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Original scientific paper

STUDY OF NATURAL ADDITIVES' INFLUENCE ON THE MICROBIOLOGICAL STATUS OF CEREALS

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A b s t r a c t: The antioxidant, antibacterial and antifungal effects of the natural plant additives such as turmeric powder, dry extract of geranium *(Geranium sanguineum)* and oregano oil on the contents of bacteria and moulds as well as the changes of pH and peroxide values in maize, wheat, barley and triticale during storage were studied. These additives have in a different extent, an inhibitory influence on the bacteria and moulds in the cereals chosen for the study, which depends on their composition and micro-organisms' strain activity. Their influence is directed mainly to the representatives of *Aspergillus* and *Penicillinum* species, whereas they have almost no influence on those of the genus *Fusarium*. Oregano oil achieved the highest antibacterial and antifungal effect. Turmeric powder showed a pronounced antifungal effect in wheat, barley and triticale. Geranium's dry extract had a strong antibacterial effect, at the same time achieving only a weak antifungal effect. On 58th day and 136th day after the addition of these herbal additives, pH values were higher than those in untreated grains. The highest pH value was measured in the grains treated with oregano oil. All the pH values measured are within the normal range for maize and cereals, showing values between 5.41 and 6.44. Peroxide values obtained in the whole period of the trial don't exceed 8 mEq O_2/kg. These values are lower in the treated cultures than in the untreated ones.

Key words: turmeric powder; *Geranium sanguineum*; oregano oil; cereals; mycological status

СТУДИЈА ЗА ВЛИЈАНИЕТО НА ПРИРОДНИТЕ АДИТИВИ ВРЗ МИКРОБИОЛОШКИОТ СТАТУС НА ЖИТАРКИТЕ

А п с т р а к т: Испитувани се антиоксидантните, антибактериските и антифунгалните ефекти на природни растителни адитиви (куркума во прав, сув екстракт од гераниум (*Geranium sanguineum*) и масло од оригано) врз содржината на бактерии и мувли, како и промените на вредностите на pH и на пероксидот кај пченката, пченицата, јачменот и тритикалето за време на складирање. Овие адитиви во различна мера имаат инхибиторно влијание врз бактериите и мувлите во житарките избрани за студијата во зависност од нивниот состав и активноста на микроорганизмите. Тие влијаат главно врз претставниците на видовите *Aspergillus* и *Penicillinum*, додека речиси и не влијаат на оние од родот *Fusarium*. Маслото од оригано го постигна највисокиот антибактериски и антифунгален ефект. Правот од куркума покажа изразит антифунгален ефект кај пченицата, јачменот и тритикалето. Сувиот екстракт на гераниум имаше силен антибактериски ефект, истовремено постигнувајќи само слаб антифунгален ефект. На 58-иот и 136-иот ден по додавањето на овие растителни адитиви, вредностите на pH беа повисоки од оние во нетретираните зрна. Највисоката pH-вредност беше измерена во зрната третирани со масло од оригано. Сите измерени pH-вредности беа во рамките на нормалниот опсег кај пченката и житните култури, покажувајќи вредности помеѓу 5,41 и 6,44. Пероксидните вредности добиени во целиот период на испитувањето не надминуваа 8 mEq O_2 / kg. Овие вредности се пониски кај третираните отколку кај нетретираните култури.

Клучни зборови: прав од куркума; *Geranium sanguineum*; масло од оригано; житарки; микробиолошки статус

INTRODUCTION

Proper storage of cereals is a prerequisite for the production of good-quality compound feeds. Grain is a natural environment for the development of various micro-organisms – bacteria and mould fungi which, if the grain is not properly stored, proliferate rapidly causing changes in its chemical composition and nutritional value (Adams, 2008) as well as possible accumulation of mycotoxins (Tankov, 2000). The main species of mould fungi having an important role in the microbial decomposition of the raw materials, are those of the genera *Aspergilus*, *Penicilium*, *Fusarium*, *Mucor*, *Cladosporium*, *Alternaria* (Chang-Yen et al., 1992; Valcheva & Grigorova, 2005). Treatment of grain for mould fungi growth's limitation and minimization mycotoxins' negative effects, can be made by chemical, physical and biological methods.

Physical methods are: heat treatment, cleansing, separation, technological treatments, γ radiation and UV irradiation.

During chemical treatments all kinds of chemical compounds are used: bentonite, zeolite, organic acids (formic, propionic, lactic, fumaric, citric), inorganic acids (phosphoric), H_2SO_4, H_2O_2, $NaHCO_3$, NH_4OH or gaseous NH_3, which have the capacity to destroy or deactivate the mycotoxins (Valcheva, 2005). However, the feed manufacturers more and more often avoid the use of certain chemicals, such as ammonia, which despite their strong antimycotic effect are potentially dangerous for the human health and pollute the environment.

The latest generation of examined and implemented in the practice methods (biological) for deactivation of the mycotoxins have been elaborated on the basis of various enzymes, live yeast cultures, micro-organisms of the digestive tract, glucomannans, and other (Lizarraga et al., 2013, Valcheva, 2005). Such product from a completely new generation is Mtox+ created on the basis of nanotechnologies and containing nanoclays, extracts of aquatic plants and *Saccharomyces cerevisiae* (www.agroyug.ru/htmledit/download/ 09.pdf).

Concerns about possible residuals of chemical fungicidal and bactericidal preparations in animal products like meat, milk and eggs have caused great caution in their use in feed for livestock and poultry (Rahimi et al., 2011). For this reason, the people engaged in the field of feed industry have united their efforts in the search for natural additives having antimicrobial action, as an alternative to the synthetic products. In this direction a subject of interest are herbs, spices and ethereal oils. They are natural feed and food additives, containing biologically active substances (BAS) with multiple action, also they are not toxic and enjoy public approval (Abadjieva & Kistanova, 2011; Grigorova, 2014; Wenk, 2002). A lot of tests are described in the literature, where herbal essential oils with pronounced antibacterial and antifungal effects have been used. However, this approach needs further studies.

The objective of the current research is to examine the influence of natural additives turmeric powder, dry extract of *Geranium sanguineum* and oregano oil on the contents of mould fungi spores and bacteria, as well as on pH and the peroxide values in maize, wheat, barley and triticale during storage.

MATERIALS AND METHODS

In this study, the following natural additives have been used:

– Turmeric powder (origin – India). It contains 3.5% curcumin (the main biologically active substance, to which the antibacterial, antifungal and antioxidant action of turmeric is owed);

– Dry extract of geranium (*Geranium sanguineum*), a product of the company Vemo 99 Ltd, Sofia, Bulgaria. The extract contains the polyphenols catechin (22.5%–27.5%) and tannin (25%), as well as 0.3% of antocyanides (water-soluble pigments), which are its BASs;

– Oregano oil, a product of the company Kandimin, Finland. It contains 80% carvacrol, the main active substance of this additive.

Representative mean samples were taken from each of the tested cereals (maize, wheat, barley, triticale) harvested in 2013 and kept in the grain store in the Institute of Animal Science – Kostinbrod, Bulgaria. Six replicates (300 g each) from each of the studied grain cultures were made for each of the examined naturel additives (turmeric powder, geranium and oregano oil) and for control group (without additives). All the samples were put in glass jars and homogenized well. Then in the experimental samples were added 2% turmeric powder, 1% geranium dry extract and 0.6% oregano oil. All the samples were kept at room temperature. The changes were checked for on the 58^{th} and 136^{th} day of the trial.

For determination of total viable count and aerobic spore forming bacteria, 10 g of samples were put in 90 ml sterile physiological solution. Decimal

dilutions were made also by using sterile physiological solution. Total viable counts were analyzed by using Plate count agar after incubation on 30˚C for 48 h. The determination of the number of aerobic spore-forming bacteria was made by pipetting 2×10 ml of basic food solution into sterile tubes. The tubes were then heat in hot water bath (81˚C). After that tubes with basic food solution were cooled and the further decimal dilutions were made. The Plate count agar was used for counting the spore-forming bacteria after incubation on 30˚C for 48 h.

For determination of moulds 10 g of samples were put in 90 ml sterile physiological solution and the further decimal dilutions were performed. Czapek Dox agar was inoculated with the sample and incubated in a thermostat at the temperature of 25°C, in a period of 5–7 days (Stankushev et al., 1971). The developing colonies were counted.

For detection of the moulds that may develop inside the examined grain samples the following procedure was performed: 100 seeds of subsequent seed samples were disinfected with concentrated solution of sodium hypochlorite and washed three times with sterile water. 25 of disinfected seeds were put into Petri dishes filed with Czapek Dox agar and incubated at the temperature of 25°C, in a period of 5–7 days. The number of seeds grown with moulds is count and the results were presented in percents.

At the beginning of the trial the following parameters of all cereals samples were determined: pH values (using pH-meter Stirrer, Type OP-95); total lipid content (TLC) by the method of Bligh and Dyer (1959); peroxide values according to Bulgarian State Standard EN 3960/2001. These indicators were also determined on days 58th and 136th after the addition of 2% of turmeric powder, 1% of Geraniums dry extract and 0.6% of oregano oil.

The results obtained were statistically processed by Excel 2000, single factor, ANOVA program. The results are presented as means with their standard errors.

RESULTS AND DISCUSSION

In Table 1 are presented the changes in the number of bacteria and moulds, occurred in the tested samples containing maize, wheat, barley and triticale with the addition of turmeric powder, dry extract of *Geranium sanguineum* and oregano oil.

Table 1

Changes in number of bacteria and moulds in maize, wheat, barley and triticale grains before disinfection, number per g (X ± SE)

Additives / Items	Control n = 6	Turmeric n = 6	Geranium n = 6	Oregano oil n = 6
Maize				
Total bacterial count	139500±219.09	32500±196.07***	14000±76.38***	21500±102.4***
Spore forming bacteria	68500±60.09	7500±48.30***	13500±65.83***	20500±65.83***
Moulds	17000±53.23	45500±48.16	72000±285.19	1500±100.82***
Wheat				
Total bacterial count	40000±306.40	55500±152.75	20000±57.74***	2000±48.30***
Spore forming bacteria	7500±88.50	51500±100.06	16500±53.87	2000±48.30***
Moulds	500±48.30	500±38.73	500±38.73	–
Barley				
Total bacterial count	37000±246.31	74500±263.31	66500±264.57	5000±123.15***
Spore forming bacteria	8000±77.45	35500±189.30	17500±78.05	3000±54.10***
Moulds	8000±87.97	2500±99.16	1500±64.54***	1500±60.52***
Triticale				
Total bacterial count	39000±278.80	48000±356.83	3500±188.57***	2500±139.40***
Spore forming bacteria	3500±169.31	3000±139.04	1500±96.61***	500±43.60***
Moulds	4500±146.06	3000±152.75*	2000±140.20**	–

Significant in each row: * = P < 0.05; ** = P < 0.01; *** = P < 0.001

Turmeric powder suppresses significantly ($P < 0.001$) the development of the bacteria disposed in the maize (the total number of bacteria dropped four-fold – from 139.500 to 32.500 units/g). Geranium reduces the amount of bacteria in maize approximately 10 times ($P < 0.001$), in wheat 2 times ($P < 0.001$) and in triticale 11 times ($P < 0.001$). Oregano oil significantly ($P < 0.001$) reduces the number of both sporogenic and moulds in all the examined samples. The moulds in wheat and triticale grains were completely destroyed.

In Table 2 are presented the changes in the mould contents in the grain cultures treated with turmeric, *Geranium sanguineum* dry extract and oregano oil, after treatment with sodium hypochlorite.

In the experiment on antifungal effect of the turmeric in the maize was registered, while in the barley was registered a complete liquidation of the fungi of the genus *Aspergillus* and a huge reduction of those of the genus *Penicillium* (reduced from 40% to 1%). In barley, the turmeric reduces (from 78% to 15%) the quantity of moulds of the genus *Aspergillus* and of the genus *Penicillium* (from 56% to 6%). In triticale, the turmeric reduces (from 56% to 6%) the quantity of mould fungi of the genus *Aspergillus*, while in a much lesser extent of those of the genus *Penicillium* (from 12% to 6%).

The dry extract of *Geranium sanguineum* has a weaker antifungal effect. Namely, in maize it caused a reduction of the quantity of moulds of the genus *Penicillium* from 72% to 36%. In wheat, the quantity of moulds of the genus *Aspergillus* decreased from 20% to 9%, and of those of the genus *Penicillium* decreased from 40% to 1%. The geranium extract reduced the quantity of mould fungi of the genus *Aspergillus* from 80% to 44% in barley, and from 12% to 4% in wheat.

In our study, the oregano oil showed the strongest antibacterial and antifungal effect. As a consequence, in maize the moulds of the genus *Aspergillus* were completely eliminated, and those of the genus *Penicillium* were significantly reduced, from 72% to 3%. In wheat, the members of the genera *Aspergillus* and *Penicillium* were completely eliminated. In barley, the amount of moulds decreased considerably for both *Aspergillus* and *Penicillium* species (from 78% to 9% for the former, and from 56% to 2% for the latter). In triticale, the amount of *Aspergillus* genera decreased from 80% to 2%, and of *Penicillium genera* – from 12% to 4%. Similar results were obtained by Ibrahim et al. (2012); Shimoni et al. (1993). According to Rasha et al. (1995), oregano oil has inhibiting action not only on the members of *Aspergillus* and *Penicillium* genera, but also of those of the genera *Alternaria*, *Cladosporium*, *Helmintosporium*, *Mucor* and *Rhisopus*. The natural additives examined in our study don't have any inhibitory action only on the members of the genus *Fusarium.*

Table 2

Changes in the content of moulds after treatment of maize, wheat, barley and triticale grains, %

Moulds		Maize				Wheat				Barley				Triticale			
		C	T	GS	O	C	T	GS	O	C	T	GS	O	C	T	GS	O
Fusarium	•	2	2	3	3	8	14	18	28	3	5	5	5	10	18	16	13
	••	6	6	2	3	18	24	26	10	–	3	2	2	11	15	14	7
Aspergillus	•	8	100	117	37	22	38	41	–	73	19	42	13	87	18	53	3
	••	8	8	75	4•••	20	–	9	–	78	15	50	9	80	10	44	2
Penicillium	•	33	17	20	2	45	28	6	–	50	5	25	4	15	5	2	1
	••	72	75	36	3	40	1	1	–	56	6	52	2	12	6	3	4
Mucor	•	51	90	75	–	36	43	25	–	22	13	28	15	18	28	30	3
	••	52	44	56	6	40	35	17	10	30	–	16	11	16	5	20	12
Rhizopulus	•	–	70	70	–	–	10	5	–								
	••	3	27	35	–	5	–	–	–								
Alternaria	•					7	19	18	5	–	3	5	–	–	10	6	–
	••					11	15	22	–	2	2	7	–	–	11	10	–
Cladosporium	•					19	13	21	5	5	7	9	–	47	27	13	6
	••					21	9	26	–	3	2	9	–	36	13	11	2

C = Control, T = Turmeric, GS = *Geranium sanguineum*, O = Oregano oil;
• = registered on day 58, •• = registered on day 136, ••• = heavily suppressed, without micelle

The results obtained in our study allow us to draw a conclusion that the natural additives' antibacterial and antifungal action depends in the first place on their composition, as well as on the species of the cereals they are implemented in and the strain specifics of the particular micro-organism they act on.

Simultaneously with the microbiological examinations, the available moisture in both the treated and untreated cultures was also followed up. Namely, the available moisture decreased insignificantly ($P > 0.05$) in all the cultures (from 14.5% to 11% in maize, from 12.65% to 11.64% in wheat, from 11.63% to 10.64% in barley and from 13.02% to 11.63% in triticale), therefore this decrease was not a cause for the microbiological changes that occurred.

In Table 3 are given total lipid contents, peroxide values and pH values in the examined cereals, before and after their treatment with the tested natural additives. Concerning TLC there are not significant differences ($P > 0.05$) between control and treated cereals. The peroxide value is an indicator for the presence of peroxides which are a product of the initial oxidizing processes in the fats. If the peroxide value is above 20 mEq O_2/kg, the fats are oxidized, while if it is 10 mEq O_2/kg or lower there are no substantial changes in the organoleptic properties of the fats. Since the grain cultures used in our study are not rich in fats, peroxide values obtained in the course of the experiment didn't exceed 8 mEq O_2/kg, being lower than in the untreated grain cultures.

Table 3

Total lipid content (TLC), peroxide values (PV) and P values of maize, wheat, barley, triticale samples at the beginning of the trial without antioxidant ingredients; at 58th day and 136th day after addition of the tested additives (X ± SE) (n=6)

Periods items	At the beginning of the trial			At 58th day			At 136th day		
Grain culture +, – additive	TLC g/100 g	PV mEq O_2/kg	pH	TLC g/100 g.	PV mEq O_2/ kg	pH	TLC g/100 g	PV mEq O_2/ kg	pH
Maize	3.27±0.009	8.91±0.013	5.65±0.027	3.25±0.01	8.57±0.014^{ABC}	5.72±0.06^{abc}	3.26±0.009	8.59±0.013^{A_2B_2C_3}	6.06±0.08
Maize + GS				3.24±0.012	1.32±0.008^{A}	5.94±0.09^{a}	3.25±0.009	2.30±0.04^{A2}	5.98±0.08
Maize + turmeric				3.26±0.01	1.55±0.01^{B}	6.05±0.1^{b}	3.26±0.013	2.35±0.06^{B2}	6.25±0.12
Maize + oregano oil					1.50±0.009^{C}	6.17±0.09^{c}	3.53±0.011	2.05±0.05^{C3}	6.10±0.06
Wheat	0.89±0.012	6.56±0.07	5.62±0.014	0.89±0.013	6.56±0.07^{A1B1C1}	5.62±0.09$^{e\ f}$	0.90 ±0.011	2.17±0.10^{A3}	6.15±0.07^{b1}
Wheat + GS				0.90.±0.012	2.42±0.04^{A1}	5.98±0.12	0.91±0.012	1.52±0.06^{A3}	6.19±.04
Wheat + turmeric				0.91±0.013	4.01±0.1^{B1}	6.23±0.07^{e}	0.93±0.014	1.98±0.08	6.44±0.07^{b1}
Wheat + oregano oil					2.60±0.11^{C1}	6.25±0.11^{f}	1.45±0.013	2.12±0.07	6.16±0.06
Barley	1.18±0.01	3.21±0.14	5.85±0.022	1.18±0.014	3.21±0.13	5.85±0.09	1.17±0.011	2.56±0.10^{e2}	6.00±0.07
Barley + GS				1.19±0.012	4.10±0.23	5.63±0.09	1.19±0.011	2.83±0.07	5.84±0.08
Barley + turmeric				1.20±0.011	3.78±0.23	5.73±0.08	1.20±0.012	3.10±0.10^{e2}	6.07±0.07
Barley + oregano oil					2.90±0.18	5.66±0.08	1.57±	2.15±0.08	5.93±0.08
Triticale	1.15±0.012	3.98±0.21	5.86±0.015	1.15±0.013	3.98±0.22	5.86±0.10^{e1C2}	1.16±0.012	2.72±0.08$^{A4\ B3}$	6.30±0.06^{f1}
Triticale + GS				1.16±0.013	4.28±0.16	6.19±0.10	1.16±0.012	2.07±0.10^{A4}	6.25±0.07
Triticale + turmeric				1.16±0.011	4.10±0.18	6.30±0.10^{e1}	1.16±0.013	1.87±0.08^{B3}	6.40±0.08
Triticale + oregano oil					3.70±0.19	6.37±0.09^{C2}	1.16±0.012	3.00±0.07	6.00±0.05^{f1}

Significant: a, b,b_1,c – $P < 0.05$; e, $e_{1\text{-}2}$, f, f_1 – $P < 0.01$; A, $A_{1\text{-}4}$, B, B_1, C, $C_{1\text{-}2}$ – $P < 0.001$

The initial pH values ranged between 5.44 in maize and 5.86 in triticale. On the 58th day of the experiment, the pH had higher values in all the treated cultures than in the untreated cultures, the highest pH value being that in the cultures treated with oregano oil. The situation was same on 136th day. All the pH values measured in the experiment are within the normal for grain cultures, ranging between 5.41 and 6.44.

CONCLUSIONS

- The natural additives turmeric powder, dry extract of *Geranium sanguineum* and oregano oil, examined in our experiment, exhibit, in a various degree, an inhibiting action on the bacteria and moulds in the grain cultures used in our study. Their action is directed mainly to the members of the genera *Aspergillus* and *Penicillium*. They have no influence on the moulds from the genus Fusarium.
- Turmeric powder suppresses significantly ($P < 0.001$) the development of the total number of bacteria in maize (their amount dropped four-fold – from 139.500 to 32.000 units/g). Geranium reduces the amount of bacteria in maize approximately 10 times ($P < 0.001$), in wheat 2 times ($P < 0.001$) and in triticale 11 times ($P < 0.001$). Oregano oil significantly ($P < 0.001$) reduces the amount of sporogenic bacteria.
- The highest antibacterial and antifungal effect is that of the oregano oil, used in our study in the concentration of 0.5 μg/ml.The turmeric powder showed a well pronounced antifungal effect in wheat, barley and triticale.
- The dry extract of *Geranium sanguineum* has a strong antibacterial and a weak antifungal effect.
- On the 58th and 136th day after the addition of the herbal additives, the pH values were higher than those in the untreated cultures, the highest pH value being the one measured in the grain cultures treated with oregano oil. All the pH values we have registered in the experiment are within the normal for maize and cereals, ranging from 5.41 to 6.44.
- The peroxide values we have obtained in the experiment don't exceed 8 mEq O_2/kg. These values are lower in the treated cultures than in the untreated ones.

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