OPEN ACCESS
Potravinarstvo® Scientific Journal for Food Industry
Crossref Similarity Check Powered by iThenticate

potravinárstvo®

Potravinarstvo, vol. 10, 2016, no. 1, p. 612-618
doi:10.5219/682
Received: 7 October 2016. Accepted: 5 December 2016.
Available online: 19 December 2016 at www.potravinarstvo.com

ISSN 1337-0960 (online)

VERIFICATION FOR THE PRESENCE OF INHIBITORY SUBSTANCES IN POULTRY MEAT AFTER THE CONSUMPTION OF THE FEED MIXTURE SUPPLEMENTED WITH FERMENTED FEED

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ABSTRACT

The European Union has an obligation to ensure that feed produced in the European Union is safe for animals and also humans by ensuring food of animal origin is safe and wholesome. An increasing demand for safe, wholesome and nutritious animal products has led to the search for alternative substances in animal feed. Fermented feed has gained a lot of popularity in many animal diets today. They meet the demand for animal nutrition due to the formation of target substances with the desired properties. As some of them are attracting attention as potential antimicrobial agents that inhibit the growth of certain microorganisms, and the products of animal origin are controlled for the presence of residues of inhibitory (antimicrobial) substances, the aim of this work was to verify the presence of inhibitory substances in poultry meat (muscle, heart, liver, kidneys of broiler chickens) after the consumption of the feed mixture with addition of fermented feed (wheat bran fermented with the strain *Umbelopsis isabellina* CCF 2412) in a dose of 10 % of the total amount of the feed. The detection of residues was performed by two approved microbiological screening methods, the screening test for the detection of antibiotic residues (STAR) and the Premi®Test. Both methods detected the positive results and pre-identified the presence of residues of the inhibitory substances not only in the meat of broiler chickens but also in the investigated fermented feed. Due to the antimicrobial potential of the fermented feed and the possible presence of the false positive results, each positive result must be confirmed by a confirmatory analysis.

Keywords: meat; feed; residues; screening

INTRODUCTION

Animal production occupies a very important place in farming in the European Union (EU) and satisfactory results in terms of public and animal health depend to a large extent on the use of appropriate good quality feedingstuffs **(Directive 2002/32/EC)**. Feed shall not be placed on the market or fed to any food-producing animal if it has an adverse effect on human or animal health and makes the food derived from food-producing animals unsafe for human consumption **(Regulation (EC) No 178/2002)**.

Action by the EU relating to public and animal health are based on the precautionary principle. One of these actions is to ban the use of antibiotics as growth promoters in food-producing animals according to **Regulation (EC) No 1831/2003** with effect from 1 January 2006. The ban of in-feed use of antibiotics has brought unintended impacts on animal production industries in the EU, such as the increase of infections in animals and the decrease of animal production **(Cheng et al., 2014)**. On the other hand, this ban has opened up a space for the development of alternative substances and alternative feeding methods to decrease animal diseases and increase the production of safe, wholesome and nutritious animal products intended for human consumption. The subject of active search for alternatives have become probiotics, prebiotics, organic acids, minerals, enzymes, herbs (plant extracts), propolis extract, phytogenic feed additives, aromatic phenolic components, or fermented feed **(Niba et al., 2009; Bobko et al. 2016; Haščík et al. 2016)**.

Fermented feed has gained a lot of popularity in animal production today for enhancing the nutrient accessibility in some feed and developing and/or improving the flavour and texture of many fermented products **(Ivey et al., 2013; Čertík et al., 2013a)**. The extensive rise of biotechnology over the past years has opened several strategies that could be applied for the formation of functional food/feed. Biotechnological approach based on solid state fermentations (SSF) is one of the most perspective techniques to enrich cereals (rice bran, wheat bran, oat flakes, peeled barley, etc.) used in animal nutrition with desired metabolites. During this process, useful microorganisms (lower filamentous fungi) grow on various cereal substrates by utilization of nutrients from these substrates. As a result, a cereal-based functional biomaterial with the demanded properties is formed. An advantage of the SSF process is that fermented materials

can be directly used for food/feed applications without any downstream process **(Čertík and Klempová, 2016)**.

One of the most valuable fungus able to grow on various cereal substrates is *Umbelopsis isabellina*. This fungal strain belongs to a group of oleaginous microorganisms able to accumulate large amount of lipids in their cells and is characterized by the simultaneous formation of both polyunsaturated fatty acids and a mixture of carotenoid pigments **(Klempová et al., 2013)**. Polyunsaturated fatty acids and carotenoid pigments are essential compounds for animal and human health. They are important modulators of a number of vital physiological processes, they have a high antioxidant activity and beneficial (protective) effects in the prevention and widespread of the modern civilization diseases **(Klempová et al., 2013; Fiedor and Burda 2014)**. However, the recent studies have shown that polyunsaturated fatty acids are attracting attention as potential antimicrobial agents due to their safety, high efficiency, wide spectrum of activity and the lack of resistance mechanisms **(Huang et al., 2010; Desbois and Lawlor 2013; Sayegh et al., 2016)**.

Due to the fact that the animal products and animal feed are carefully controlled and monitored for the presence of the residues of antimicrobial substances and their derivatives in accordance with **Council Directive 96/23/EC** and due to the formation of natural bioactive agents with potential antimicrobial activity in fermented cereals, the aim of this work was to verify the presence of inhibitory substances in poultry meat after the consumption of the commercial feed mixtures with addition of wheat bran fermented with the strain *Umbelopsis isabellina* in a dose of 10% of the total amount of the feed.

MATERIALS AND METHODS

Sample material

A total of 8 tissue samples and 3 feed samples were used for residues analysis. Tissue samples (muscle /2/, heart /2/, liver /2/, kidneys /2/) were obtained from broiler chickens (ROSS 308) from two (experimental /1/ and control /1/) groups slaughtered at the age of 37th days after elapse of the withdrawal period by legally permitted method. Feed samples were obtained from the feed supplied to broiler chickens (medicated feed mixtures BR2 containing a polyether ionophoric antibiotic salinomycin authorised according to **Commission Regulation (EC) No 167/2008** for the prevention of coccidiosis in chickens for fattening at a minimum/maximum content of active substance in complete feed 60 – 70 mg.kg^{-1} /1/ and unmedicated feed mixture BR3 /1/ both commercially produced by De Heus a.s., Czech Republic, and fermented feed /wheat bran fermented with the fungal strain *Umbelopsis isabellina* CCF 2412 produced according to **Čertík et al. (2013b)**.The strain *Umbelopsis isabellina* CCF 2412 was obtained from Culture Collection of Fungi (Charles University, Czech Republic).

Experimental animals

The feeding regime for the control group consisted of the administration of BR1, BR2 and BR3 according to the age of broiler chickens and the feeding regime for the experimental group consisted of the administration of BR1, BR2 and BR3 with the addition of fermented wheat bran in a dose of 10% of the total amount of the feed. The animals were housed in accredited premise of the Clinic for birds, exotic and free-living animals at the University of Veterinary Medicine and Pharmacy in Košice fulfilling the conditions for the preservation of the health and welfare of the animals. All broiler chickens had free access to feed and water. Tissue and feed samples were individually packed and stored in a freezer at -20 °C until the analysis.

Sample analysis

For the verification of the presence of inhibitory substances in the tissues of broiler chickens after the consumption of the feed mixture supplemented with fermented wheat bran, the screening test for the detection of antibiotic residues (STAR) **(R–25, 2006)** as the plate method and the Premi®Test **(R–26, 2006)** as the tube method both developed on the principle of inhibition of growth of the test organism by an antimicrobial substance presents in the sample were used. Both methods are officially approved for screening food-producing animals and their products for residues of antimicrobial substances in many EU member states, including Slovakia.

STAR protocol

Culture media, test strains (bacteria) and standards were purchased from Merck (Germany), Oxoid (UK), Difco (USA), Sigma-Aldrich (USA) and American Type Culture Collection (USA). Test plates (*Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 /addition of trimethoprim at a concentration of 0.005 μg.mL^{-1} agar medium/, *Bacillus subtilis* BGA, *Escherichia coli* ATCC 11303, *Kocuria rhizophila* ATCC 9341, *Bacillus cereus* ATCC 11778) were prepared according to the procedure set by the method. A cylindrical core obtained from each frozen tissue sample (muscle, heart, liver, kidneys) using a sterile cork borer (diameter 9 mm) was cut into slices of 2 mm in thickness with a sterile lancet and placed in parallel in each of five test plates. For analysis of feed samples, a clear supernatant obtained by centrifugation of 10 g of feed sample dissolved in 30 mL of sterile demineralized water was used. The clear supernatant was applied on the surface of the test plates using a paper disc (diameter 9 mm, 30 μL, Albet Lab Science, Germany). The test plates were incubated under the conditions set by the method.

Premi®Test protocol

100 μL of the juice obtained by thawing the tissue sample in a microwave oven on defrost setting and 100 μL of clear supernatant obtained by preparation of feed samples as described above in Section STAR were pipetted onto the agar in the ampoule inoculated with *Bacillus stearothermophilus* var. *calidolactis.* The ampoules were subjected to pre-incubation and further incubation according to the manufacturer's instructions (R-Biopharm AG, Germany*).*

Reading the method results

Diffusion of any antimicrobial substance from the sample into an agar medium seeded with a sensitive test organism present results in the formation of an inhibition

zone around the sample, in which the growth of the test organism is inhibited (STAR) or from a delayed or absent colour change of the agar medium due to impaired growth of the test organism (Premi®Test). The size of the inhibition zone and the colour change of the agar medium is directly proportional to the concentration of the antimicrobial substance in the sample.

After incubation of the STAR test plates, the diameters of clear inhibition zones around the tissue samples or the filter paper discs moistened with clear supernatant were measured in mm from the edge of the sample (disc) to the outer limit of the inhibition zone using a digital caliper with a precision of 0.01 mm (Mitutoyo, Japan). The samples were considered positive, if they gave the inhibition zone equal or superior to 2 mm in width on the *Bacillus subtilis* BGA, *Escherichia coli* ATCC 11303, *Kocuria rhizophila* ATCC 9341 and *Bacillus cereus* ATCC 11778 test plates, and equal or superior to 4 mm in width on the *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 test plates. To verify that the operating conditions were systematically respected, a quality control on each test plate was performed.

After incubation of the Premi®Test ampoules with *Bacillus stearothermophilus* var. *calidolactis* test strain, a colour from the bottom 2/3 part of the ampoule was read. A clear colour change from purple to yellow indicated that a sample was considered negative (the absence of antimicrobial substance above the limit of detection). No clear colour change from purple to yellow indicated that a sample was considered positive (the presence of antimicrobials above the limit of detection). The samples with the light shades of purple colour were considered dubious (the presence of antimicrobials at the limit of detection).

RESULTS AND DISCUSSION

The results of screening for the presence of inhibitory substances in the examined animal and feed matrices are presented in Table 1 and Table 2. The photos documenting the positive, dubious and negative results detected by the STAR and the Premi®Test are presented in Figure 1 and Figure 2.

As shown in Table 1 and Table 2, both microbiological screening methods detected the presence of inhibitory substances in the animal and feed matrices. Evaluating the results after the screening of tissues samples of broiler chickens from the experimental and control group by the STAR, comparable results were detected. The inhibition of the growth of the test strain was observed only in two of five test strains, namely *Kocuria rhizophila* ATCC 9341 and *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149. No inhibition zones were observed on the *Bacillus cereus* ATCC 11778, *Bacillus subtilis* BGA and *Escherichia coli* ATCC 11303 test plates. The only inhibition zone observed around the kidney sample from the experimental group was not considered positive. Muscle yielded a positive result on the *Kocuria rhizophila* ATCC 9341 test plates, heart on the *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 test plates, and liver and kidneys on both the *Kocuria rhizophila* ATCC 9341 and *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 test plates.

Evaluating the results after the screening of samples of commercially produced feed mixtures BR2 and BR3 and fermented wheat bran by the STAR, all feed samples showed inhibition on the several test plates. Medicated BR2 yielded a positive result on four *(Bacillus cereus* ATCC 11778, *Bacillus subtilis* BGA, *Escherichia coli* ATCC 11303, *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149) of the five test plates. unmedicated BR3 yielded a positive result on two (*Bacillus subtilis* BGA, *Escherichia coli* ATCC 11303) of the five test plates, and fermented wheat bran yielded a positive result on the test plates identical with examined animal matrices (the *Kocuria rhizophila* ATCC 9341 and *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 test plates).

Evaluating the results after the screening of animal tissue and feed samples by the Premi®Test with *Bacillus stearothermophilus* var. *calidolactis* test strain, only medicated BR2 and fermented wheat bran yielded the positive results.

Table 1 The results of screening for the presence of inhibitory substances in the examined matrices by using the STAR.

		STAR				
Group of broiler chickens	**Matrix / No**	***B. cereus*** IZ (mm ±SD)	***B. subtilis*** IZ (mm ±SD)	***E. coli*** IZ (mm ±SD)	***K. rhizophila*** IZ (mm ±SD)	***B. stearothermophilus*** IZ (mm ±SD)
Control	**Muscle /9/**	-	-	-	**2.13 ±0.42**	-
	Heart /10/	-	-	-	1.56 ±0.58	**7.89 ±0.77**
	Liver /11/	-	-	-	**5.00 ±0.43**	**11.57 ±0.67**
	Kidneys /12/	-	-	-	**2.35 ±0.31**	**7.93 ±0.44**
Experimental	**Muscle /5/**	-	-	-	**2.43 ±0.17**	-
	Heart /6/	-	-	-	1.00 ±0.10	**6.60 ±0.39**
	Liver /7/	-	-	-	**4.34 ±0.33**	**10.69 ±1.01**
	Kidneys /8/	-	1.53 ±0.20	-	**2.26 ±0.61**	**9.17 ±1.30**
Feed	**BR 2 /13/**	**3.30 ±0.70**	**6.33 ±2.24**	**3.05 ±0,78**	0.87 ±0.07	**6.70 ±0.77**
	BR 3 /14/	-	**3.79 ±1.52**	**2.65 ±0,44**	-	-
	FWB /15/	-	-	-	**2.40 ±0.76**	**5.98 ±0.82**

Note**:** IZ – inhibition zone**,** bold numerals indicate positive results, SD – standard deviation, FWB – fermented wheat bran.

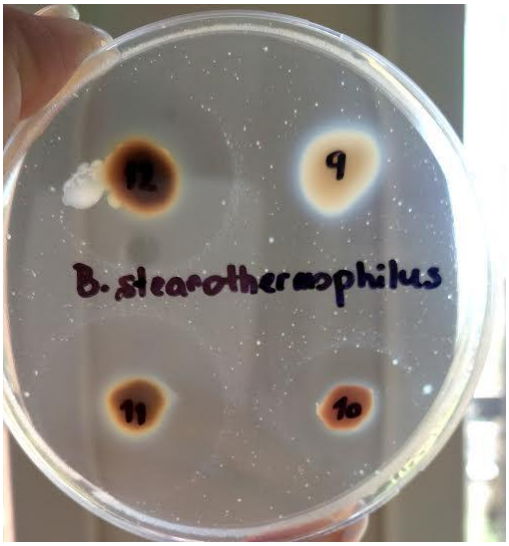

Figure 1 A visual demonstration of the results detected by the STAR.

Table 2 The results of screening for the presence of inhibitory substances in the examined matrices by using the Premi®Test.

Group of broiler chickens	Premi®Test Matrix / No	Result
Control	**Muscle /9/**	±
	Heart /10/	-
	Liver /11/	±
	Kidneys /12/	-
Experimental	**Muscle /5/**	±
	Heart /6/	±
	Liver /7/	±
	Kidneys /8/	-
Feed	**BR 2 /13/**	+
	BR 3 /14/	-
	FWB /15/	+

Note: IZ – inhibition zone, + positive results, - negative results, ± dubious results. FF – fermented wheat bran.

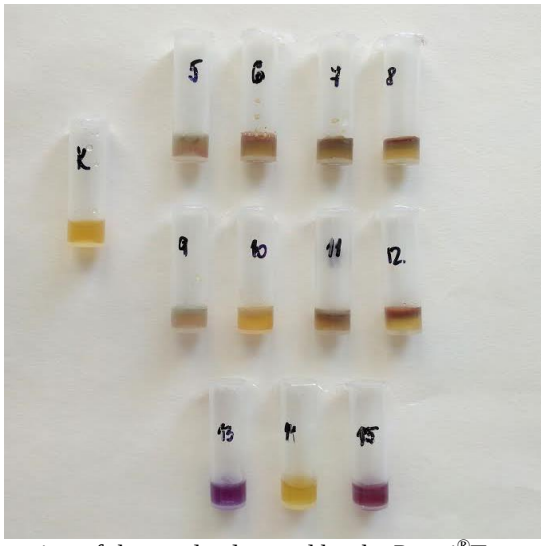

Figure 2 A visual demonstration of the results detected by the Premi®Test.

The muscle and liver from the control animals and the muscle, heart and liver from the experimental animals yielded a dubious result.

No positive result was observed in kidneys obtained from both animal groups and unmedicated BR3 feed mixture.

Summarizing the results of screening for the presence of inhibitory substances in the tissues of broiler chickens after the consumption of the feed mixture without addition of fermented wheat bran (control group) and with addition of fermented wheat bran (experimental group) we can state that both microbiological screening methods detected the positive results and pre-identified the presence of residues of the inhibitory substances not only in the meat of broiler chickens from the control group but also in the meat of broiler chickens from the experimental group. Both microbiological screening methods also revealed the presence of the inhibitory substances in wheat bran fermented with the strain *Umbelopsis isabellina*.

Microbiological methods based on the inhibition of bacterial growth by antimicrobial residues are an interesting screening step because they are capable of detecting a wide range of antimicrobial residues with one single method. They generally give results in less than 4 h (tube methods) or in 18-24 h (plate methods) **(Gaudin et al. 2008)**. The STAR which was developed at the European Union Reference Laboratory in *Fougères* (France) and the Premi®Test which was developed by DSM Gist B.V. (The Netherlands) have been most widely used in residue screening **(Gaudin et al. 2004; Stead et al., 2004; Stead et al., 2005; Gaudin et al. 2008; Kožárová et al. 2009; Pikkemaat et al. 2009; Gaudin et al. 2010; Kožárová et al., 2011; Magalhães et al., 2012, Gondová et al., 2014)**. The Premi®Test allows for the detection of broad spectrum of most relevant antimicrobial compounds simultaneously while the STAR allows for distinguishing (pre-identify) the families of antimicrobials, i.e. each test plate is sensitive to one or two families of antimicrobials. *Bacillus cereus* ATCC 11778 test plates are specific for tetracyclines, *Bacillus subtilis* BGA test plates for aminoglycosides, *Escherichia coli* ATCC 11303 test plates for quinolones, *Kocuria rhizophila* ATCC 9341 test plates for macrolides, and *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 test plates for b-lactams and sulphonamides.

According to the declared sensitivity of the respective test strain of the STAR to the respective family of antimicrobials we can assume that the positive animal samples were suspected for the presence of ß-lactams, sulphonamides and macrolides, and the positive feed samples, except medicated BR2, for the presence of all antimicrobial families. If the sample exhibits the positive result on more than one test plates, the confirmation should be directed onto specific family/families of antimicrobials detected.

Due to the fact that all screening methods have defined a false compliant rate of <5 % (β-error) by the **Commission Decision 2002/657/EC**, the false compliant (positive) results may be affected by inhibitory substances other than antimicrobials. These inhibitors (naturally occurring inhibiting substances presented in the food or feed matrices) can influence the test result and inhibit the growth of the test strain/strains. To prevent these inhibitors interfering with the test result, a special sample-procedure required by the Premi®Test for kidneys and feed involving the pre-incubation (heat pre-treatment of the sample at 80 °C for 10 min.) step was applied for all investigated matrices.

Currently, there are no published studies dealing with the screening of residues of inhibitory substances in meat of broiler chickens fed with fermented feed or feed supplemented with various bioactive substances. To reach a final conclusion regarding the presence of residues of antimicrobial substances such as antibiotics, sulphonamides, and quinolones or the presence of bioactive agents possessing antimicrobial activity in fermented cereals in meat of broiler chickens, further research is needed.

CONCLUSION

Screening methods have established performance criteria. They must detect the presence of a substance or class of substances at the level of interest (in compliance with legislation) with a false compliant rate of <5 %. Considering the results obtained in our work and growth inhibition of two of the five test strains after the screening of meat of broiler chickens fed with feed mixture with addition of fermented wheat bran, it is necessary for reaching true scientific conclusion and confirmation or exclusion of false positive and false negative results to perform a confirmatory analysis providing information on a substance/substances that cause/causes the inhibition of growth of the respective test strain.

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Acknowledgments:
This study was supported by grants APVV-14-0397, VEGA-1/0457/14 and VEGA 1/0574/15.

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