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MICROBIAL BIOFILMS AND MICROBIAL CONTAMINATION OF FEED FOR LIVESTOCK ANIMALS: CHALLENGES AND WAYS TO OVERCOME THEM

Kolchyk O. V. [1], Levytskyi T. R. [2], Buzun A. I. [1], Çelik E. [3], Hrynchenko D. M. [4], Korovin I. V. [1], Orda Yu. V. [1]

[1] National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv, Ukraine, e-mail: kolchyk-elena@ukr.net
[2] State Scientific Research Control Institute of Veterinary Medicinal Products and Fodder Additives, Lviv, Ukraine
[3] Kafkas University, Kars, Turkey
[4] State Biotechnological University, Kharkiv, Ukraine

Summary. The article describes the problem of microbial contamination of feed in animal husbandry and the microflora that causes mastitis in lactating cows. The microbial contamination of 52 commercial batches of fodder from 5 farms of 3 regions of Ukraine (barley, corn silage, oat haylage, alfalfa hay, sunflower meal) has been determined. *Pasteurella multocida* in association with *Neisseria lactamica*, *Actinobacillus pleuropneumonia*, *Clostridium perfringens* was isolated from 61.5% of barley, 66.7% of corn silage, 60.0% of alfalfa hay, and 50.0% of sunflower meal. 262 samples of milk from cows with mastitis have been studied. *Aspergillus candidus*, *Aspergillus niger* were most often isolated in association with *Mycoplasma bovis*, *Streptococcus agalactiae*, *Candida albicans*, *Neisseria sicca*, *Clostridium perfringens*. High film-forming activity of microorganisms in feed was determined, by optical density: *Pasteurella multocida* + *Actinobacillus pleuropneumonia* D_{620} = 3.76 and *Pasteurella multocida*, *Actinobacillus pleuropneumonia*, *Neisseria lactamica* D_{620} = 3.62. While from the milk of cows with mastitis we isolated associations of microorganisms that were strong producers of biofilms by the optical densities D_{620} = 4.02 and 4.23

Keywords: cows, mastitis, bacteria, fungi

Introduction. It is known that microbial biofilms are a dynamic complex biological system for protecting microorganisms from adverse environmental factors (Flemming and Wingender, 2010; Bednarska et al., 2013).

Pathogenic bacteria in microbial biofilms increase resistance to antimicrobial drugs by 100–1,000 times compared to planktonic (free-floating) cells (Austin et al., 1998; Dewachter, Fauvart and Michiels, 2019; Pu et al., 2016) The ability of microorganisms to form biofilms (film-forming activity) is now considered as a factor in their pathogenicity (Roy et al., 2018; O'Loughlin et al., 2013; Gostev and Sidorenko, 2010; Uruén et al., 2020).

Therefore, the study of the composition of microorganisms in feed and their biofilm-forming activity is important for preventing the development of associated animal diseases and, consequently, microbial contamination of the human food chain. According to the European Union's strategy regarding the development of animal husbandry, the biosafety of feed production and animal feeding is one of the key factors in preventing epizootics and, consequently, microbial contamination of the human food chain (EC, 2007).

Therefore, in the European Economic Community (EEC), based on the doctrine of 'One Health', the requirements for the sanitary quality of feed are formulated almost stricter than for food products (EP and CEU, 2017). Unfortunately, feed production in Ukraine, in terms of control of microbial contamination of raw materials and final product, is still regulated by old standards (MAPFU, 2012), which do not take into account new scientific knowledge, including the existence of bacterial associations in the form of microbial biofilms.

The **study aimed** to determine the microbiological contamination of the feed chain in animal husbandry and milk from lactating cows with biofilm-forming bacteria.

Materials and methods. Microbiological studies of feed were conducted in the Laboratory for the Pig Diseases Study of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine) following modern methods.

Isolation, cultivation and study of cultural and morphological properties of feed microorganisms were performed on nutrient media: meat-peptone broth (MPB) with a pH of 7.2–7.4; Hottinger broth; Martin medium; Edward medium, 2.5% meat-peptone broth (MPB) with the addition of 2.0%, meat-peptone agar (MPA) with a pH of 7.2–7.4; Endo agar; modified Kitt–Tarozzi medium; Saburo agar; Olkenytsky medium; Simons citrate; acetate agar.

Biofilm formation was studied by determining the ability of consortium isolates and individual microorganisms to adhere to the surface of a 96-well polystyrene plate (O'Toole and Kolter, 1998). Microorganisms were cultured in meat peptone broth (MPB) at a temperature of 37 ± 0.5°C for 48 h. According to the standard protocol, planktonic cells were removed from the wells of the plate and the microbial biofilms were stained with crystalline violet.

To do this, 150.0 µl of distilled water and 20.0 µl of 1% crystal violet were added to the well and incubated for 45 min at room temperature. After washing three

times with distilled water, 200.0 µl of 96% ethanol was added to the wells to extract the paint from the biofilm, and the optical density of the solution was measured on an ELISA reader at an optical wavelength of 620 nm (D_{620}).

52 commercial batches of fodder from 5 farms of 3 regions of Ukraine (barley, corn silage, oat haylage, alfalfa hay, sunflower meal) were studied. Feed selection was carried out both in the feed shop and in the livestock facility where the animals are kept. 262 samples of milk from lactating cows with mastitis were taken.

Results and discussion. According to the results of microbiological studies, plant feeds for cattle, regardless of the place of sampling, had a high level of microbial contamination, and their species composition was very diverse (Table 1). 52 samples of 5 types of feed at a dilution of 1:100,000 (10^{-5}) and 10^{-2} formed stable associations.

Table 1 — Associations of bacteria isolated from commercial batches of feed for cattle (4 repetitions each, $p < 0.005$)

Type of feed	Total, batches	Isolated microorganisms		Contaminated batches***
		10^{-5}, n*	10^{-2}, n**	
Barley	13	*Clostridium perfringens*, n = 3	*Pasteurella multocida*, *Clostridium perfringens*, n = 5	8
Corn silage	9	*Pasteurella multocida*, n = 2	*Pasteurella multocida*, *Neisseria lactamica*, n = 4	6
Oat haylage	17	*Pasteurella multocida*, n = 5	*Pasteurella multocida*, *Actinobacillus pleuropneumonia*, n = 6	11
Alfalfa hay	5	*Clostridium perfringens*, n = 1	*Pasteurella multocida*, *Neisseria lactamica*, *Clostridium perfringens*, n = 2	3
Sunflower meal	8	*Actinobacillus pleuropneumonia*, n = 1	*Pasteurella multocida*, *Actinobacillus pleuropneumonia*, *Neisseria lactamica*, n = 3	4

Notes: * — according to the current Decree No. 131 (MAPFU, 2012); ** — by microbial biofilms (see Table 3); *** — actual bacterial contamination.

Pasteurella multocida was isolated in associations with *Neisseria lactamica*, *Actinobacillus pleuropneumonia*, *Clostridium perfringens* from 8 contaminated batches of barley (61.5%), 6 batches (66.7%) of corn silage, 11 batches (60.0%) of alfalfa hay, and 4 batches (50.0%) of sunflower meal.

Bacterial associations in feed affect the immune system of animals. Before, during and after calving, cows experience significant stress due to the many physiological changes associated with calving and the onset of lactation. The microflora in animal feed increases this stress due to suppression of immunity and reduced feed intake, increased negative energy balance and increased risk of metabolic disorders and the development of inflammation of the mammary gland (mastitis).

Therefore, the next stage of research was the isolation of pathogens that cause mastitis in lactating cows in autumn and spring (Table 2).

Table 2 — Microorganisms that cause mastitis in lactating cows (4 repetitions each, $p < 0.001$)

Sampling period	Number of samples	Isolated microorganisms
Spring	125	*Pasteurella multocida*, *Mycoplasma bovis*, *Streptococcus agalactiae*, *Candida albicans*
Autumn	137	*Mycoplasma bovis*, *Neisseria sicca*, *Clostridium perfringens*, *Candida albicans*, *Aspergillus niger*

The pathogenic microflora of *Aspergillus candidus* and *Aspergillus niger* was isolated from cows with clinical mastitis regardless of the season, due to the use of litter and feed contaminated with spores of these fungi, as well as high humidity (> 90%), which promotes the reproduction of fungi in livestock facilities.

Aspergillus forms stable associations with mycoplasmas, streptococci, clostridia, neisseria, which can enter the udder from dairy calves infected with these microorganisms.

At the next stage of research, the film-forming ability of bacterial associations isolated from feed and mastitis milk from lactating cows was studied (Table 3). High film-forming activity of microorganisms in feed was determined, by optical density, *Pasteurella multocida* + *Actinobacillus pleuropneumonia* $D_{620} = 3.76$ and *Pasteurella multocida*, *Actinobacillus pleuropneumonia*, *Neisseria lactamica* $D_{620} = 3.62$. While from the milk of cows with mastitis we isolated associations of microorganisms that were strong producers of biofilms by the optical densities $D_{620} = 4.02$ and 4.23.

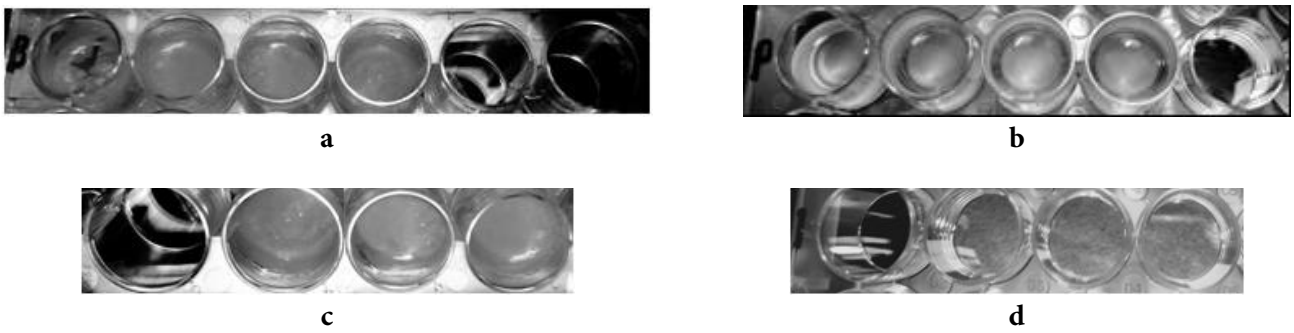
Table 3 — Estimation of the density of microorganism biofilms isolated from different types of feed and mastitis milk

Type of feed / Group of cows with mastitis	Bacterial associations	Biofilm growth time, h	Relative density of microbial biofilm (D_{620})
Bacteria associations from feed			
Barley	*Pasteurella multocida*, *Clostridium perfringens*	72	3.13 ± 0.57*
Corn silage	*Pasteurella multocida*, *Neisseria lactamica*	72	3.48 ± 0.46*
Oat haylage	*Pasteurella multocida*, *Actinobacillus pleuropneumonia*	72	3.76 ± 0.51*
Alfalfa hay	*Pasteurella multocida*, *Neisseria lactamica*, *Clostridium perfringens*	72	3.35 ± 0.49*
Sunflower meal	*Pasteurella multocida*, *Actinobacillus pleuropneumonia*, *Neisseria lactamica*	72	3.84 ± 0.52*
Control	Nutrient medium without biofilms	72	0.12 ± 0.04
Bacteria associations from mastitis milk			
Experimental 1	*Pasteurella multocida*, *Mycoplasma bovis*, *Streptococcus agalactiae*, *Aspergillus candidus*, *Candida albicans*	72	4.23 ± 0.61*
Experimental 2	*Mycoplasma bovis*, *Neisseria lactamica*, *Clostridium perfringens*, *Candida albicans*, *Aspergillus niger*	72	4.02 ± 0.58*
Control	Nutrient medium without biofilms	72	0.10 ± 0.03

Notes: * — $p \leq 0.05$ relative to the control. Scale for assessing film-forming activity: Optical density of biofilms up to ≤ 2×OD — low; from > 2×OD up to ≤ 4×OD — moderate; > 4×OD — expressed.

In this case, the yeast-like fungi *Candida albicans* and the fungi *Aspergillus candidus*, *Aspergillus niger* are a matrix that has a high degree of tolerance to antibacterial substances (Borgersen et al., 2018; Garrett, Bhakoo and Zhang, 2008; Karatan and Watnick, 2009; Vorobey, Voronkova and Vinnikov, 2012), thus protecting pasteurella, mycoplasmas, neisseria, clostridia from the action of antibiotics, which explains the multidrug resistance of these biofilms to 47 antibiotics (macrolides, fluoroquinolones, cephalosporins, aminoglycosides, lincosamides) and complicates the course of the disease and has a chronic course.

In Fig. 1 you can see the difference in shape and consistency between microbial biofilms from bacteriological cultures from different objects. Thus, Fig. 1a and Fig. 1b show the biofilms characteristic of mastitis cows' milk plated on Hottinger medium in dilutions of 1:50 and 1:1,000, respectively.



a **b** **c** **d**

Figure 1. Typical microbial biofilms in platings of milk from cows with mastitis (a and b) and hay (c) and straw (d) extracts. Wells without biofilms — control of the bacterial medium

Obviously, in the second case, the microbial biofilm is thinner and more fragile. According to the results of bacterioscopy, this is due to the absence of such a concentration of fungal microflora and most of its species, as well as higher volumes of anaerobic bacteria compared to the same sample studied at a dilution of 1:50.

Fig. 1c and Fig. 1d show examples of microbial biofilms of plating of samples (1:50), fresh hay from the feeder, and straw from the litter in the calf house, respectively. According to the results of bacterioscopy in the microbial biofilms of calf litter, the content and biodiversity of fungal microflora are much higher, compared to the platings of hay extracts. This may explain why these biofilms are more massive and colorful in appearance.

Thus, according to the results of our own research, it was found that various associations of bacteria that contaminate animal feed and milk from cows with mastitis have high film-forming activity. This indicates the probable formation of microbial biofilms of different composition and physical properties on the surface of grain, granules and other feed components, as well as on

the surfaces of containers, feeders, etc. These biofilms may contain and promote the survival of infectious agents dangerous to livestock (Lazăr and Chifiriuc, 2010; Santos-Lopez et al., 2019; Magana et al., 2018).

A contributing factor in the development of mastitis is the use of disinfectants for the treatment and prevention which kill bacteria. This provokes a violation of the microflora on the skin of the nipples. This fact contributes to the development of multidrug resistance of bacteria to antibiotics, disinfectants, the formation of bacterial biofilms in which the main role (matrix) is played by fungi *Aspergillus candidus*, *Aspergillus niger*, *Candida albicans*, due to which the bacteria in the biofilm remain viable in the environment for a long time (Zhang et al., 2020; Guzmán-Soto et al., 2021).

The results of research indicate a high probability that the current norms of feed contamination control in Ukraine do not provide an objective assessment, as they do not take into account the presence in feed of strong microbial biofilms, of which according to current procedures it is almost impossible to extract sufficient amounts of microflora, so that it is manifested in the dilution of extracts 10^{-5}.This is clearly beneficial to feed manufacturers, but it poses a real and great danger to livestock and the human food chain under the doctrine of 'One Health'.

Conclusions. 1. Microbial biofilms in cows with mastitis, as well as in animal feed, pose a serious biosafety threat when they are inhabited by opportunistic pathogens, and even more, pathogenic microflora. Therefore, their monitoring and study of properties should be given much more attention than is currently the case.

2. It is highly probable that this will lead to a revision of the current methods and standards of control of feed bacterial contamination, as well as the spectrum of antibiotic resistance of pathogenic bacteria — i. e. basic veterinary and sanitary indicators.

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