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EVALUATION OF MICROORGANISMS AND MOLECULAR VARIABILITY OF SOME OLD VARIETIES OF *Malus domestica* L.

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Abstract

Even if the Romanian market has been overrun in recent years by non-indigenous hybrids, the high degree of adaptability of the Romanian varieties helps them to persist in the struggle for survival. The demands of organic consumption have determined a segment of the country's population to refocus on the consumption of indigenous apples or originating from other areas, but grown for a very long time in our country.
The interest for these varieties with tasty fruits and special flavors led us to make a microbiological and molecular assessment. Molecular variability was investigated using ISSR (Inter Single Sequence Repeats) and SCoT (Start Codon Targeted) markers. The microbiological methodology is based on techniques for the isolation and determination of mesophilic bacteria, molds, yeasts, the presence of faecal bacteria and Escherichia coli, Salmonella and Shigella species. Escherichia coli was observed in some samples and enterococci were highlighted in only one sample. The results indicate a variation of the microbial groups in the varieties analyzed and the absence of species that can disturb serious the digestive tract (for example Salmonella and Shigella).

Key words: *Escherichia coli, Malus domestica L., microorganisms, molecular variability.*

INTRODUCTION

The period 2016-2025 has been called the "decade of nutrition", as in this time the implementation of government measures will be followed to eliminate all forms of malnutrition in emerging or developing region. According to the available data, one person out of three suffers from "malnutrition, micronutrient deficiency and obesity". In response, measures have been proposed to support sustainable food systems and healthy food (Goncearov et al., 2004; Petcu et al., 2007). This is mainly possible by stimulating and sustaining the agriculture and horticulture sectors (United Nations 2016-2025/https://www.un.org/nutrition/sites/).
In this context, the UN (United Nations) has declared 2021 as the "International Year of Fruits and Vegetables", to emphasize their importance in human nutrition and health. It is known that fruits are a source of vitamins, minerals, antioxidant compounds and fibers (Slavin & Lloyd, 2012). Thus, the contribution of fruits and vegetables in solving the above-mentioned problem is very significant. FAO (2015) recommended 400 grams of fruit per day/person. According to statistics, in 2019, in Romania, food spending in each household represented 61.0% of total spending, including 6.1% for fruit. Fruit consumption for each person was 366.3 grams per year in 2019 (Dobre-Baron, 2020).
Also, recent studies have shown that there is a correlation between non-communicable diseases and diets poor in fruits and vegetables (Jhee et al., 2019). According to WHO data (2019), in such cases, the incidence of mortality is 41 million persons per year. The consumption of fruits and vegetables is beneficial and promoted all over the world, but the availability and quality of fruits depends on

their interaction with microorganisms. Therefore, knowledge of the diversity of microorganisms on fruits is essential, as it influences the health of consumers (https://www.fao.org/).

Knowledge of the microbial load and their diversity can give us a better idea of their sustainability over time and help us improve food security, because research has shown that microbial degradation (Janisiewicz & Korsten, 2002; Savu & Petcu, 2002) and contamination (Petcu et al., 2019) of agrifood products is a global problem. The consequences of microbial spoilage are: food waste (Snyder & Worobo, 2018) and the negative effect on the social and economic environment (Jeswani et al., 2021).

Over the past few years, the "eco" and "organic" fruit and produce market has diversified.Unfortunately, Romania has turned from an exporting country to a consumer market for fruit from abroad (Ministry of Agriculture and Rural Development (2017), especially from the EU. However, there are still old varieties with special aromas and tastes. These varieties are not chemically treated and can be found in family households or orchards. According to data from the National Institute of Statistics (INS) (cited by Dobre-Baron, 2020) plum, apple and cherry plantations are dominant in Romania.

The apple originates from Central Asia and has been present in Europe and Asia since antiquity. *Malus domestica* L. grows in temperate zones and is part of the genus *Malus,* family *Rosaceae,* class *Magnoliopsida,* division *Manoliophyta* (Li et al., 2022). The Malus genus includes over 7500 varieties (Koseoğlu & Al-Taie, 2022). Apples are most commonly consumed in Romania (Dobre-Baron, 2020) and around the world (Wassermann et al., 2019).

Experts in the field appreciate that apples have a health benefit because they are sources of flavonoids, procyanidines and pectins (Shoji & Miura, 2014; Samout et al., 2016; Shtriker et al., 2018). It is known that flavonoids and procyanidins have antioxidant and antitumor potential (He & Liu, 2007; Shoji & Miura, 2014; Ribeiro, 2014; Zielinska, 2019; Azizah et al., 2020).

In Romania, in rural households or orchards in more isolated areas, there are genotypes of apples, grown for a very long time, adapted to local environmental conditions, the origin of which is uncertain. It may be considered that some of them originate from known varieties because of specific traits.

For this reason the present research aimed to assess the degree of relatedness between these forms, cultivated for a very long time in the same geographical region. In order to assess the genetic diversity between the apple genotypes studied, molecular markers were used to establish genetic fingerprints. Inter Simple Sequence repeats (ISSR) markers that amplify regions between adjacent opposite microsatellite sequences were used (Pradeep et al., 2002), because they do not require prior knowledge of the sequences analyzed. Besides, we used Start Codon Targeted (SCoT) markers, which amplify sequences in the gene region, the used primers having sequences complementary to the start codons (Colard et al., 2009). These markers are widely used for genetic evaluation in various plant species, including apple (Stepanov et al., 2021; Yao, 2022, Raja et al., 2022).

Microbial diversity in fruits and vegetables (Wassermann, 2019) is not widely known, because most studies have focused on food safety, food-borne pathogen germs and food poisoning (WHO, 2015) and relatively few with the study of the apple microbiome.

In this context, our studies have focused on apple varieties, cultivated for a long period in Romanian households, which meet the requirements regarding aroma, taste, but also the category "organic apple".

Our research focused on the study of microbiological load (in several ecophysiological groups), starting from the assumption that a "healthy apple" should also be safe from a hygienic and health point of view.

MATERIALS AND METHODS

Microbiological analysis of apple varieties

The microbiological determinations were performed on 6 varieties of apples, grown in the territory of our country during a very long time, from orchards located in the county of Hunedoara (varieties 1-5), respectively Bihor (variety 6).

The apples were harvested from the soil and were transported to the Microbiology laboratory, within the University of Life Sciences "King Mihai I" from Timisoara, in October. From each variety, four apples were chosen. The varieties studied were: Biotype similar with Starkinson originally from the United States of America, named Bot de iepure (Rabbit snout), 2 - Poinic, known as an ancient autochthonous variety, 3 - Biotype similar with Jonathan originally from the United States of America, 4 - Piros, an old variety with unknown origin; 5 - Biotype similar with Basil, an old variety originally from France, named Rosu busuioc (Red Basil) and 6 - Variety Golden Delicious, originally from the Netherlands.

Most of the microorganisms found in fresh fruit are saprophytes, such as: lactic bacteria, sporulant microorganisms, coliforms, micrococcis and pseudomonades, derived from soil, air and water. Due to the acidity of raw fruit, the primary spoilage microorganisms are fungi, predominantly molds and yeasts, such as *Sacharomyces cerevisiae, Aspergillus niger, Penicillum* spp., *Byssochlamys fulva, B. nivea, Coletotrichum gloesporoides,* but also sporulated and non-sporulated bacteria (Alzamora et al., 2000).

We were interested to identify the microorganisms that are represented by the faecal contamination indicators, belonging to the enterobacteria family – total coliforms, *Escherichia coli* and possibly *Salmonella, Shigella* and *Enterococcus faecalis* respectively, since the fruits tested were also harvested from the soil.

For this reason, we analyzed epicarp and mesocarp separately in terms of contamination by coliform microorganisms and fecal streptococci.

The isolation and determination of microorganisms was carried out using conventional group-specific methods (Misca 2011; Jeddi et al., 2014).

All phases were carried out under sterile conditions.The microbial groups were isolated using the successive dilution method.Three serial dilutions were prepared. For the epicarp, the 10^{-3} dilution was inoculated on media specific to each individual microbial group. For the mesocarp, the dilution of 10^{-1} was inoculated. MacConkey medium was used for coliform isolation, and bile-esculin-azide (BEA) agar was used as isolation medium for enterococci.To confirm the pathogenic species, the so-called faecal contamination indicators were used concomitantly and the chromogenic media together with the biochemical culture media for confirmation.Thus, for coliform bacteria, as biochemical confirmation media, we simultaneously use Triple Sugar Indol (TSI), SIM and Citrate Simmons (CS) media.

For the isolation of mesophilic bacteria, the 10^{-3} dilution corresponding to the epicarp was inoculated on nutrient medium Plate Count Agar (PCA) (ISO 4833-1:2013), respectively on Sabouraud+Chlormphenicol (SC), for the isolation of molds and yeasts.The SC medium replaced the Sabouraud dextrose agar medium (Jeddi et al., 2014).Cultivation conditions varied depending on the microbial group, the mesophilic bacteria, molds and yeasts were incubated at a temperature of 28°C, for 24-48 hours for the bacteria, respectively for 5 days for the last two microbial groups.

Molecular analysis

DNA extraction was performed on young leaves collected in early spring using the modified CTAB method (Doyle et al., 1987). 10 ScoT and 9 ISSR primers were initially used for amplification, from which 6 SCoTand 2 ISSR primers were selected, generating the most complex and clear fingerprints (Table 1).

Table 1 The primers sequences

Primer name	Primer sequence
SCoT 1	5'CAACAATGGCTACCACCA3'
SCoT 14	5'ACGACATGGCGACCACGC3'
SCoT 24	5'CACCATGGCTACCACCAT3'
SCoT 34	5'ACCATGGCTACCACCGCA3'
SCoT 35	5'CATGGCTACCACCGGCCC3'
SCoT 36	5'GCAACAATGGCTACCACC3'
UBC808	5'$(AG)_8$C3'
UBC811	5'(GA)8C3'

The amplification was carried out in 25 µl with the following components: 150 ng DNA, GoTaq® Green Master Mix 1x (Promega, USA), 20 pmol primer, 10pmol supplementary $MgCl_2$. The amplifying program followed the known steps, the annealing temperature being 55ºC. The amplification products were separated by electrophoresis in 1.8% agarose

gel. The results obtained were evaluated using Vison Works software and statistically interpreted with UPGMA software (unweighted pair group method with arithmetic mean) (http://genomes.urv.cat/UPGMA/index.php).

RESULTS AND DISCUSSIONS

Evaluation of the microbial load and faecal contaminants

In quantitative terms, we found a very large difference between the microbiological load of the epicarp and the microbiological load of the mesocarp. Both coliform and *E. coli* bacteria and *Enterococcus fae*calis species were absent in the mesocarp while on the epicarp, a consistent load of total coliforms between $4.5x10^3$ and $95x10^3$ germs/10 grams of product was identified, and for *Enterococcus faecalis*, $3x10^3$/10 grams of product, only in the Piros variety.

From a qualitative point of view, we identified the presence of the species *E. coli, Proteus* sp., *Klebsiella* sp. and *Enterococcus faecalis.* We present the appearance of each species on chromogenic and Mac Conkey culture media respectively (Figure 1). *Salmonella* and *Shigella* species were absent.

Figure 1. Coliforms and enterococci isolated on selective nutrient media

On the basis of these results, we observe that the microorganisms of faecal origin are concentrated on the surface of the fruit without penetrating the mesocarp, which is beneficial, because their titre may be significantly reduced by washing them before consumption. It can also be said that the source of contamination may be telluric, as the apples were also harvested from the soil.

Transportation conditions, harvesting and storage methods, soil, water, animals and feces can lead to contamination of fruit (Lopez-Camelo, 2007; NM ISO 6888-1., 2008; Sperber and Doyle, 2009; Ofor et al., 2009). It is obvious that at the time of harvest, the fruits harvested from the tree must be separated from those harvested from the soil, in order to avoid contamination of the production. But at the same time, it is necessary to consider the integrity of the epicarp, to prevent the entry of pathogenic microorganisms into the mesocarp, in order to prevent the consumption of contaminated fruit, which could be the basis of food poisoning.

These measures are also recommended in other studies that examine the status of faecal germs in fruits and vegetables (Erahioui et al., 2021).

According to our research, the same authors report that the level of fecal contamination is variable. The species emphasized by them include *Escherichia coli* and *Staphylococcus aureus*, however, *Salmonella* and *Shigella* have not been isolated, similar to the case we presented.

Some research shows that in the microbiome of unprocessed apples, the orders *Betaproteobacteriales* and *Enterobacteriales* dominate (following molecular analyses, the values were 51.3% and 20.4%, respectively) (Wicaksono et al, 2022b), but some authors have also shown that a substantial fraction of the apple microbiome is beneficial (Abdelfattah et al., 2021).

The statistical evaluation of our data emphasized the relation between different types of detected microorganisms. The number of coliforms was correlated with the number of Yeast (Figure 2).

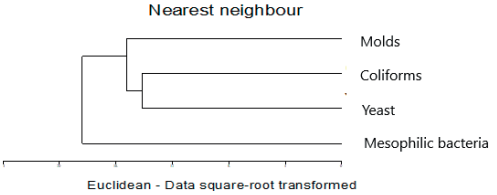

Figure 2. Cluster analysis based on Euclidian Distances

The results regarding the load of microorganisms isolated from the epicarp of the 6 varieties of apples are presented in the Figure 3.

The prevalence of the most important mesophilic bacterial load on apples was on the epicarp of the Poinic and Rosu busuioc (Red Basil) varieties, followed in descending order by the Bot de iepure (Rabbit snout) >Piros> Biotype Jonathan > Golden varieties.

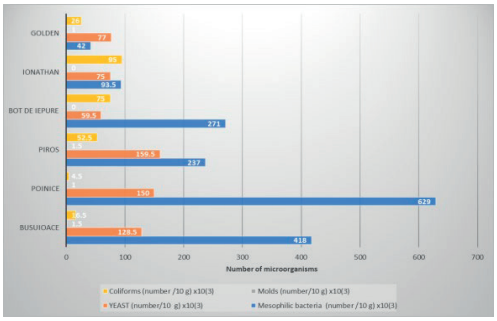

Figure 3. Abundance of microorganisms on the apples epicarp

The high bacterial abundance was also proven by other authors (Wicaksono et al., 2022b). Research shows that microbial abundance and diversity are dependent on the growth system (wild plantations, conventional systems, fruit trees in the garden) and the area. The same authors state that the microbial load is higher in natural plantations and on fruits from the garden (Wicaksono et al., 2022a). The difference between microbial diversity in the conventional and organic system is also confirmed by others (Lupatini, 2017; Wassermann et al., 2019). The abundance can also be explained by its origin, because it is known that the microorganisms present on fruits, plants and flowers come from the soil, an ecosystem that is a microbial reservoir (Zarraonaindia et al., 2015; Massoni et al., 2021). The soil increases its microbial diversity through organic fertilization.

In our study, there were differences according to the area, such as the Golden variety, which comes from a conventional plantation and a different zone, compared to other varieties, has a lower microbial load (Figure 4).

Yeast levels were high in the Piros, Poinic and Rosu busuioc (Red Basil) varieties. The lowest number of yeasts was isolated from the epicarp of the Bot de iepure (Rabbit snout) variety. In general, the load of bacteria and yeasts was high in the Rosu busuioc (Red Basil), Poinic and Piros varieties (Figure 5).

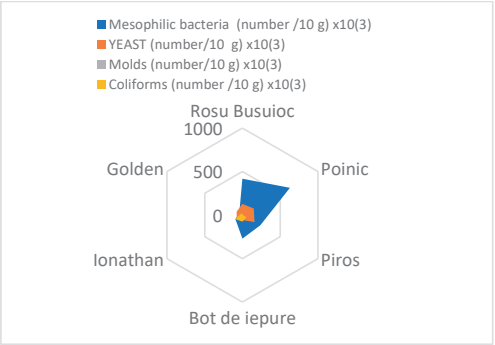

Figure 4. Apple Microorganisms Chart

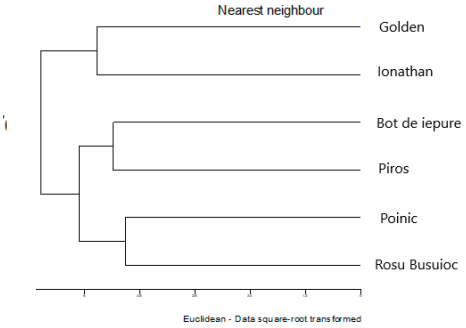

Figure 5. Cluster analysis of apple varieties based on microorganisms number

Compared to other authors who observed a significant growth of molds (1.6x105) on apples (Wicaksono et al., 2022a), our research demonstrated that molds have a lower growth (1.5x103). Their growth was absent in the Bot de iepure (Rabbit snout) and Biotype Jonathan varieties.

Molecular analysis

For biotypes originated from the same region (Hunedoara County), genetic studies were conducted to assess their degree of relatedness using ScoT and ISSR molecular markers.

Once the primers that produced the clearest fingerprints were selected, they were used to amplify the 5 DNA samples extracted and purified. Agarose gel electrophoresis analysis of the amplification products with primers ScoT 34, SCoT 35 and SCoT 36 is shown in Figure 6.

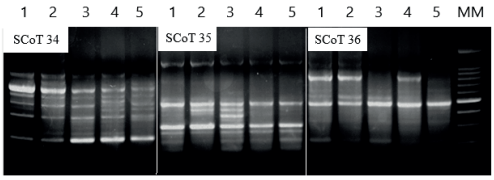

Figure 6. The results of the amplification with ScoT 34, SCoT 35 and SCoT 36 markers

Legend: 1- Piros; 2- Rosu busuioc (Red Basil); 3- Bot de iepure (Rabbit snout); 4- Poinic; 5-Ionathan biotype

A total of 55 alleles were amplified (6.9 alleles/primer) with the selected primers of which 42 were polymorphic (76.4%). Analysis of the amplified fragments with UPGMA software allowed the establishment of similarity indices based on Jaccard coefficients (Table 2). Based on the similarity coefficients, a dendrogram was established showing the degree of relatedness between the genotypes analyzed (Figure 7).

Table 2 Similarity Matrix computed with Jaccard coefficient

Genotype	1	2	3	4	5
1	1.000	0.745	0.696	0.680	0.640
2		1.000	0.814	0.750	0.673
3			1.000	0.702	0.696
4				1.000	0.714
5					1.000

Legend: Bot de iepure (Rabbit Snout); 2-Poinic; 3-Ionathan biotype; 4- Piros; 5- Rosu busuioc (Red Basil)

The average similarity index between the genotypes studied was high (0.711). The highest similarity index was found for the Poinic and Ionathan varieties (0.814). In addition to these two genotypes in the same cluster was the Piros biotype.

The lowest similarity index was found between genotype Bot de iepure (Rabbit Snout) and Rosu busuioc (Red Basil) (0.640).

It appears that genotypes of American origin and those with unknown origin were located in a common cluster, whereas the genotype Rosu Busuioc (Red Basil) of French origin differed from them, having the lowest degree of similarity.

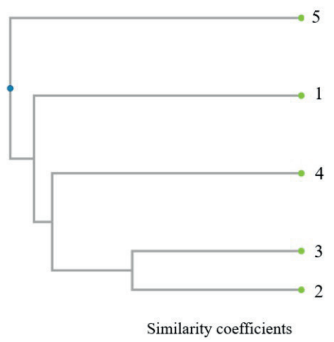

Figure 7. Dendrogram of the relatedness degree based on ISSR and SCoT analysis

Legend: 1- Bot de iepure (Rabbit snout); 2-Poinic; 3-Ionathan biotype; 4- Piros; 5- Rosu busuioc (Red Basil)

CONCLUSIONS

In conclusion, indicators of fecal contamination, belonging to the *Enterobacteriaceae* family, were highlighted on the epicarp of the analyzed apple varieties. Faecal enterococci were observed only on apples belonging to the Piros variety. *Escherichia coli* was isolated from some apple varieties, while *Salmonella and Shigella* were absent. The source of the contamination is telluric, because the apples were harvested from the soil, so compliance and promotion of hygienic-sanitary measures are essential in these cases. The load of mesophilic bacteria and yeasts remained high, especially in the varieties Rosu busuioc (Red Basil), Poinic and Piros, on the other hand, the number of molds was reduced or even absent.

From a genetic point of view, the genotypes analyzed had a high similarity index, differing according to their origin. The Red Basil genotype, with French origin, showed the highest degree of diversity compared to genotypes with USA or unknown origin.

Taking into account the importance and high consumption of apples, but also the limited research on the microbiome of these fruits. Future studies will assess the microbiome using methods for harvesting, handling and storing apples.

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