PAPER

QUALITY AND MYCOBIOTA COMPOSITION OF STORED EGGS

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ABSTRACT

The aim of this study was to monitor the quality and mycobiota composition of table eggs during storage period. The most significant changes in the egg weight and water activity were observed on Day 7. To identify the mycobiota present on the eggshell by PCR method, a newly designed procedure for the extraction of fungal DNA based on a combination of commercial isolation kit, proteinase K and ultrasound was implemented. Identified mold genera included *Penicillium* spp., *Cladosporium* spp., *Fusarium* spp. and *Alternaria alternata* group. Their ratio varied considerably during storage with the dominance of *Penicillium* spp. on Day 14.

Keywords: egg quality, Haugh units, micromycetes, PCR, storage, table eggs

1. INTRODUCTION

Egg is one of the most nutritious low-energy foods possessing all the proteins, vitamins and minerals needed for human health (TOLIK *et al*., 2014). Because of its beneficial composition, it may also be used as nutraceuticals and protein ingredients for food applications (ZAMBROWICZ *et al*., 2015). However, during egg collection, there is significant risk of contamination of the shell surface with microscopic filamentous fungi. By laying out, an egg gets to the external environment, which becomes its source of contamination. The majority of egg microbiological contamination studies deal primarily with microorganisms of bacterial origin (BAHOBAIL *et al*., 2012; JONES *et al*., 2015; ERKMEN and BOZOGLU, 2016; KARIMIAZAR *et al*., 2019), secondarily with fungal contamination (TOMCZYK *et al*., 2019). Micromycetes are able to grow under conditions which are unsuitable for bacteria (extreme pH, low a_w, wide range of enzymatic activity). Spores of various mold genera are regularly found on the shell surface and can furher penetrate into the egg contents (TOMCZYK *et al*., 2018). Contamination of the eggshell may be initiated by ambient conditions during egg collection and storage, which may be as a result of the quality of litter and feed, the presence of dust, air temperature and humidity. Therefore, the presence of numerous fungal genera (*Aspergillus*, *Cladosporium*, *Drechslera*, *Penicillium*, *Stemphylium* and *Fusarium*) on the eggshell has been reported in many studies (KOKKONEN *et al*., 2010; ROHWEDER *et al*., 2011; TOMCZYK *et al*., 2019).
Fungi not only degrade the substrate on which they occur, they also cause numerous diseases due to the presence of spores in the air (SKÓRA *et al*., 2015). Mycotoxins, which are secondary metabolites produced by some fungal strains, are of the greatest risk for consumers (SYPECKA *et al*., 2004).
Freshness, the characteristic most commonly related to egg quality, declines with time and temperature after laying (HIDALGO *et al*., 2006). Several chemical and physical changes occur during egg storage such as increase in albumen pH, thinning of the thick albumen, water evaporation through the shell (LUCISANO *et al*., 1996), enlargement of air cell, development of Maillard reaction (HIDALGO *et al*., 2006). The most common indices used to evaluated egg freshness are air cell height (EU, 2007) and thick albumen height, expressed as Haugh units (USDA, 1995).
Storage conditions (especially the temperature) also have a great impact on mycological contamination and the production of fungal exoproducts on the shell surface. Temperatures below 5°C slow the aging process, but primarily reduce the development of microorganisms. If the refrigerator temperature decreases below –2°C, the egg contents would start to freeze. According to the European legislation (EC, 2008), shelleggs must not be exposed to refrigeration at temperatures lower than 5°C. Another significant parameter in egg storage is the relative air humidity which is adjusted to reduce as much as possible the losses caused by water evaporation. This results in a weight decrease and changes in individual egg quality indicators. In general, the lower the storage temperature, the higher the relative humidity (STEINHAUSEROVÁ *et al*., 2003). Within the storage period, diversified mycoflora, including potentially toxinogenic and toxinogenic species of micromycetes, is found in the eggs. Therefore, the identification of micromycetes is necessary (THRANE *et al*., 2004). Current routine methods used for the detection and identification of microscopic filamentous fungi often require culture isolation and further morphological and physiological characterisation (SIMMONS, 2007). However, it does not provide sufficient distinction among species. The limitation of these techniques is unstable micromycete cell morphology, which may vary within the species and also dependent on ambient conditions (RAINIERI *et al*., 2003). The development of molecular techniques has

enabled the identification of not only the genus, but also the individual species of micromycetes. Currently, numerous identification methods are generally in use. DNA reassociation techniques are used to detect DNA complementarity. The disavantage of these methods is inability to distinguish closely related species. Gene sequence comparison no longer has this limitation. However, detection of rRNA genes, such as the internal transcriptional region (ITS), is the preferred method (KURTZMAN *et al*., 2011). For proper identification of micromycetes by PCR methods, sufficient amount and high purity of the DNA are necessary.
The aim of this study was to detect changes in quality of table eggs during the storage period of 28 days and to identify mold genera present on the eggshell using the PCR method. For correct and accurate identification it is necessary to obtain DNA which is often very complex in micromycetes. To extract the fungal DNA, the cell wall, cytoplasm, and nuclear membrane must be first effectively lysed. However, the structure of fungal cell wall itself prevents lysis and sufficient nucleic acid isolation (Čmoková *et al*., 2014). Therefore, a new effective procedure for lysing fungal cells and further DNA extraction has been designed and implemented in this study.

2. MATERIALS AND METHODS

Micromycetes were isolated from 120 brown-shell table eggs, laid by the *Lohmann Brown* crossbreed laying hens in cages, weight category M (EC, 2008). The eggs were graded, labeled and stored in the breeding stock at an average temperature of 14.3°C and a relative humidity of 61%. Sets of 30 sample units were taken every 7 days. Among them, water activity (a_w) of the eggshell was determined in 10 eggs, another 10 eggs were checked for selected egg quality indicators and the enumeration of microscopic filamentous fungi according to STN ISO 21527-1 (2010) and STN ISO 21527-2 (2010) was carried out using the last 10 eggs. Since molds are aerobic organisms and colonize mainly the shell, enumeration of molds was focused on the eggshell surface.

2.1. Determination of water activity (a_w) of eggshell and egg quality indicators

Water activity of the eggshell was determined by the LabMASTER-a_w (Novasina AG, Lachen, Switzerland) at a constant temperature (25°C) for 20 min±2.48 min. Measurements were repeated three times for individual egg. Eggshell breaking force was measured in accordance with the manufacturer's instructions by the Egg Force Reader (Orka Food Technology Ltd., Ramat HaSharon, Israel) - a compact system for automatic measuring of eggshell rupture point. The unit of strength measurement (kilogram-force, kgf) is calculated as gently applied force on the eggshell until it cracks.
Egg Analyzer™ (Orka Food Technology Ltd., Ramat HaSharon, Israel) was used to determine the egg weight, Haugh units (HU), quality grade and yolk color. The device is able to measure egg weight (g), height of the thick albumen (mm), and color of the yolk. The first two measurements were used for calculation of Haugh units that indicate egg quality. The equation for working out the rating is shown below:

$$HU = 100 \log (h - 1.7w^{0.37} + 7.6),$$

where:
HU = egg quality in Haugh units
w = egg weight in grams
h = height of the thick albumen in mm (NAGY *et al*., 2011)

Yolk color intensity was compared to the Roche Yolk Color Fan (RCF) ranging from 1 to 15 (1 means pale yellow and 15 dark yellow).
After evaluation, the egg contents were transferred to large Petri dishes with diameter of 200 mm each, they were then placed on a dark flat surface and both the height and the width of the egg yolk as well as those of the thick albumen were measured using a micrometer gauger and a ruler. Subsequently, the yolk index (YI) and albumen index (AI) were calculated according to NAGY *et al*. (2011) as follows:

$$\text{Albumen index} = \text{Height of the thick albumen (mm)} \times 100 / [\text{Length of the thick albumen (mm)} + \text{Width of the thick albumen (mm)}/2]$$

$$\text{Yolk index} = \text{Height of the yolk (mm)} / \text{Yolk diameter (mm)} \times 100$$

2.2. Fungal strains and culture condition

Microscopic filamentous fungi were removed from the eggshell surface as described by CUPÁKOVÁ *et al*. (2010). For this purpose, ten eggs of each experimental group were tested every week. Individual egg was transferred aseptically into a sterile plastic bag and sterile peptone water (0.1 wt%) in a volume of 100 mL was added. The sample was then shaken for 15 minutes using the Orbi-ShakerTMJR (Benchmark Scientific Inc., Sayreville, USA). Further decimal dilutions were prepared in accordance with STN EN ISO 6887-1 (2017). As described by the standard procedure, the first two decimal dilutions were spread in parallel on the surface of Dichloran Glycerol agar medium (DG-18; OXOID, Basingstoke, UK) and Dichloran-Rose Bengal Chloramphenicol agar medium (DRBC; OXOID, Basingstoke, UK) in a volume of 0.1 mL and incubated for 5 days at 25°C (STN ISO 21527-1 and STN ISO 21527-2, 2010). Colonies were further subcultured individually on the surface of Czapek agar medium (OXOID, Basingstoke, UK) and incubated at 25°C for another 7 days. After incubation, 1 to 5 colonies were subjected to macroscopic and microscopic identification.
Macroscopic evaluation included the growth rate, color, texture and topography of fungal colonies. Microscopic study of different mold genera was carried out by preparing slides stained with lacto phenol cotton blue and observed under light microscope.

2.3. DNA isolation

Because the cell wall of micromycetes is difficult to break, there was a challenge to obtain pure and concentrated fungal DNA. Therefore, the following three pre-isolation techniques were designed:
1. Mycelium in a quantity of 10-50 mg was taken with a sterile scalpel from the surface of Czapek agar medium, transferred into 1.5 mL eppendorf, exposed to liquid nitrogen for 5 min and then heated to 95°C for 5 min. Freezing and heating was repeated three times. The DNA isolation was then performed with the help of commercially available E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Norcross, USA).

2. Proteinase K (Macherey-Nagel, Düren, Germany) in a volume of 10 μL was added to eppendorfs with 10-50 mg mycelium and incubated for 30 min at 37°C. After that, the DNA isolation procedure was carried out in accordance with instructions of the commercially available E.Z.N.A.® Fungal DNA Mini Kit manufacturer (Omega Bio-Tek, Norcross, USA).
3. Both zircon and glass beads (1:1) in a volume of 0.2 mL and Proteinase K (Macherey-Nagel, Düren, Germany) in a volume of 10 μL were added to fungal mycelium (10-50 mg) in the eppendorf. Samples were incubated at 37°C for 30 min. After that, 800 μL of lysing solution FG1, which was a part of the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Norcross, USA) was added. Samples were then incubated at 65°C for 10 min with ultrasound waves of 500 Hz. Further DNA isolation was performed according to recommended procedure for the commercially available E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, USA, Norcross, USA).
The purity and concentration of DNA was measured using spectrophotometer (SHIMADZU, Wien, Austria). The obtained supernatant was used as a source of DNA in PCR reactions.

2.4. Primer design

The forward primer ITS 212d and the reverse primer ITS 549 amplified a specific DNA fragment for *Penicillium* spp. (336 bp; Table 1).

Table 1. DNA sequences of primers used in this study.

Primer name	Primer sequence (5′-3′)	Annealing temperature (°C)	PCR product	GenBank-EMBL accession number	Reference
	***Penicillium* spp.**				
ITS 212d	AAATATAAATTATTTAAAACTTTC	46	336 bp	LC 317718.1	Pedersen *et al.*, 1997
ITS 549	CTGGATAAAAATTTGGGTTG				
	***A. alternata* group**				
18S-F	AGGATCCATTGGAGGGCAAGT	61	99 bp	AY 563301	Pavón *et al.*, 2010
18S-R	TCCAACTACGAGCTTTTTAACTGCA				
Altsp-F	GAGAACAGCTTCATGGACTTCTCTTT	61	195 bp		
Altsp-R	CGCGGCAGTAGTTGGGAA				
Alt A–F	CGCATCCTGCCCTGTCA	60	118 bp		
Alt A–R	GTTGGTAGCCTTGATGTTGAAGC				
	***Cladosporium* spp.**				
MS1	CAGCAGTCAAGAATATTAGTCAATG	51	370 bp	AY 291273	Zeng *et al.*, 2006
MS2	GCGGATTATCGAATTAAATAAC				
Clado-PF	TACTCCAATGGTTCTAATATTTTCCTCTC	51	87 bp		
Clado-PR	GGGTACCTAGACAGTATTTCTAGCCT				
	***Fusarium* spp.**				
ITS-Fu-f	CAACTCCCAAACCCCTGTGA	55	410	MK 156681.1	Abd-Elsalam *et al.*, 2003
ITS-Fu-r	GCGACGATTACCAGTAACGA				

These primers were designed based on ITS region and *5.8S* rRNA sequences from *Penicillium* spp. available in the GenBank-European Molecular Biology Laboratory database (GenBank-EMBL; PEDERSEN *et al*., 1997).
Alternaria-specific and *Alternaria alternata*-group-specific primer pairs were designed based on *Alt a 1* gene sequences from *Alternaria* spp. available in the GenBank-EMBL database (Table 1; PAVÓN *et al*., 2010).
Both the forward primer AltspF and the reverse primer AltspR amplified a DNA fragment of 195 bp. in all *Alternaria* spp. The primer pair AltAF and AltAR amplified a specific DNA fragment (118 bp) for *Alternaria alternata* group. Finaly, the primer pair 18SF and 18SR, based on conserved *18S* rRNA gene, was used as positive amplification control of the assay (Table 1; MARTÍN *et al*., 2009; PAVÓN *et al*., 2010).
Mitochondrial (mt) small subunit rRNA (SSU rRNA) of *Cladosporium* was amplified by PCR using the universal fungal mitochondrial primers MS1 and MS2. Two specific primers Clado–PF and Clado–PR were designed for multiplex PCR assay (Table 1). The expected amplicon size for primer pair Clado-PF/R was 87 bp (ZENG *et al*., 2006).
Two primers were designed based on sequence information obtained to amplify specific fragments within the *ITS* regions of *Fusarium* spp. The initial tests for specificity revealed that the primer pair ITS-Fu-f and ITS-Fu-r were highly specific for *Fusarium* genus (Table 1; ABD-ELSALAM *et al*., 2003). All the primers were synthesized by the same commercial company (Ecoli s.r.o., Bratislava, Slovakia).

2.5. PCR amplifcation

The amplification was done in a volume of 20 μL containing 1 ng to 10 ng of DNA, 0.5 μL of each primer (concentration 10 pmol/μL), 4.0 μL HOT Firepol® Blend Master Mix (Solis BioDyne, Tartu, Estonia) in the thermal cycler (TC-512, Techne UK, Staffordshire, United Kingdom) using the same cycling conditions for each primer sets, with an initial cycling step at 95°C for 12 min, followed by DNA denaturation at 95°C for 20 s, annealing at diferent temperatures depending on the type of primer used (Table 1) for 60 s, and elongation at 72°C for 2 min. The amplification was terminated by cooling to 6°C.
The following reference strains were used as positive controls in this study: *Penicillium chrysogenum* CCM F-362, *Fusarium sporotrichioides* CCM F-352, *Alternaria alternata* CCM F-397, *Cladosporium cladosporioides* CCM F-348 (Czech Collection of Microorganisms, Brno, Czech Republic).

2.6. Sensitivity of specific detection assays

To determine the minimum detectable amount of fungal DNA in three established PCR assays, variable quantities of fungal genomic DNA ranging from 10 ng to 100 ng were used as DNA template. The PCR products were size fractionated in agarose gels (1.5%) and visualized using GelRed™Nucleic Acid gel stain (Biotium INC., Fremont, USA). Amplicons were visualized by UV transillumination using the Mini Bis Pro® (DNR Bio-Imaging Systems Ltd., Neve Yamin, Izrael).
The identity of PCR products with the selected primers was confirmed by a commercial company (GATC Biotech AG, Cologne, Germany). The DNA sequences obtained from fungal strains were searched for homology to those available at the GenBank-EMBL database using the BLAST program (NCBI software package).

2.7. Statisical analysis

Two-way analysis of variance (ANOVA) and Tukey test for multiple comparison of means with a confidence interval set at 95% was conducted with R - statistics software (R CORE TEAM, 2018). The storage period was set as the main factor. Multiple factor analysis was conducted in R - statistics software (R CORE TEAM, 2018) with "FactomineR" (SEBASTIEN *et al*., 2008) and "factoextra" package (KASSAMBARA and MUNDT, 2007) according to SEMJON *et al*. (2018).

3. RESULTS AND DISCUSSION

Pre-market table eggs must be sorted, packed and stored under appropriate conditions to maintain temporary shelf life. According to Commission Regulation (EC) No. 589/2008, the minimum shelf life of fresh eggs is 28 days from the day it is laid. Storage conditions are limited by storage temperature where the lower limit is 5°C. However, the upper limit is not defined, it only requires maintaining the optimum egg quality.

3.1. Changes in egg quality indicators

Quality parameters of table eggs change during storage. Within the entire storage period, the average values of egg weight ranged from 60.47±2.838 to 57.83±3.368. The most significant egg weight loss (2.64 g±0.87) in this study was recorded on Day 7.
The decrease in egg weight was accompanied by a decrease in water activity (a_w) in the eggshell. Similarly, the most significant decrease in water activity was recorded on Day 7. On the same day, the average shell firmness achieved its maximum value. Shell firmness is related to water evaporation and subsequent drying of the eggs. The rate of water evaporation is influenced by the permeability of the shell and the number of pores in the shell (SIMEONOVOVÁ and MÍKOVÁ, 2003).
As already reported, the above-mentioned three egg indicators are interrelated. Due to egg drying, the increased water evaporation has a negative effect on egg weight (MATUŠOVIČOVÁ *et al*., 1986), which has also been confirmed in this study. Similar results were published by DE LEO *et al*. (2018). The authors reported an average weight loss of 1.67% after 35 days of egg torage.
According to SIMEONOVOVÁ *et al*. (1999), evaporation of water from egg contents depends on the environment, in particular temperature and humidity, as well as on the storage period. ALLEONI and ANTUNES (2004) also reported a reduction in egg weight during storage.
The HU indicates egg quality and the calculation is based on both the height of the thick albumen and the egg weight (CANER and YÜCEER, 2015). The initial value of HU represents the main marker for evaluating egg quality, and its expression provides an indication of the egg shelf-life as well as the storage conditions (FIGUEIREDO *et al*., 2014). As seen in Table 2, mean Haugh unit values ranged between 53.74 and 59.80 during egg storage in this study. DE LEO *et al*. (2018) also confirmed a HU decrease during the storage period of 42 days, which corresponds to similar earlier studies (CANER, 2005; CANER and YÜCEER, 2015; MORSY *et al*., 2015).
In general, Haugh units decrease during egg storage. This can be explained by changes in protein structure resulting in albumen thinning (SIMEONOVOVÁ *et al*., 1999). Results of JIN *et al*. (2011) were consistent with those obtained in this study. In contrast, lower values

within the entire storage period were reported by SAMLI *et al*. (2005) and AKYUREK and OKUR (2009). All of the cited authors stated that Haugh units were decreasing over time. As also reported by TOMCZYK *et al*. (2019), longer egg storage period resulted in a more noticeable decline in Haugh unit values.
A similar decrease was observed in the albumen index, which is based on both the width and height of the thick albumen. The values ranged from 1.370 to 10.500, depending on the storage period. Albumen index was decreasing with prolonged egg storage. The most significant decline occurred on Day 7, when the maximum weight loss and shell firmness, as well as minimum eggshell breaking point, water activity and Haugh units were all determined. Similarly, LAZAR (1990) also reported a decrease in albumen index value with increase in storage time and MÍKOVÁ and DAVÍDEK (2000) ranked the albumen index among the main indicators of egg freshness.

Table 2. Changes in physico-chemical parameters during egg storage.

Storage period (days)	1	7	14	21	28
Water activity (a_w)	0.91±0.00^{a}	0.91±0.00^{a}	0.91±0.01^{a}	0.91±0.00^{a}	0.91±0.00^{a}
Weight (g)	60.47±2.84^{a}	58.20±3.71^{a}	58.23±2.60^{a}	59.00±2.14^{a}	58.23±2.26^{a}
Color	8.33±1.63^{a}	9.17±2.23^{a}	9.50±1.64^{a}	9.33±0.52^{a}	9.00±1.26^{a}
Haugh units	59.80±23.03^{a}	57.85±11.91^{a}	58.33±14.28^{a}	56.15±13.83^{a}	54.57±9.76^{a}
Albumen index (%)	10.50±1.30^{a}	6.86±3.49^{b}	2.25±1.44^{c}	1.23±0.26^{c}	1.42±0.77^{c}
Yolk index (%)	36.70±2.90^{a}	35.51±2.59^{a}	32.92±2.63^{ab}	34.85±1.67^{ab}	31.02±2.99^{b}
Eggshell breaking force (kgf)	3.55±1.06^{a}	4.97±1.08^{a}	4.69±0.73^{a}	4.21±1.25^{a}	3.75±1.53^{a}
Egg quality grade	AA-B^{a}	AA-B^{a}	AA-B^{a}	AA-B^{a}	B^{a}

$^{a-c}$ Different superscripts in each row indicate significant differences between the mean values (Tukey´s, $p<0.05$).

The yolk index is also an excellent indicator of egg freshness, which is based on the measurements of the yolk height and width (YÜCEER and CANER, 2014). Yolk index ranged from 30.74% to 36.90% depending on the length of storage. Yolk width increased during storage as the vitelline membrane lost its elasticity due to aging. Similarly, in a study by DE LEO *et al*. (2018), the yolk index ranged from 30.99% to 39.96% during 42 days of egg storage.
As reported by NEDOMOVÁ (2012), the yolk index decreases with prolonged egg storage. During storage, the structure of vitelline membrane is changed, its strenght decreases, which may, in a combination with water gained from the egg albumen, lead to the enlargement and deformation of the yolk (SIMEONOVOVÁ *et al*.,1999). Similar decrease in yolk index during egg storage was also observed in other studies (SAMLI *et al*., 2005; AKYUREK and OKURK, 2009; NEDOMOVÁ and SIMEONOVOVÁ, 2010), where the storage period was identified as the limiting factor.
Another indicator of egg quality is the yolk color. The yolk color is a consumer factor without any nutritional significance. In this study, the yolk color ranged from 8 to 10 La Roche scale and became more intense as the egg aged. Similar to other studies (MÍKOVÁ and DAVÍDEK, 2000; JINN *et al*., 2011), it becomes more intense during egg storage.

Changes in egg quality parameters during storage may also be accompanied by egg contamination with microscopic filamentous fungi. Therefore, not only the temperature, but also the relative humidity is of great importance during egg storage. Microscopic filamentous fungi need sufficient moisture to grow. Thus, eggs are more frequently contaminated in high relative humidity environments. DE REU *et al*. (2006) and MESSENS *et al*. (2007) reported that a higher level of eggshell contamination led to a better possibility for penetration of micromycetes into the egg contents. Shell pores allow a potential route of entry for fungi and this can lead to both health hazards and a foul smell and taste of an egg. Therefore, detecting egg contamination is an important aspect of public health concern. In addition, volatile organic compounds (VOCs) are produced by micromycetes as they proliferate.These chemicals are emitted back into the environment through the eggshell. Currently, 69 fungi are known as volatile emitters (LEMFACK *et al*., 2014).

The fact that table eggs are often contaminated with fungal spores was also confirmed by TOMCZYK *et al*. (2019) during three weeks of egg storage under various storage conditions. NEAMATALLAH *et al*. (2009) also reported the presence of microscopic filamentous fungi in 38% of eggs in their study with an average count of 3.53 log CFU/mL. The authors have identified the following mold genera: *Aspergillus* (14%), *Penicillium* (9%), *Fusarium* (1%), *Mucor* (6%), *Rhizopus* (4%), and *Cladosporium* (5%).

In this study, micromycetes were isolated from the shell surface using DG–18 and DRBC selective culture media. According to STN ISO 21527-1, DRBC culture medium is used for enumeration of yeasts and molds in food products with water activity greater than 0,95 (e. i. egg contents). However, the measurements in this study gave a value of $a_w \leq 0.95$ in the eggshell which requires the use of DG–18 medium (STN ISO 21527-2). Some mold genera and species are xerophilic and need lower a_w to grow. Therefore, mycological examination of the eggshell was performed using both the above-mentioned culture media in this study. For genus identification, macroscopic evaluation of colony size and characteristics on special culture media is of great importance. Czapek agar medium often provides useful information with regards to growth rates on low water activity media. However, experience has shown that the brand of agar medium used may affect the appearance of colonies that grow on it (SAMSON and PITT, 2000). Therefore, microscopic evaluation of fungal structure is used for more accurate genus identification. Phenotypic key identification is difficult, because these characteristics are unstable and micromycetes sometimes appear to be atypical with slow spore formation and aberrant production of conidiophores (CHEŁKOWSKI and VISCONTI, 1992).

As seen in Table 3, fungal isolates in this study belonged to *Penicilium* spp., *Fusarium* spp., *Alternanria* spp. and *Cladosporium* spp. After inclusion of mold isolates into individual genera, the ability of both culture media to capture these genera was evaluated (Tables 2 and 3).

Despite the fact that STN ISO 21527 recommends the use of two selective culture media depending on water activity of particular food samples, different capture ability has been confirmed for both media. DG-18 medium was more suitable for isolation of *Cladosporium* spp. and *Alternaria* spp. On the contrary, capture of *Fusarium* spp. and to a lesser extent *Penicillium* spp., was significantly higher on DRBC medium. Similar results have been published by WEIDENBORNER *et al*. (1995) and PEREIRA *et al*. (2010). The authors reported that DRBC medium permited the isolation of a wider range of fungal genera/species, regardless of the type of food under study.

In this study, the counts and composition of mycoflora on the eggshell varied during the storage period. Similar to changes in egg quality indicators, changes in numbers of micromycetes were also confirmed between 7 and 28 days of egg storage. However, these

changes did not show any statistical significance (Table 2). With the use of DRBC medium, significant changes in the composition of mycoflora have been noticed on Day 21, when *Cladosporium* spp. was no more detected and a significant increase in *Fusarium* isolates (57%) has been observed. On Day 28, *Cladosporium* accounted for only 16% of the total mycological representation. Regarding the DG-18 medium, changes in the genus composition were first noticed on Day 14 with the dominance of *Penicillium* spp. (Table 3). The presence of *Alternaria* spp. (14%) was confirmed on Day 21 of egg storage.

Table 3. Mycological contamination of eggshells during the entire storage period.

Day	log CFU/mL	Percentage of isolates
		DRBC
1	1.95±1.98	*Cladosporium* spp. (50%) *Penicillium* spp. (25%) *Fusarium* spp. (25%)
7	2.23±2.88	*Cladosporium* spp. (50%) *Penicillium* spp. (33%) *Fusarium* spp. (17%)
14	1.85±1.65	*Cladosporium* spp. (42%) *Penicillium* spp. (16%) *Fusarium* spp. (42%)
21	1.85±1.76	*Penicillium* spp. (43%) *Fusarium* spp. (57%)
28	2.15±1.73	*Cladosporium* spp. (16%) *Penicillium* spp. (42%) *Fusarium* spp. (42%)
		DG-18
1	1.95±1.87	*Cladosporium* spp. (50%) *Penicillium* spp. (40%) *Fusarium* spp. (10%)
7	1.90±1.96	*Cladosporium* spp. (86%) *Penicillium* spp. (14%)
14	1.90±1.88	*Cladosporium* spp. (20%) *Penicillium* spp. (60%) *Fusarium* spp. (20%)
21	1.48±1.43	*Cladosporium* spp. (58%) *Penicillium* spp. (14%) *Fusarium* spp. (14%) *Alternaria* spp. (14%)
28	1.95±1.85	*Cladosporium* spp. (56%) *Penicillium* spp. (44%)

The results are expressed as the (average±standard deviation) of six independent measurements.

All the physico-chemical and mycological parameters of table eggs during the storage period of 28 days were further analyzed statistically using the method of multiple factor analysis (MFA) where the storage period was the main qualitative factor.

The results of MFA showed four selected components that explained 63.39% of the total variation in the results of experimental eggs. The first dimension (Dim1) explained 18.66% of variation, dimension 2 (Dim2) 17.22%, dimension 3 (Dim3) 15.20%, and dimension 4 (Dim4) 12.92%.

Contribution of the analyzed data in Dim1 was mainly related to storage period (51.36%, r=0.95) and physicochemical variables (46.70%, r=0.94). The first two dimensions explained 35.88% of variance (Fig. 1). The highest contribution in Dim 1 included albumen index (r=0.92), yolk index (r=0.62) and egg weight (r=0.41). Dim2 was characterized by the contribution of the effect of storage period (48.78%, r=0.89) and microbial parameters (37.25%, r=0.80) on analyzed variables. Microbiota count analyzed on DRBC agar (r=0.78) with water activity (r=-0.49) were correlated in Dim2. In the first two dimensions, these variables were correlated on statistical significant level of alfa<0.05 (Fig. 2).

Dim3 was mostly related with the storage period, as well as with combination of physicochemical and microbial parameters of experimental eggs. Correlation coefficients for microbial variables in Dim3 were determined as follows: CFU analyzed on DG–18 agar (r=0.57), water activity (r=0.45), yolk index (r=-0.39) and eggshell breaking force (r=-0.47).

The highest contribution on Dim4 was related with physicochemical variables (43.38%). However in Dim4 only the correlated CFU analysed on DG–18 agar (r=0.39) was statistically significant.

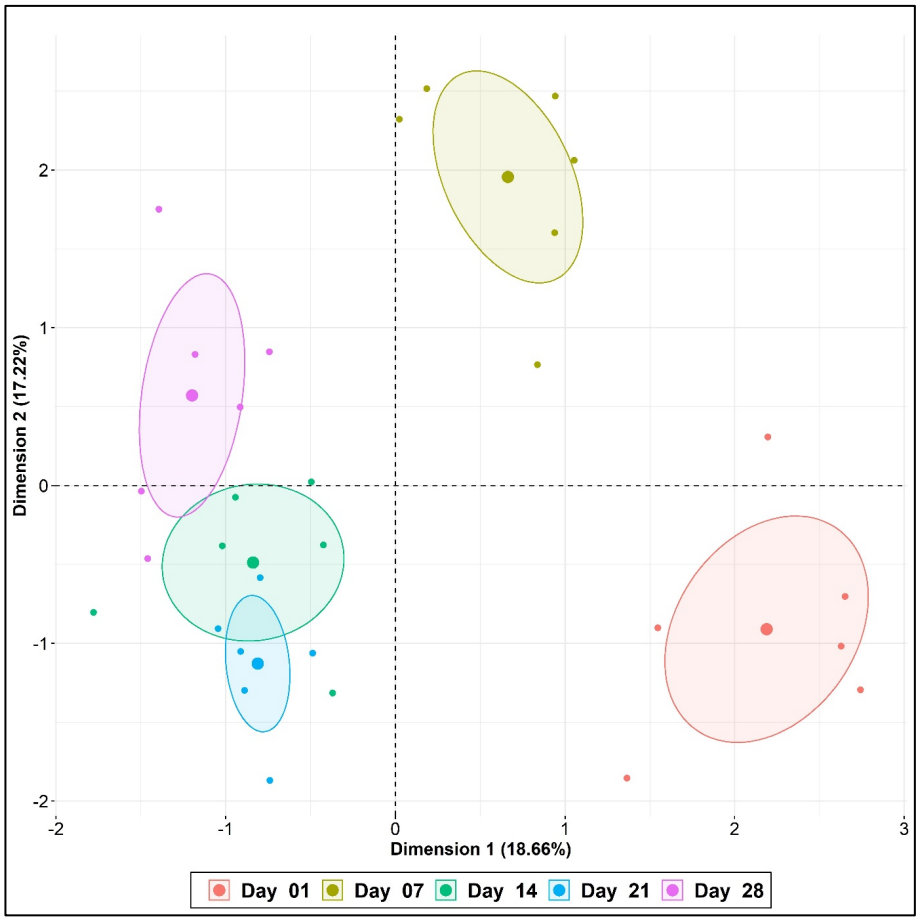

Figure 1. Plot of individuals in the first and second extracted dimensions during egg storage period.

From the results obtained by the MFA method, it can be concluded that the effect of storage conditions on experimental eggs was significant. The MFA method showed differences between experimental egg groups during the storage period. Analyzed variables of experimental eggs were more similar to each other on Days 14, 21 and 28 than on other days during the storage period. On experimental days 1 and 7, the eggs were significantly different from each other as well as from experimental eggs analyzed on Days 14, 21 and 28 in all monitored variables.

3.2. Isolation of fungal DNA

To overcome the poor diagnostic sensitivities and long turnaround times associated with the detection and identification of fungal pathogens in samples by cultivation, non-cultivation methods including the polymerase chain reaction (PCR) are increasingly being used for exact confirmation and more accurate identification of micromycetes. The

ultimate sensitivity of any PCR assay for the detection of fungal pathogens depends on the efficient lysis of fungal cells in the tissue sample and the purification of DNA that is free of PCR inhibitors. Fungi have cell walls that impede lysis and the recovery of nucleic acids. Furthermore, highly sensitive and specific nucleic acid-based methods for the detection of fungi necessitate the use of DNA extraction reagents that are free of contaminating fungal nucleic acids (FREDRICKS *et al.*, 2005).

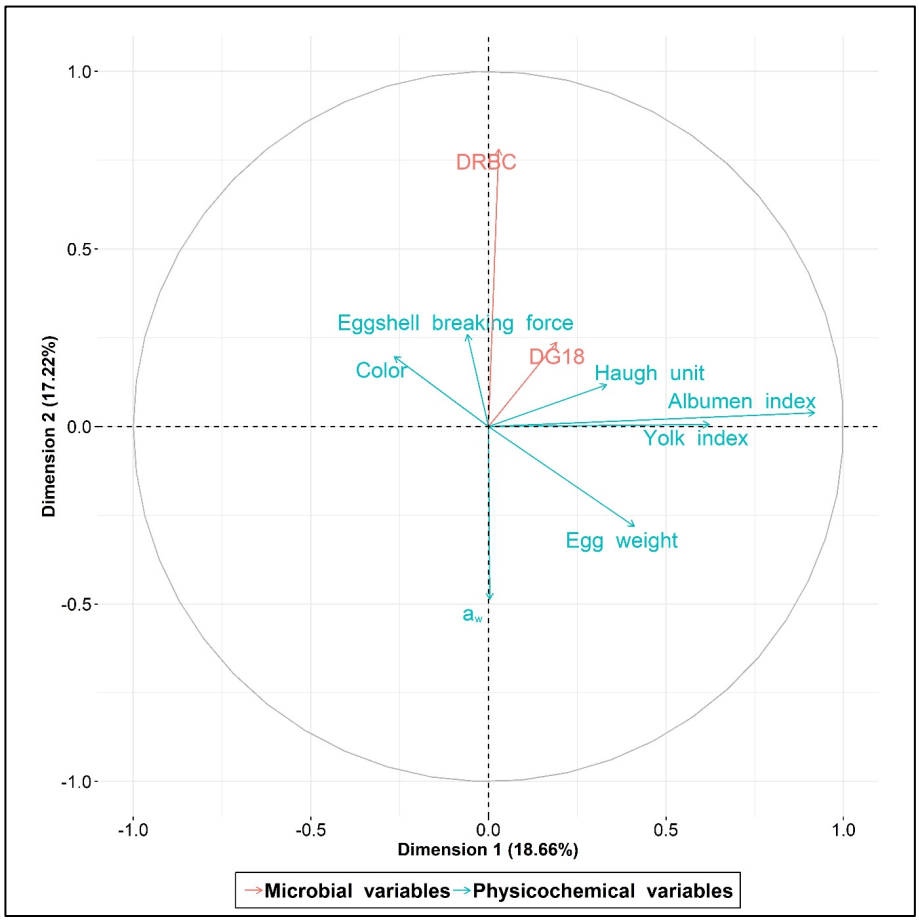

Figure 2. Correlation plot of variables in the first and second extracted dimensions during egg storage period.

In the case of the direct detection of molds in food samples (especially eggshell), DNA yield and purity depends primarily on the quantity and quality of the material taken. To isolate fungal DNA, it is first necessary to effectively disrupt the cell wall, lyse the cytoplasm and nucleus membrane, precipitate the proteins and remove the DNA from a number of inhibitors that can reduce the effectiveness of PCR. The individual steps can be executed using chemicals by known methodological procedures, or using commercial kits to facilitate isolation. In this case, however, it may be a problem to optimize the methodological process, because some chemicals in diagnostic kits are subject to corporate secrecy (ČMOKOVÁ *et al.*, 2014). The cell walls can be disrupted by homogenising of frozen sample in mortar using liquid nitrogen (GARG *et al.*, 2009; UCHIDA *et al.*, 2009), by

vortexing with glass or zircon beads (EBIHARA *et al*., 2009; SATO *et al*., 2010), or by repeated freezing (LITZ and CAVAGNOLO, 2010).
Membrane lysis is made with detergents (e.g. Triton X-100, sodium laurysulfate) followed by nucleic acids release into a buffered solution which contains chelators, most commonly EDTA and bonding calcium cations serving as a nucleases cofactor to prevent cleavage of the released DNA. To increase the nucleic acid purity, proteinase K is sometimes added into the lysis solution to cleave proteins including DNA-bound histones. A commonly used method is the incubation with proteinase K and subsequent completion of isolation using a commercial kit (BERGMANS *et al*., 2010; ALEXANDER *et al*., 2011; BEIFUSS *et al*., 2011; WISSELINK *et al*., 2011). In this study, three isolation procedures of fungal DNA were used and compared: 1. Combination of liquid nitrogen and heat 95°C; 2. Proteinase K and FG1 lysis solution; and 3. Zircon and glass bead isolation with simultaneous effects of proteinase K and ultrasonication of 50 Hz. Measurements of DNA concentration (Table 4) provided useful information on which of the three test procedures is the most effective for cell wall lysis and DNA extraction. DNA samples with the highest concentration and purity were used for further analysis. After PCR identification, the effectiveness of isolation procedures in relation to individual mold genera was re-evaluated.

Table 4. Comparison of DNA concentrations (ng/μL) yielded by three extraction procedures (average±SD).

	Procedure 1	Procedure 2	Procedure 3	One-Way ANOVA *P* value
Penicillium spp.	36.372±17.272^{C}	49.146±26.657^{B}	97.669±12.225^{A}	<0.001
Cladosporium spp.	12.462±4.807^{B}	35.585±11.433^{A}	38.175±20.410^{A}	<0.001
Fusarium spp.	22.335±9.134^{C}	64.222±18.859^{B}	71.554±17.674^{A}	<0.001
Alternaria alternata group	10.867±0.103^{C}	29.017±0.618^{B}	49.650±0.812^{A}	<0.001

$^{A-C}$ Different superscripts in each row indicate significant differences between the mean values (Tukey´s, $p<0.05$).

As can be seen in Table 4, the lowest average values for all identified genes were obtained by isolation procedure using a combination of thermal shock and liquid nitrogen. Among four mold genera, this procedure was most effective for *Penicilium* spp. isolates, where the average DNA concentration achieved a value of (36.372±17.272 ng/μL). The first procedure proved to be the worst for isolates of the genus *Cladosporium*. The second isolation procedure involving the use of proteinase K and a lysing solution FG1 appeared to be sufficient for isolates of the genus *Fusarium* (average DNA concentration was 64.222±18.859 ng/μL). The third method of isolation with a combination of zirconium and glass beads, proteinase K and ultrasonic waves, appeared to be the most effective for all four mold genera tested (Figs. 3-5). However, the highest average DNA concentration was recorded in *Penicillium* spp. (97.669±12.225 ng/μL). The last procedure was also sufficient for *Cladosporium* spp. (38.175±20.410 ng/μL) where the other isolation methods did not yield satisfactory results. In general, the purity of DNA obtained by all the three isolation procedures was very high (1.75-1.91).

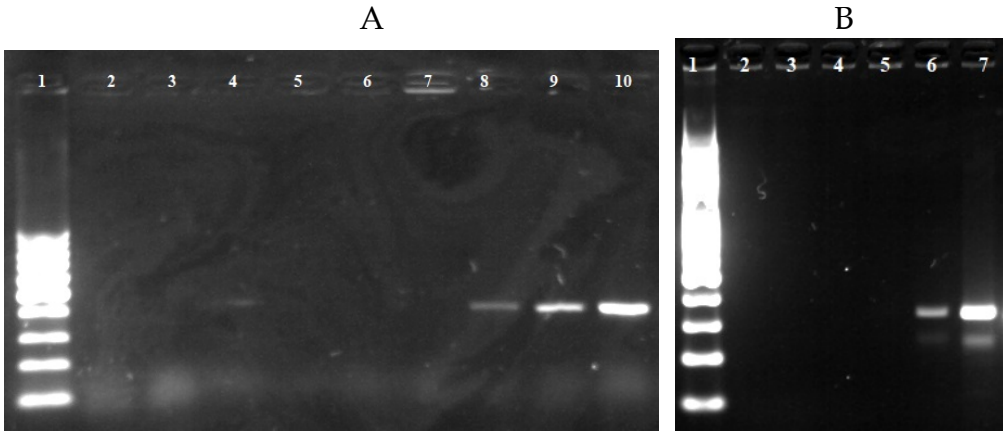

Figure 3. Identification of *Penicillium* spp. (A) and *Fusarium* spp. (B) - comparison of three DNA isolation procedures (1 - 3).
Figure A: Line 1 - 100 bp ladder; Lines 4,7,10 - reference strain *Penicillium chrysogenum* CCM F-362; Lines 2,3,5,6,8,9 - isolates of *Penicillium* spp. (336 bp). Lines 2,3,4 - Procedure 1; Lines 5,6,7 - Procedure 2; Lines 8,9,10 - Procedure 3.
Figure B: Line 1 - 100 bp ladder; Lines 2,4,6 - isolates of *Fusarium* spp.; Lines 3,5,7 - reference strain *Fusarium sporotrichioides* CCM F-352 (410 bp). Lines 2,3 - Procedure 1; Lines 4,5 - Procedure 2; Lines 6,7 - Procedure 3.

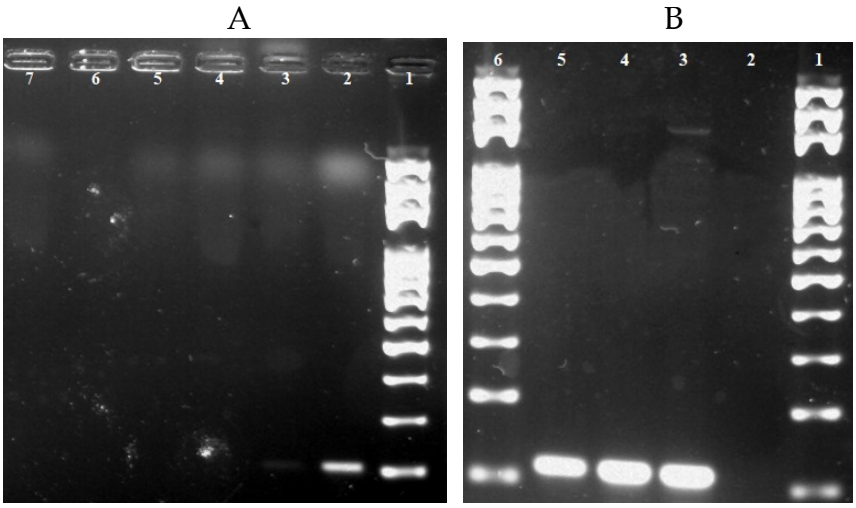

Figure 4. Identification of *Alternaria alternata* - comparison of three DNA isolation procedures (1 - 3).
Figure A: Line 1 - 100 bp ladder; Lines 2,5 - reference strain *Alternaria alternata* CCM F-397; Lines 3,4,6,7 - isolates of *Alternaria alternata* (118 bp). Lines 2,3,4 - Procedure 2; Lines 5,6,7 - Procedure 1.
Figure B: Line 1 - 100 bp ladder; Line 2 - negative control; Lines 3,4,5 - isolates of *Alternaria alternata*; Line 5 - reference strain *Alternaria alternata* CCM F-397 (118 bp). Lines 3,4,5 -Procedure 3.

Statistically significant differences (p<0.001) in the DNA concentrations obtained by three isolation procedures were observed in *Penicillium* spp., *Fusarium* spp. and *Alternaria alternata* group (Table 4). In *Cladosporium* spp., statistically significant difference was only recorded for the first isolation procedure where the minimum average concentration of DNA was obtained. The effectiveness of fungal DNA isolation using a combination of mechanical and chemical actions was also confirmed by LIU *et al*. (2000). However, other authors have reported various methods of cell wall destruction. In the most common

method, fungal mycelium is milled with liquid nitrogen or glass beads (LEE *et al*., 1988, WU *et al*., 2001). Some researchers also used dry ice (GRIFFIN *et al*., 2002), glass or magnetic beads (FAGGI *et al*., 2005), enzymatic cleavage (LI *et al*., 2002), or benzyl chloride (XUE *et al*., 2006). Although these techniques generally provide DNA of satisfactory quantity and quality, the greater problem arises with the *Cladosporium* DNA.
Melanized cell walls contain complex of polysaccharides and various secondary metabolites, including complex phenolic compounds, which hamper successful isolation of DNA (ADAMS *et al*., 1994). Due to the complexity of the fungal cell wall, conventional methods are often not appropriate for DNA extraction (KARAKOUSIS *et al*., 2006) and, compared to mammalian cells (WONG *et al*., 2007), the use of rigorous techniques is required (YANO *et al*., 2003).

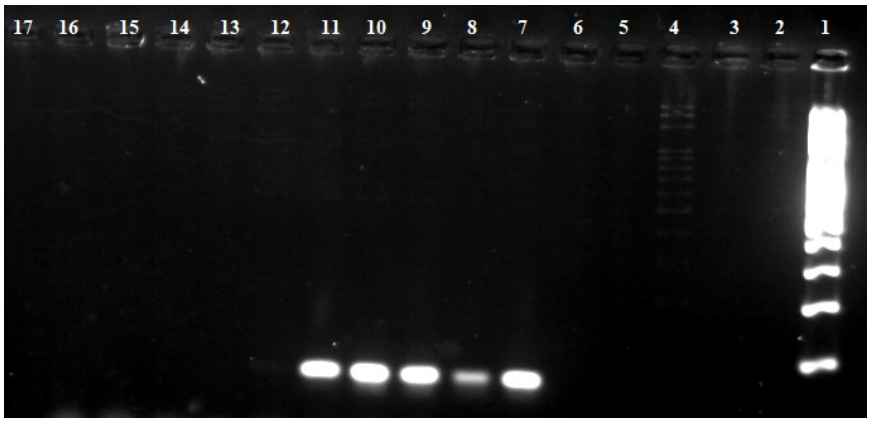

Figure 5. Isolation of *Cladosporium* spp. - comparison of three DNA isolation procedures (1 - 3).
Line 1 - 100 bp ladder; Lines 2,3,4,5,7,8,9,10,12,13,14,15,16 - isolates of *Cladosporium* spp.; Lines 6,11,17 - reference strain *Cladosporium cladosporioides* CCM F-348 (87 bp). Lines 2,3,4,5,6 - Procedure 1; Lines 7,8,9,10,11 - Procedure 3; Lines 12,13,14,15,16 - Procedure 2.

In this study, the combination of glass and zircon beads, proteinase K, ultrasound, and a commercially distributed insulating kit, yielded DNA in sufficient concentration and quality to identify the genus *Cladosporium* by PCR method. The PCR products obtained in this study were further separated by agarose gel electrophoresis. This procedure verified the DNA integrity required for reliable identification of fungal isolates at the genus level by the PCR method.

3.3. Identification of micromycetes

Identification of fungal isolates from table eggs was performed with the help of PCR method. The main advantage of this method lies in its high sensitivity and detection rate (MEHLIG *et al*., 2014) ranging from several hours to 2-3 days. Methods that allow the detection of genus without futher specification (pan-dermatophyte PCR) are focused on conservative DNA segments (KANO *et al*., 2003). Most current methods used to identify major fungal species are targeted at the ribosomal DNA domains (including 18S, ITS1, 5,8S, ITS2 and 28S). Specifically, the ITS region provides sufficient differentation among micromycete species. Methods based on classical PCR are most frequently used to detect fungal genus as a whole without more precise determination (BRILLOWSKA *et al*., 2007,

2010a,b; KONDORI *et al*., 2010; KIM *et al*., 2011). By this method, 30 isolates of *Fusarium* spp. (Fig. 6A) and 27 isolates of *Penicillium* spp. (Fig. 6B) were identified in this study, where the ITS region and 5.8S rRNA were used as the target site.

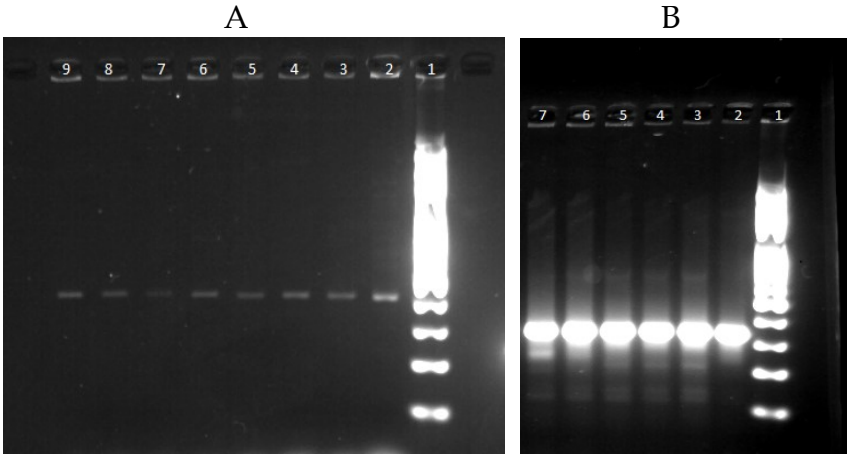

Figure 6. Identification of *Fusarium* spp. (A) and *Penicilium* spp. (B) by PCR method.
Figure A: Line 1 - 100 bp ladder; Lines 2,3,4,5,6,7,8,9 - isolates of *Fusarium* spp. (410 bp). Figure B: Line 1 - 100 bp ladder; Lines 2,3,4,5,6,7 - isolates of *Penicillium* spp. (336 bp).

Unlike classical PCR, multiplex PCR offers identification of major mold species (LI *et al*., 2002). Multiplex PCR was used to identify the prevalent species within *Alternaria alternata* group and *Cladosporium* spp. (Figs. 7A and 7B). *Alt a 1* gene sequence was selected as the target site for identification of *Alternaria alternata* group. Strains of *Cladosporium* spp. (35 isolates) were identified by specific sequence in mitochondrial (mt) small subunit rRNA (ZENG *et al*., 2006).

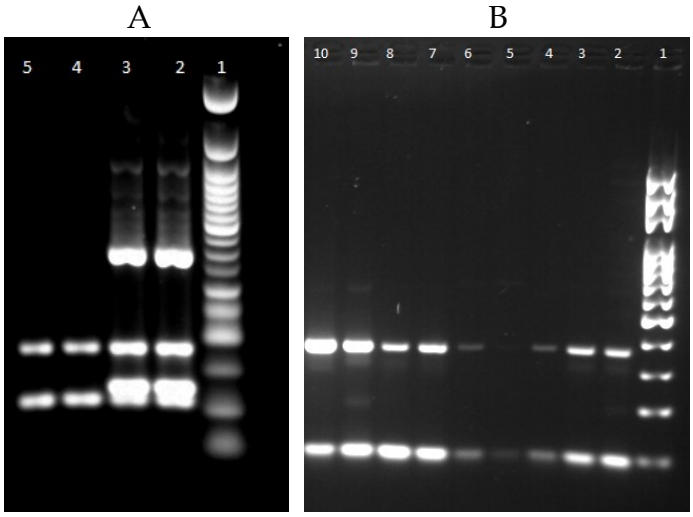

Figure 7. Identification of *Alternaria alternata* group (A) and *Cladosporium* spp. (B) by mutliplex PCR method.
Figure A: Line 1 - 50 bp ladder; Lines 2,3 - isolates of *Alternaria alternata* group (400 bp, 195 bp, 118 bp), Lines 4,5 - unidentified isolates.
Figure B: Line 1 - 100 bp ladder; Lines 2,3,4,5,6,7,8,9,10 - isolates of *Cladosporium* spp. (370 bp, 87 bp).

The PCR products obtained in this study were further sequenced and the homologues of the amplified DNA sequences were searched for in the GenBank-EMBL database. PCR genus identification correlated perfectly with the results of both macroscopic and microscopic identifications. Based on these results, fungal isolates from the eggshells were identified as *Cladosporium* spp. (35 isolates), *Fusarium* spp. (30 isolates), *Penicilium* spp. (27 isolates), and *Alternaria alternata* group (1 isolate). With the exception of *Cladosporium* spp., the remaining three fungal genera are potentially toxinogenic common pathogens of food crops. Their increasing occurrence in table eggs was reported by NEAMATALLAH *et. al*. (2009). The authors found that 38% of the examined eggs were contaminated by potentially toxinogenic micromycetes of the genus *Aspergillus* (14%), *Penicillium* (9%), *Fusarium* (1%), *Mucor* (6%), *Rhizopus* (4%) and *Cladosporium* (5%). GILARDI *et al*. (2015) confirmed the presence of the same species on the eggshell as found in this study (*Alternaria alternata* group), which was manifested as black spots on the inner shell membrane. Based on these findings, there is a high probability that the contaminating toxinogenic micromycetes on the eggshell may serve as a potential source of mycotoxins found in the egg contents. This theory has already been confirmed by EL MALT (2015).
Mycological analysis of stored eggs also indicated the presence of micromycetes on the shell surface in other studies. In Nigeria, micromycetes of the genus *Penicillium* were found in 82.5% of the examined egg samples (OBI and IGBOKWE, 2009). GRECO *et al*. (2014) reported that *Penicillium* is the second most common genus of microscopic filamentous fungi that contributes to the contamination of eggs in Buenos Aires, La Pampa and the province of Rio Negro. This genus was also placed at the forefront by EL MALT (2015). The prevalence of *Penicillium* spp. has also been demonstrated in this study.
Problems with the occurrence of microscopic filamentous fungi in stored eggs concern the entire world, but especially African countries which account for around 5% of the world egg production (MOTTET and TEMPIO, 2017). In these countries, environmental conditions are combined with very poor hygiene, resulting in the survival and proliferation of microorganisms (SALIHU, 2015).

4. CONCLUSION

The results of this study confirmed that storage period has significant impact on egg quality. Therefore, commercial freshness of table eggs can be extended by maintaining appropriate storage period and adequate storage conditions. In this study, changes in egg quality and the counts of micromycetes were observed during egg storage at an average temperature of 14.3°C and a relative humidity of 61%. The composition of eggshell mycoflora indicates a risk araising from the presence of potentially toxinogenic micromycetes belonging to *Fusarium* spp., *Penicillium* spp. and *Alternaria alternata* group. Rapid and reliable identification of mold genera by specific PCR methods requires high quality and purity of fungal DNA. For that purpose, a new effective method of DNA extraction from fungal cells based on the combination of a commercial isolation kit, proteinase K and ultrasound was designed and implemented in this study.

ACKNOWLEDGEMENTS

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