.g. cooling of table egg. *EFSA J*. 957:1–29.

Giaccone V., Ferri M., and Colavita G. (2002). Quantitative risk assessment, methodological aspects. *Rivist ra Di Coniglicoltura* 39:27-35.

Gottselig, S.M.; Dunn-Horrocks, S.L.; Woodring, K.S.; Coufal, C.D.; Duong, T. Advanced oxidation process sanitization of eggshell surfaces. *Poultry Science*, v.95, p.1356-1362, 2016. DOI: 10.3382/ps/pev450.

Hald, T. 2013. Pathogen update: Salmonella. *In Advances in Microbial Food Safety. Elsevier*, Cambridge, UK.

Hazard analysis and critical point (HACCP). 2001. Procedures for the safe and sanitary processing and importing of juice. Final rule. *Fed. Regist.* 66:6137–6202.

Ikai, H., K. Nakamura, M. Shirato, T. Kanno, A. Iwasawa, K. Sasaki, Y. Niwano, and M. Kohno. 2010. Photolysis of hydrogen peroxide, an effective disinfection system via hydroxyl radical formation. *Antimicrob. Agents Chemother*. 54:5086-5091.

Iso, I. 2007. 21348: 2007 (E). Space environment (natural and artificial)-Process for determining solar irradiances, 2007:5-6.

Keita, A., A. Huneau-salaun, A. Gulliot, P. Galliot, M. Tavares, and J. Puterflam. 2016. A multi-pronged approach to the search for an alternative to formaldehyde as an egg disinfectant without affecting worker health, hatching, or broiler production parameters. *Poult. Sci*. 95:1609-1616.

Linley, E., S. P. Denyer, G. Mcdonnell, C. Simons, and J. Y. Maillard. 2012. Use of hydrogen peroxide as a biocide new consideration of its mechanisms of biocidal action. *J. Antimicrob. Chemother.* 67:1589–1596.

Mielcke J., and Ried A. (2004). Current state of application of ozone and UV for food processing. *In proceedings of the food protection international conference* 20-22 of May 2004; Monte da Caparica, Portugal.

Mantouanelli A., Marino M., Comi G., Vallavanti W., and Dolzani L. (2001). Use of microbial analysis to test HACCP systems in food industries. *Industrie Alimentari* 40: 853-865.

Musgrove, M. T., D. R. Jones, J. K. Northcutt, M. A. Harrison, N. A. Cox, K. D. Ingram, and A. J. Hinton, Jr. 2005. Recovery of Salmonella

from commercial shell eggs by shell rinse and shell crush methodologies. *Poultry Science*. 84:1955-1958.

Okayama T., Iwanaga S., Mitsui Y., Isayama T., Houzouji T., and Muguruma M. (2002). Effect of ozone treatment on metmyoglobin formation and lipid oxidation on beef, 48 th ICOMST Rome; 1.

Rehault-Godbert, S., N. Guyot, and Y. Nys. 2019. The golden egg: nutritional value, bioactivities, and emerging benefits for human health. Nutrients. 11:684.

Solomon, S. E. 2010. The eggshell: Strength, structure and function. Br. *Poult. Sci*. 51:52-59.

Wells, J.B.; Coufal, C.D.; Parker, H.M.; Kiess, A.S.; Purswell, J.L.; Young, K.M.; Mcdaniel, C.D. Hatchability of broiler breeder eggs following eggshell sanitization by repeated treatment with a combination of ultraviolet light and hydrogen peroxide. *International Journal of Poultry Science, v.10*, p.421-425, 2011. DOI: 10.3923/ijps.2011.421.425.

Wells, J.B.; Coufal, C.D.; Parker, H.M.; Mcdaniel, C.D. Disinfection of eggshells using ultraviolet light and hydrogen peroxide independently and in combination. *Poultry Science*, v.89, p 2499-2505, 2010. DOI: 10.3382/ps.2009-00604.

Zeweil, H.S.; Rizk, R.E.; Bekhet, G.M; Ahmed, M.R. Comparing the effectiveness of egg disinfectants against bacteria and mitotic indices of developing chick embryos. *The Journal of Basic & Applied Zoology, v.70*, p.1-15, 2015. DOI: 10.1016/j.jobaz.2014.12.005.

PHYTOEXTRATION OF NICKEL FROM INDUSTRIAL AND MING WASTE: A REVIEW

Marilda OSMANI
Department of Chemistry, Faculty of Natural Sciences, University of Elbasan, Aleksandër Xhuvani, Albania
Aida BANI
Department of Agro-Environment and Ecology, Agricultural University of Tirana, Albania
Belinda HOXHA and Armela MAZRREKU
Department of Chemistry, Faculty of Natural Sciences, University of Elbasan, Aleksandër Xhuvani, Albania

---

ABSTRACT

Mining, metallurgy, food processing, textiles, lumber, and cement were among the leading industries in Albania under the previous regime, when heavy industry was a priority and some factories were capable of exporting. After 1989, the sector declined due to the lack of new technology and financing and the dilapidated condition of the factories. Anthropogenic pollution results from daily human activities such asindustrial, mining, agricultural and domestic make exposure to heavy metal release possible. Exposure to heavy metals release poses a threat to human health. Consequently, the removal of heavy metals from soil is unavoidable. There is a range of physical, chemical and biological techniques used to remove heavy metals and metalloids from soils, but the phytoextraction method appears to bean effective and economical technique. Phytoextraction is the removal of metals from soil using plants and relies on the ability of a plant crop to remove and concentrate heavy metals from either naturally metalliferous or contaminated soils. This paper aims to evaluate the concentration of total major (Ca, Mg, and Fe) and trace elements (Ni, Co, Cr, Zn, Pb) in industrial polluted and mining soils and the Ni phytoextraction or phytomining potential of the hyperaccumulator *A. murale* Waldst. & Kit in soil. Given the phytoextraction benefits and the capacity of *Alyssum murale* to accumulate nickel in soil, it could be concluded that *Alyssum murale* could bea candidate for phytoremediationof contaminated soilsatex-industrial sites and industrial and mining waste sites.

Keywords: phytoextraction, phytomining, hyperaccumulator plants, heavy metal, ex-industrial site, mining sites

1. INTRODUCTION

Accumulation of heavy metals in soil, water and air is one of the major environmental concerns worldwide, which mainly occurs due to anthropogenic activities such as industrialization, urbanization and mining. With the increment of urbanization and industrialization, the cases of soil contaminated by heavy metals have increased rapidly and become a threat to food safety, ecological environment, and sustainable development of the agriculture sector (Yao *et al*., 2012). Conventional remediation strategies involving physical or chemical techniques are not cost-effective and/or eco-friendly, emphasizing the need for novel approaches. Phytoextraction is a developing technology that uses plant species to accumulate elements from contaminated or mineralized soils and transport them to their shoots, which may then be harvested as a crop to remove them from the land (Chaney *et al*., 2007). It is a type of phytoremediation, while the term phytomining has been applied to the latter case in which the economic value of the recovered metal is the primary objective. Effective phytoextraxtion requires both plant genetic ability and the development of optimal agronomic management practices. These species have the genetic potential to remove and metabolize contaminants (Li *et al.*, 2000). Many studiesprovide information about the great variability in phytoextraction potential in different Albanian populations of *A. murale*depending on collection site (Shallari *et al*., 1998; Bani *et al*., 2009; Bani *et al*., 2010; Bani *et al*., 2013; Osmani *et al*., 2015; Osmani *et al*. 2018a).

Brooks *et al*., (1977) first used the term hyperaccumulators to describe plants containing >1000 µg/g (0.1%) nickel in their dried tissues. Hyperaccumulators are species capable of accumulating metals at levels 100 fold greater than those typically measured in shoots of the common non accumulator plants. The largest number of Ni-hyperaccumulators is found in the Brassicaceae family in temperate climates, especially Mediterranean Europe and Turkey (Reeves and Adigüzel, 2008). The genus Alyssum (Brassicaceae) contains the greatest number of reported Ni hyperaccumulators, many of which can achieve 30 g kg^{-1} Ni in dry leaf biomass (Baker and Brooks 1989). The Balkans has the highest diversity in Ni hyperaccumulator plants in Europe and is home to the widespread plant *A. murale*, one of the most studied species worldwide for phytomining (Nkrumah *et al*., 2016). The Albanian flora contains a wide range of Balkan endemic taxa, including some serpentine-obligate (Stevanovi´c *et al*., 2003) among which, the most efficient Ni-accumulator individuals of the species *A. murale* (Bani *et al*., 2009; 2010). *A. murale* occurs widely on these ultramafic Vertisols (Bani *et. al*., 2009) and is a spontaneous weed to other crops. The

use of nickel hyperaccumulator plant species for nickel phytominig in Albanian ultramafic soil is a reality. Bani*et al*., (2015b; 2019) showed that the phytoextraction potential of *A. murale* (syn. *Odontarrhena chalcidica*) under different agronomic practices in Albanian vertisol can be 112 – 145 kg Ni ha^{-1}. *A. murale* Waldst. & Kit is the most efficient Ni hyperaccumulator plants in Albania (Bani *et al*., 2013; 2015a).

Thepresent paper evaluatesthe concentration of total major (Ca, Mg, and Fe) and trace elements (Co, Cr, Ni, Zn, Pb) in soil in ex-industrial polluted areas, in industrial and mining wastes sites and the Ni phytoextraction or phytomining potential of the hyperaccumulator *A. murale*Waldst. & Kit in soils.

2. MATERIALS AND METHODS

In addition to the Përrenjas dumpsite (41°4’0, 99”N, 20°32’20, 72”E), the present investigation was carried out in the metallurgical plant (ex-industrial site) and dumpsite, located in Elbasan (41°4’58, 54”N, 20°1’24, 51”E) as well. The metallurgic plant in Elbasani is the largest one in the country (4 km far from the city), with a surface of 155 hectares and a treatment capacity of 800 thousand tons/year of iron-nickel and produced an estimated 44.8 tons of toxic dust. The main plants, which have been operating (1967- 1990), are nickel-cobalt plant (Ish-Uzina12), metallurgy electrolysis plant and ferro-chrome plant (Shehu, 2009). This is the most polluted region in the country and produces a considerable amount of toxins, as at least 11 hectares of soil is polluted by the ferrochrome wastes. Industrial activity is the main source of heavy metals contamination in the region (Shallari *et al*., 1998; Sallaku *et al*., 1999; Osmani *et al*., 2015; Osmani and Bani 2017; Osmani *et al*., 2018 a,c). In Përrenjas, the biggest iron, nickel and cobalt mine is located 500 m far from the city center. A considerable amount of ferro-nickel mineral was extracted (500 thousand tons/year). 350 thousand tons was processed in the metallurgical complex of Elbasan and 150 thousand tons was exported to Europe. The metallurgical complex of Elbasani is the main source of soil contamination with heavy metals (Osmani *et al*., 2017; Osmani *et al*., 2018b).

Soil analysis

Soil samples were collected from a overlaying deposit, up to 30 cm when possible. Once collected, they were air-dried and laboratory investigated. The microwave digester was involved for the mineralization process as a means to address the determination of trace metals. Conditions for mineralization were as following: 6 ml HCl, 2 ml HNO3, and 3 ml H_2O_2, per 0.5 g soil. Total major (Ca, Mg, and Fe) and trace elements (Co, Cr, Ni, Zn, Pb) in digestion solutions were measured via atomic absorption spectrophotometry (AAS).

A DTPA–TEA extractant (0.005 M diethylenetriaminepentaacetic acid (DTPA) with 0.01 M $CaCl_2$ and 0.1 M triethanolamine (TEA)) at pH 7.3 was employed for the availability of Ni. A ratio of 1 g soil: 10 ml DTPA-TEA solution was shaken for 2 h, and then the suspension was centrifuged for 20 min. Once centrifuged, it was filtered through a 0.2 μm pore size cellulose nitrate filter (Echevarria*et al*., 1998). Ni concentrations in the soil extracts were determined spectrochemically using the atomic absorption spectrophotometer (Nov AA-350).

Plant analysis

All plant samples were washed, dried and ground to a fine powder. Nickel concentrations in plants were determined by plasma emission (ICP) spectrometry after microwave digestion of plant samples. A 0.25gdry matter plant aliquot was digested by adding 8 ml of 69% HNO_3 and 2 ml of H_2O_2. The final solution was filtered and made up to 25 ml with deionized water. The atomic absorption spectrophotometry (AAS) was used to measure the nickel concentrationdigestion solutions.

Nickel phytoextraction

The efficiency of phytoextraction depends on the level of soil contamination and the amount of metals accumulated by plants. Metal phytoextraction is determined by biomass production and heavy metals bio concentration degree found at high levels (Mc Grath and Zhao, 2003). The biomass (dried) was weighed in each plot to calculate the nickel phyto-extraction yield (η) as the product of plant biomass (B) with the concentration of nickel in the cultivated hyperaccumulator plant (C_P) (mg kg^{-1}).

$$\eta = B \times C_P$$

3. RESULTS

Concentrations of chemical elements in the soil

Table 1 reports the results for pH and the concentration of the total major (Ca, Mg, and Fe) and trace elements (Ni, Co, Cr, Zn, Pb). Both dumpsites have a similar pH, ranging from 8.4 to 8.6, which shows a similarity to the mineralogical composition. Results obtained show that the dumpsites soils recorded higher metal concentrations than soil in ex-industrial site, where the pH is lower (7.9).

The Përrenjas dumpsite has higher levels of magnesium concentration (25267 mg kg^{-1}) than the Elbasan dumpsite (9941 mg kg^{-1}), which corresponds to the serpentine soil materials and ultramafic originated residues. The ferromagnetic minerals are very rich in Mg (Shallari *et al*.,

1998; Bani *et al*., 2014). Accordingly Mg:Ca ratio varied 2.7 in Elbasan Dumpsite soil and 5.8 in Përrenjas dumpsite soil, a range that is reported in ultramafic materials (Proctor, 1971; Shallari *et al*., 1998; Bani *et al*., 2014). The plant species growing on this ex mining area must be physiologically adapted to cope with the high Mg/Ca ratio and Ca deficiency.

Table 1. The pH and the concentration of elements in study soils (mg kg^{-1})

Soil type	pH	Ca	Mg	Fe	Ni	Co	Cr	Pb	Zn
Ex-industrial	7.9	13957	10916	23242	700	112	525	103	142
Përrenjas Dumsite	8.4	4286	25267	36715	6859	286	5458	25.8	117
Elbasan Dumpsite	8.6	3710	9941	34853	1842	245	7185	42.2	135

Iron, nickel and cobalt concentrations are higher in Përrenjas dumpsite (36715, 6859 and 286 mg kg^{-1}) than in Elbasan dumpsite (34853,1842 and 245 mg kg^{-1}) and soil in ex-industrial site (23242,700 and 112 mg kg^{-1}). As a result of the industrial activity, iron-nickel plant and other plants around in Elbasan and the iron, nickel and cobalt mining in Përrenjas, this metal concentrations are higher in all type of soils. Since cobalt naturally occurs in nickel bearing laterites and nickel-copper sulphide deposits, it is most often extracted as a by-product of nickel and copper. Kapusta (2007) said that based on the Cobalt Development Institute about 48% of cobalt production originates from nickel ores. Even though the minerals of ultramafic rocks are the source of soils pollution in theex-industrial sites, the total Ca:Mg ratio was higher than 1 (1.3), differently from that in ultramafic soils.

The Dutch standards define the permissible limit for chrome as 100 mg kg^{-1}. Chrome content in all samples was greater than the permissible limits (Ministry of Housing, Netherland, 1994). Sources of chromium contamination include releases from electroplating processes and disposal of chromium containing waste.

Lead and zinc concentrations in soil were within the permissible limits. The maximum intervention limit in soil for Pb is 50-300 mg kg^{-1}, and for Zn is 150-300 mg kg^{-1}as defined in(86/278/EEC). Anthropogenic activites might be the source of higher Zn concentration (142 and 135 mg kg^{-1}) in the ex-industrial and Elbasan dumpsite soils, than in Përrenjas dumpsite (117 mg kg^{-1}). However, Bani*et al*., (2009) stated that this element could be sometimes found at a high concentration in serpentine soil

Biomass, Ni concentration in plant, Ni yields and DTPA extractable Nickel

The biomass production of metal hyperaccumulators depends on productivity of the soil, harvesting time, climatic conditions. Plant biomass is higher in the soils in ex-industrial site (660 g), than in both dumpsite soils, because this soil is used for agricultural purposes and it is fertilizer with organic and chemical fertilizer. The biomass is negatively correlated with Ni concentration in *A. murale*. In fertilized soils the biomass is higher, while the nickel concentration is lower (Osmani and Bani, 2017; Osmani *et al*., 2018a). Fertilizers increase the biomass and dilution the Ni concentration in plant tissues. Nickel concentrations in plants (Table 2) were higher in Përrenjasi dumpsite soil (2189 mg kg^{-1}) than in Elbasan dumpsite (735 mg kg^{-1}) and soils inex-industrial sites (452mg kg^{-1}), due to chemical availability of Ni in soil (Figure 1).

As it has been already reported, *A. murale* can hyperaccumulate up to 1% of nickel. In the present investigation, *A. murale* could not hyperaccumulatenickel more than 2000 mg kg^{-1} nickel since the available nickel content in soil is very low, and the Caamount in the soils in the soil of ex industrial sites is high. These data are in line with (Bani *et al*., 2010) showing that in *A. murale*, there appears at leastan inverse relationship between the Ni uptake and the Ca concentration in the soil.

Table 2. Plant biomass, nickel concentration in plant and Ni phytoextraction

Soil type	Plant biomass (g)	Ni concentration in plants (mg kg^{-1})	Nickel phytoextraction
Ex-industrial site	660	452	289 mg Ni/m^2
Përrenjas Dumsite	4.2	2189	8.77 mg Ni/pot
Elbasan Dumpsite	4.1	735	3.01 mg Ni/pot

Considering the biomass production and Ni accumulation, *A. murale* can be a potential candidate for phytoextraction of Ni in metal contamination sitein ex industrial site.

The Nickel concentration in soils polluted by anthropogenic activities (maily ultramafic minerals) had low concentration of available Ni as previously shown. Massoura *et al*., (2006) said that the soil is poor in smectite, rich in high-Ni goethite and slightly rich in alkaline.

The amount of the available Nickel in the soil, called Ni_{DTPA}, significantly decreased with time of *A. murale* cultivation. DTPA-extractable Ni in the soil was lower after the harvest of *A. murale*, mainly in the ex-industrial site, than in both dumpsite soils. It decreased from 3.8 to 3.1 mg kg^{-1} in ex-industrial

soil and in Përrenjas and Elbasan dumpsite, from 4.8 to 3.4 mg kg^{-1} and 4.7 to 3.6 mg kg^{-1}, respectively.

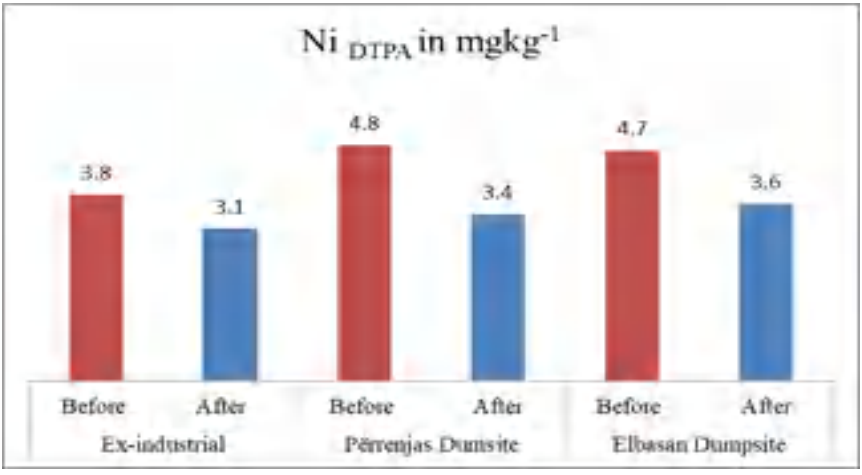


Fig. 1: DTPA extractable Nickel before and after *A. murale* harvest.

The results suggest that *A. murale* takes up Ni from a pool of soil Ni that can be at least partly quantified using DTPA, because the DTPA Ni decreased after the cultivation of *A. murale*. This is confirmed by DTPA previously having shown to extract isotopically-the exchangeable Ni, i.e. Ni from the labile pool (Shallari *et al*., 2001). The contamination potential of the waste resulting from mining materials or metallurgical waste was reduced as the DTPA-extractable pool of Ni in the soil after the successive culture of *A. murale* was reduced.

Ni_{DTPA} here reported is in full accordance with (Shallari *et al*., 2001; Bani *et al*., 2015).

4. CONCLUSION

The data here reported show that industrial activities are the main source of heavy metals pollution. In addition, metal concentrations are higher in the Elbasani dumpsite soil due tothe industrial activity (iron-nickel plant and other plants) and in the Përrenjas dumpsite soil which is rich in iron, nickel, cromium and cobalt mining wastes.

The term “hyperaccumulator” describes a number of plants that belong to distantly related families, but share the ability to grow on metalliferous soils and to accumulate extraordinarily high amounts of heavy metals in the aerial organs, far in excess of the levels found in the majority of species, without

suffering phytotoxic effects. The increase of biomass quantity under the influence of fertilization shows that nickel plays a strong role in plant growth. The ability of hyperaccumulative plants to keep the hyperaccumulation ability also in the non-mineralized contaminated soil shows the basic tolerance and adapter priority for the ability of hyperaccumulator. The low concentration of available nickel in soil and the high content of calcium compared to the serpentine soils where *A. murale* grows naturally limits the accumulation of nickel. The use of fertilizer has increased nutrients in the soil, which are essentials for plant growth. As a result, we have the growth of plant biomass and Ni phytoextraction. Given the phytoextraction benefits and the capacity of *Alyssum murale* to accumulate nickel in soil, it could be concluded that *Alyssum murale* could be a candidate for phytoremediation in the ex-industrial soils and industrial and mining waste.

REFERENCES

Baker AJM, Brooks RR. 1989. Terrestrial higher plants which hyperaccumulate metalic elements—A review of their distribution, ecology and phytochemistry. *Biorecovery***, 1**:81– 126.

Bani A, Echevarria G. 2019. Can organic amendments replace chemical fertilizers in nickel agromining cropping systems in Albania? *International Journal of Phytoremediation*, **21**:43–51**.**

Bani A, Echevarria G, Sulçe S, Morel JL. 2015b. Improving the agronomy of *Alyssum murale* for extensive phytomining: a five-year field study. *International Journal of Phytoremediation*, **17**:117–127 doi: 10.1080/15226514.2013.862204.

Bani A, Echevarria G, Zhang X, Laubie B, Morel JL, Simonnot MO. 2015. The effect of plant density in nickel phytomining field experiments with *Alyssum murale* in Albania. *Australian Journal of Botany*, **63**:72–77 doi: 10.1071/BT14285.

Bani A, Echevarria G, Montarges-Pelletier E, Gjoka F, Sulce S, Morel JL. **2014**. Pedogenesis and nickel biogeochemistry in a typical Albanian ultramafic toposequence. *Environmental Monitoring and Assessment*, **186**:4431–4442. doi: 10.1007/s10661-014-3709-6.

Bani A, Imeri A, Echevarria G, Pavlova D, Reeves RD, Morel J L and Sulçe S. 2013. Nickel hyperaccumulation in the serpentine flora of Albania. *Fresenius Environmental Bulletin*, **22**:1792–1801.

Bani A, Pavlova D, Echevarria G, Mullaj A, Reeves RD, Morel JL, Sulçe S. 2010. Nickel hyperaccumulation by species of *Alyssum* and *Thlaspi* (Brassicaceae) from the ultramafics of Balkans. *Botanica Serbica*, **34**:3–14.

Brooks R R, Lee J, Reeves R D and Jaffrré T. 1977. Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. *Journal of Geochemical Exploration*, **7**: 49–57.

Chaney RL, Angle JS, Broadhurst CL, Peters CA, Tappero RV, Sparks DL. 2007. Improved understanding of hyperaccumulation yields commercial phytoextraction and phytomining technologies. *Journal of Environmental Quality*, **36**: 1429–1443.

Council of the European Communities (CEC): Council Directive of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture (86/278/EEC). *Official Journal of the Europe*.

Echevarria G, Leclerc-Cessac E, Fardeau JC, Morel JL. 1998. Assessment of phytoavailability of Ni in soils. *Journal of Environmental Quality*, **27**:1064– 1070.

Kapusta JP. 2007. Cobalt Production and markets: a brief overview. Cobalt News, **07**/**1**, 9-12.

Li YM, Chaney RL, Angle JS, Baker AJM. 2000. Phytoremediation of heavy metal contaminated soilsK. In: Wise DL (ed) Bioremediation of contaminated soils. Marcel Dekker, New York, 837–884.

Massoura S T, Echevarria G, Becquer T, Ghanbaja J, Leclerc-Cessac E, Morel JL. 2006. Nickel bearing phases and availability in natural and anthropogenic soils. *Geoderma*, **136**, 28–37

Nkrumah P N, Baker AJM, Chaney R L, Erskine PD, Echevarria G, Morel JL, Van der Ent A. 2016. Element Case Studies: Nickel Current status and challenges in developing nickel phytomining: an agronomic perspective. *Plant Soil*, **406**:55–69.

Osmani M, Bani A. 2017. Heavy metals concentration of dumping site soils and their accumulation in *Alyssum murale* growing in selected dumping sites in Albania. *Thalassia Salentina*, **39**:83-98.

Osmani M, Bani A, Hoxha B. 2018a. The Phytomining of nickel from industrial polluted site of Elbasan-Albania. *European Academic Research*, Romania **5(9):** 5347-5364.

Osmani M, Bani A, Gjoka F, Pavlova D, Naqellari P, Shahu E, Duka I, Echevarria G. 2018b. The natural plant colonization of ultramafic post-mining area of Përrenjas, Albania. *Periodico di Mineralogia*, Universita degli Studi di Roma, La Sapienza. **78 (2**): 135-146.

Osmani M, Bani A, Hoxha B. 2015 Heavy Metals and Ni phytoextraction in the metallurgical area soils in Elbasan. *Albanian Journal of Agricultural Science*, **14(4):**414-419.

Osmani M, Bani A, Hoxha B, Mazrreku A. 2018c. Industrial activity and soil contamination in Elbasan, Albania. Association for Promotion of

Holistic Aproach to Environment Buzetska 25, 44000 Sisak, Republic of Croatia. *Proceedings*, 567-573.

Proctor J. 1971. The plant ecology of serpentine. III. The influence of a high magnesium/calcium ratio and high nickel and chromium levels in some British and Swedish serpentine soils. *Journal of Ecology*, **59:** 827-842.

Reeves RD, Adigüzel N. 2008. The nickel hyperaccumulating plants of the serpentines of Turkey and adjacent areas: a review with new data. *Turkish Journal of Biology*, **32**:143–153.

Sallaku F, Shallari S, Wegener HR, Henningsen PF. 1999. Heavy metals in industrial area of Elbasan. *Bulletin of Agricultural Sciences*, **3**: 85-92.

Stevanović V, Tan K, Iatrou G. 2003. Distribution of the endemic Balkan Flora on serpentine I 309 obligate serpentine endemics. *Plant Systematics and Evolution*, **242**, 149–170.

Shallari S, Echevarria G, Schwartz C, Morel JL. 2001. Availability of nickel in soils for the hyperaccumulator 9 Waldst. & Kit. *South African Journal of Science*, **97**: 568-570.

Shallari S, Hasko A, Schwartz C, Morel JL. 1998. Heavy metals in soils and plants of serpentine and industrial sites of Albania. *The Science of the Total Environment*, **209**: 133-142.

Shehu E. 2009. Teknologjia kimike dhe mjedisi. 222-251.

Yao C, Rath U, Maiato H, Sharp D, Girton J, Johansen KM, Johansen J. 2012. A nuclear-derived proteinaceous matrix embeds the microtubule spindle apparatus during mitosis. ***Molecular Biology of the Cell***, **23(18)**: 3532-3541.