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The coexistence of several microbial species at the same site of bovine mammary gland parenchyma infection and their mixed infections

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SUMMARY

The purpose of this study was to analyse both negative results of bacteriological cultures and the coexistence of several pathogens at the same intramammary inflammatory focus within bovine udder parenchyma in order to compare the health status of cows' udders in 1985 and 2021. Mastitis was diagnosed on the basis of anamnesis, clinical inspection of the udder, milk quality, and somatic cell counts (SCC on the Fossomatic 5000-FC). In 1985, joint bacteriological and mycological diagnosis was based on the guidelines established for bovine mastitis by FIL-IDF (1971), while for 2021, it was based on the National Mastitis Council (NMC) laboratory Handbook on bovine mastitis (Hogan et al., 1999). In the first study (1985), mainly the genus *Staphylococcus* was isolated, but at present other species may be involved in cases of mastitis. In both periods, both subclinical and clinical cases were registered. In addition, the milk yield of cows was observed to increase over time, with concomitant deterioration of mammary gland immunity. The phenomenon of biofilms was documented in the study, which may be explained not only by the low efficacy of antibiotic therapy against

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udder disease but also by impaired immunity in cows, as the lymphocytes trapped in the biofilm matrix were inactive with respect to both cellular and humoral responses.

KEY WORDS: bovine mastitis, microbiological diagnosis, bacteriology and mycology, pathogens of bovine mastitis

INTRODUCTION

The bovine mammary gland is continually exposed to concomitant infections by multiple bacteria classified as either pathogens or environmental bacteria. This exposure may result from contact with a variety of microbes that trigger local immunity during efforts to obtain high milk yield, which involves permanent udder irritation due to machine milking. The term 'mastitis' covers the commonly known inflammations and infections of the bovine mammary glands, leading to dynamic physical, chemical, biological and bacteriological changes in the glandular tissues and in the milk produced, which may directly influence its quantity and quality (Sharma et al., 2011). Acute clinical mastitis (CM) has clearly recognizable symptoms, such as redness of the gland, elevated temperature, and swelling of the affected lobe, while organoleptic changes take place in the milk. In contrast, in chronic cases and subclinical cases (SCM), there are no accompanying clinically visible symptoms (Sharma et al., 2004; Sharma et al., 2007). In chronic CM cases, the first stream of milk undergoes organoleptic changes, but these changes are not observed in SCM. The common feature of the various forms of mastitis is a high somatic cell count (SCC) in the milk, which may consist of various epithelial cells (living or dead whole cells and their fragments) and leukocytes. SCC levels increase in proportion to the degree of inflammation. It should be emphasized that acute and chronic CM cases may constitute only the 'tip of the iceberg' in cattle, since prevailing cases in the herd are SCM (as many as 50–60%). In Asia, various forms of mastitis (up to 94.5%) are more widespread among cows than among milking buffaloes (68.6%) (Sharma et al., 2007; Sharma et al., 2011).

Zadoks (2002) was the first to report that in the last two decades before 2002, a diminished proportion of contagious forms of mastitis was accompanied by the expansion of environmental and opportunistic infections (Fig. 1).

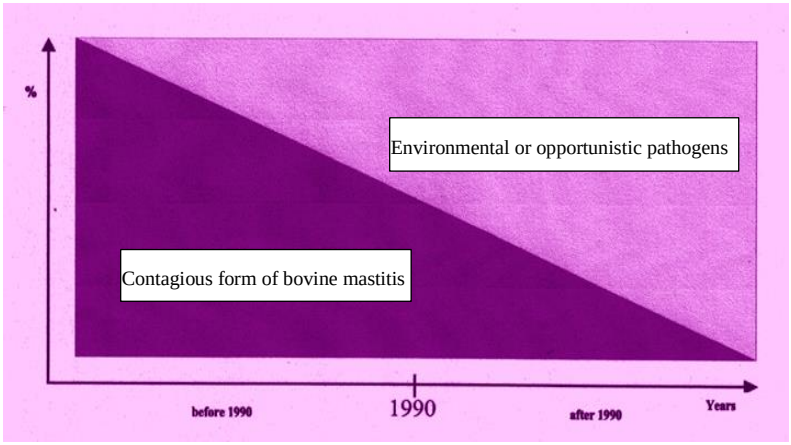


Figure 1. Probable transformation of the population of pathogens inducing clinical cases of mastitis (CM) over time, according to Zadoks (2002)

Zadoks (2002) implemented two mathematical models. The first, developed by Reed-Frost, described a situation in which mastitis has been spread in the herd between cows, with the milking machine identified as a possible vector of infection. In this contagious mastitis model, the probability of new intramammary infections (IMI) in the herd depends on the number of cows already infected. Up to 99% of new infections take place through a teat duct. Transmission of pathogens via the blood is virtually excluded, but the frequent concomitant infection of both the uterus and udder suggest that they may be transmitted via the lymph, as both of these organs are supplied with lymph from an intestinal confluence (Glazer, 1977). The situation typical of infection with other strains (environmental or opportunistic) is described by the Greenwood model, in which the probability of infection depends on a balance between cow vulnerability and environmental pressure. Confronting mathematical models with molecular microbial diagnosis (of pathogens), Zadoks (2002) emphasized that even after bacterial strains are identified, it is difficult to determine their pathogenicity, which must therefore be established each time for a given strain and linked to the symptoms observed.

The aim of the present study is to conduct an interdisciplinary analysis of isolated pathogens cultured from milk samples collected from cows with mastitis, since bacterial–fungal results from different decades of observations may be the subject of discussion regarding mixed infections or potential false-negative cultures.

MATERIALS AND METHODS

Plan of the study

The research was carried out in different time periods. The first survey was performed in 1985 and included 180 black and white cows with various genes characteristic of Holstein-Friesian cattle, with an average milk yield of 5500 kg in 305-day lactation. The second part of the study was performed in 2021 on a population of 372 Polish Holstein-Friesian black and white cattle with an average yield of milk of 8700 kg per standard lactation (305 days). The cows were milked

mechanically using a deLaval milking machine. Both parts of the study compared and documented microbial strains causing clinical forms of mastitis (CM) during lactation. Diagnosis of mastitis was based on interviews with cow handlers, clinical evaluation of udders, and organoleptic milk assessment, as well as measurement of somatic cell count (SCC) in milk samples using the Fossomatic 5000-FC.

Criteria of infection assessment

The bacteriological and mycological diagnosis of bovine mastitis cases in 1985 was based on a monograph on bovine mastitis written by experts from the A2 International Dairy Federation (FIL-IDF, 1971). Tests were carried out once a month. Among the 12 tests conducted during the year, two (designated A and B), at a one-month interval, were chosen for analysis in the present study. In 2021, procedures were performed according to the recommendations in the National Mastitis Council (NMC) Laboratory Handbook on bovine mastitis (Hogan et al., 1999). This time, a single examination was performed. An 'ecological' nutrient medium (containing 5% sheep blood and 0.1% aesculin) buffered to pH 6.5, 7.0 and 8.5 and mycological Sabouraud agar buffered to pH 5.5, 6.0 and 6.8 were used for the cultures. Additionally, for bacteriological cultures, a CAMP test was performed. Milk samples were plated on Edward's medium modified by Chodkowski, Chapman agar, Baird-Parker agar, and MacConkey agar. With the same media composition, sensitivity tests for pathogens were performed by the Kirby–Bauer method. For the mycological inoculations, classical methods described by Lodder, Richard and Wołoszyn or Microstix-Candida tests were used, as described in detail elsewhere (FIL-IDF, 1971). In 2021, bacteria were identified after cultivation on TSA blood agar (CMR, Krakow) and then with selective media: SAIDE (BioMerieux) for staphylococci, CPSE (BioMerieux), for gram-negative bacteria, and Sabouraud (BioMerieux) for yeasts. After a 3-day incubation period at 35°C or after 7 days at a temperature below 24°C (for yeasts), all colonies were observed, differentiated, examined under a microscope, and designated for further identification of genus and species by biochemical and molecular methods. Additionally, biochemical testing of isolated *S. aureus* strains was conducted to identify the type of haemolysis, its genomic content, proteolytic activity, and sensitivity to antibiotics. Laser desorption/ionization analysis was conducted using matrix MALDI-TOF, the PCR-RFLP method for genome typing to confirm the identification of species, and MLVA analysis to establish the phylogenetic relationship between strains. GelCompar II software (Applied Maths) was used to create a phylogenetic dendrogram of molecular similarity between *S. aureus* isolates.

Statistical analysis of the results

In the 1985 analysis, the Gauss test (Szwabe, 1987) was used to verify whether the theoretical distribution was in agreement with the experimental distribution at a significance level of $p<0.05$ for statistical classification of data (19). For a thorough comparison of relative values (percentages), their decimal fractions were analysed (n – ratio of the number of samples with a specific pathogen to the total number of samples), verifying significant differences at levels of $p<0.01$, $p<0.02$, $p<0.05$, $p<0.001$ and $p<0.0001$ (Floegel, 1984; Szwabe, 1987). Analysis of variance with Fischer's test was applied. When the arithmetic averages significantly differed ($F_o > F$), they were verified by Student's t-test (Fisz, 1969; Szwabe, 1987). Correlations between qualitative variants were estimated by the chi square test of independence (Szwabe, 1987). The frequency of coexistence of some pathogens at the same inflammatory site was analysed in four configurations (Szwabe, 1987). First, the number of coexisting pathogens was confirmed by the Euler characteristic of combinatorics. Diagrams of

coexistence were prepared on the basis of taxonomy by Czekanowski (1930), calculating Steinhaus distances and representing them with dendrites (Florek et al., 1951).

Based on SCC values measured with an electronic counter in the milk samples of the cows (quarter analysis), successive stages of mastitis in the cow udders were identified. Based on the results of the microbiological culture, the relationship between the type of microbe and the form of mastitis identified in the cows was established, after which Yates' correction and Fischer's exact test were performed for the herds. Mathematical classification of mastitis cases was conducted using Gauss's test. Significant differences were assessed at different levels of statistical significance ($p<0.01$, $p<0.02$, $p<0.05$, $p<0.001$ and $p<0.0001$). The FREQ procedure of the SAS (2019) ver. 9.4 statistical software package was used to establish the relationship between the factors analysed and the distribution of SCC values. The statistical significance of the relationships was assessed using Fisher's exact test. One-way analysis of variance (GLM-SAS procedure) was used to assess the impact of age (lactation number) on the SCC value of the milk, and the means were compared in detail using Duncan's test. The SAS (2019) ver. 9.4 statistical package was used for the analyses.

RESULTS

During the research carried out in 1985, the coexistence of several pathogenic species in the same inflammatory foci (mixed infection) was documented, with staphylococci prevalent among the pathogenic populations. Between tests A and B in the cows of the same herd, the proportion of healthy udders and SCM cases diminished, while CM cases (5.24%) increased. Comparison of the data in Table 1 and in Table 2 clearly shows that fewer cows with healthy udders (9.17%) were identified in the earlier period (1985) than in the recent period (11.02±5.11 = 16.13%). Far more CM cases were disclosed in the current study period (Table 3; 19.62+25.54=45.16%) than in the earlier period (1985; 2.18+3.06 = 5.24%). False-negative data (lack of indication of bacterial growth) were similarly obtained in both the earlier and recent period, for both CM and SCM cases. this was observed in 7.42% of cases (5.24±2.18) in the early study period and in 25.54% of cases (5.91±19.62) from the 2021 survey.

Table 1.
Health status of cows' udders in the herd examined in 1985 and types of pathogens identified in their mammary glands

Type of infection			Udder health status						Total
			Healthy udders & secretion disorders	Latent infections	False negative SCM	Confirmed SCM	False negative CM	Confirmed CM	
None		n	21	-	22	-	5	-	48
		%	9.17	-	5.24	-	2.18	-	16.59
Pure culture		n	-	96	-	15	-	5	116
		%	-	41.92	-	6.56	-	11.45	59.93
Coexistence	Mixed culture of bacteria	n	-	20	-	8	-	1	29
		%	-	7.86	-	3.49	-	2.29	13.64
	Bacteria & fungi	n	-	32	-	13	-	1	46
		%	-	14.85	-	5.67	-	2.29	22.81
Total		n	21	148	22	36	5	7	229
		%	9.17	64.63	5.24	15.72	2.18	3,06	100.00

Table 2.
Health status of cows' udders and the types of pathogen identified (in 2021)

Type of infection			Udder health status							
			Healthy udders	Secretion disorders	Latent infections	False negative SCM	Confirmed SCM	False negative CM	Confirmed CM	Total
None		n	41	19	-	22	-	73	-	155
		%	11.02	5.11	-	5.91	-	19.62	-	41.67
		% row	26.45	12.26	-	14.19	-	47.10	-	
		% col.	100.00	100.00	-	100.00	-	100.00	-	
A pure culture		n	-	-	16	-	4	-	23	43
		%	-	-	4.30	-	1.08	-	6.18	11.56
		% row	-	-	37.21	-	9.30	-	53.49	
		% col.	-	-	17.02	-	14.29	-	24.21	
Coexistence	Of some bacteria	n	-	-	57	-	19	-	47	123
		%	-	-	15.32	-	5.11	-	12.63	33.06
		% row	-	-	46.34	-	15.45	-	38.21	
		% col.	-	-	60.64	-	67.86	-	49.47	
	Bacteria & fungi	n	-	-	21	-	5	-	25	51
		%	-	-	5.65	-	1.34	-	6.72	13.71
		% row	-	-	41.18	-	9.80	-	49.02	
		% col.	-	-	22.34	-	17.86	-	26.32	
Total		n	41	19	94	22	28	73	95	372
		%	11.02	5.11	25.27	5.91	7.53	19.62	25.54	100.00
		Fisher's exact test			P					

Analysis of the results of the 1985 study revealed more mixed bacterial–fungal infections than mixed staphylococcal–streptococcal infections; moreover, only two pathogens coexisted at the same site. Figure 2 presents the proportions (over the diagonal line) and numbers (under the diagonal line) of coexisting pair types of pathogens, and Figure 3 shows the pairs that were confirmed to be correctly identified. Among 15 possible combinations in survey A, only six were in fact confirmed, while in survey B nine of 15 were confirmed. In survey A, both staphylococcal–fungal and staphylococcal–streptococci mixed infections had a real meaning, while in observation B, only staphylococci with fungi were observed.

	Gr	Pa	Ma	PG -	In	Grz
Gr		8, 85	4, 42	1, 77	0, 00	16, 81
Pa	10		0, 88	0, 00	0, 00	3, 54
Ma	5	1		0, 00	0, 00	3, 54
PG -	2	0	0		0, 00	0, 88
In	0	0	0	0		0, 88
Grz	19	4	4	1	1	

	Gr	Pa	Ma	PG -	In	Grz
Gr		8, 85	4, 42	1, 77	0, 00	16, 81
Pa	10		0, 88	0, 00	0, 00	3, 54
Ma	5	1		0, 00	0, 00	3, 54
PG -	2	0	0		0, 00	0, 88
In	0	0	0	0		0, 88
Grz	19	4	4	1	1	

Figure 2. Type of coexistence for two different pathogens in the same focus of infection during examination A (upper diagram) and B (lower diagram) of cows' udders, carried out one month apart before 1990

Types of pathogens: *Staphylococcus* (Gr), *Streptococcus* (Pa), *Corynebacterium* (Ma), gram-negative rods (PG-), other bacteria (In), pathogenic fungi (Grz)

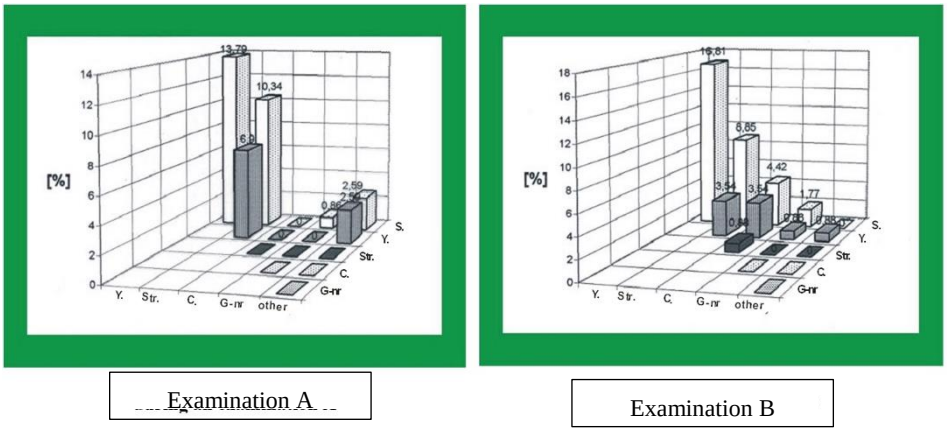


Figure 3. Possible and actual frequencies of two pathogens coexisting at the same inflammatory site in cow udders before 1990

In 2021 (Table 3), there were more bacterial infections with multiple species (2–6) and fewer overall bacterial–fungal infections. Among the 468 possibilities of pathogen coexistence (Table 3), only 25 were confirmed, and only three pair types were of practical significance: coagulase-negative staphylococci and *Corynebacteriaceae*, coagulase-negative staphylococci and other bacterial strains, *Corynebacteriaceae* and other bacterial strains.

Table 3.
Pure cultures and mixed infections with multiple (26) pathogens and calculation of the possibility of their coexistence in pairs (data from 2021)

Pure culture infections of pathogens and their coexistence in pairs (mixed infections)				
No. of pathogen	**n**	**%**	**Possible combinations**	
1	30	9.26	0	
2	51	15.74	1	
3	71	21.91	3	∑ = 468
4	81	25.01	6	
5	71	21.91	10	
6	20	6.17	15	
Total	**324**	**100.00**	**c = ½ n x n-1**	

Figures 4 and 5 are confirmed by the short arms of the Steinhaus distance values for such correlations, i.e. 3 mm for coagulase-negative staphylococci and *Corynebacteriaceae*, 17 mm for coagulase-negative staphylococci and other bacterial strains, and 20 mm for *Corynebacteriaceae* and other bacterial strains.

	Gr+	Gr-	Pa-z	Pa-s	PG-	Cor	Inn	Grz
Gr+		1,00	0,40	0,40	0,20	0,80	0,40	0,40
Gr-	5		0,80	1,81	5,62	11,45	8,63	4,02
Pa-z	2	4		0,00	0,20	0,00	0,00	0,40
Pa-s	2	9	0		0,40	0,40	0,20	0,80
PG-	1	28	1	2		5,22	3,41	2,41
Cor	4	57	0	2	26		8,03	4,42
Inn	2	43	0	1	17	40		3,21
Grz	2	20	2	4	12	22	16	

Figure 4. Type of pairwise coexistence of different pathogens at the same intramammary focus of infection during the recent research (2021). Below the diagonal: number of recorded coexistences; above the diagonal: their frequency in relation to all possible infections (n=498). Gr+ - coagulase-positive staphylococci, Gr- - coagulase-negative staphylococci, Pa/z - infectious streptococci, Pa/s - environmental streptococci, PG- - gram-negative rods, Cor - coryneform bacteria, Inn - other bacteria, Grz - pathogenic fungi.

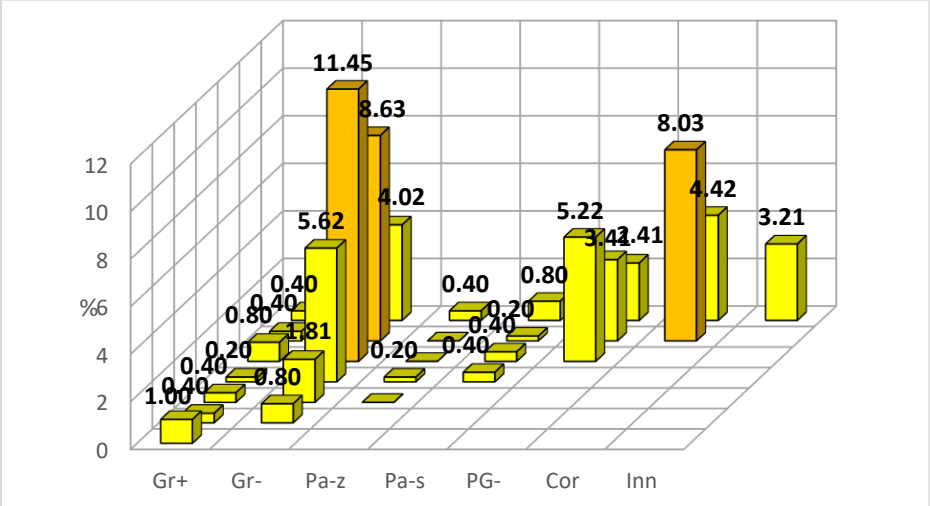


Figure 5. Current (2021) possible (∑=498) and actual frequency of the coexistence of several pathogens in pairs at the same inflammatory site during mixed infections of bovine udders

The dendrites in Figure 6 illustrate the statistical dependence between SCM and CM in the field observations performed before 1990. The dendrite in panel A describes CM cases, while the dendrite in panel B describes SCM cases. When dendrites A and B were superimposed on panel C, they could not be connected by a common arm. This is represented by a dashed line in panel C, indicating that different types of udder infections (environmental and infectious) coexisted in 1985. Moreover, the geometric means of areas B and A are slightly deformed, since the description of CM cases includes organoleptic changes in milk that are absent in SCM. To simplify, we could assume that CM in cattle can be recognized as a specific form of SCM, which is true only in the environmental model of the disease. In contrast, in Figure 7, all the lines are continuous, due to single-species infections. Figure 8 shows the current dendrite (2021).

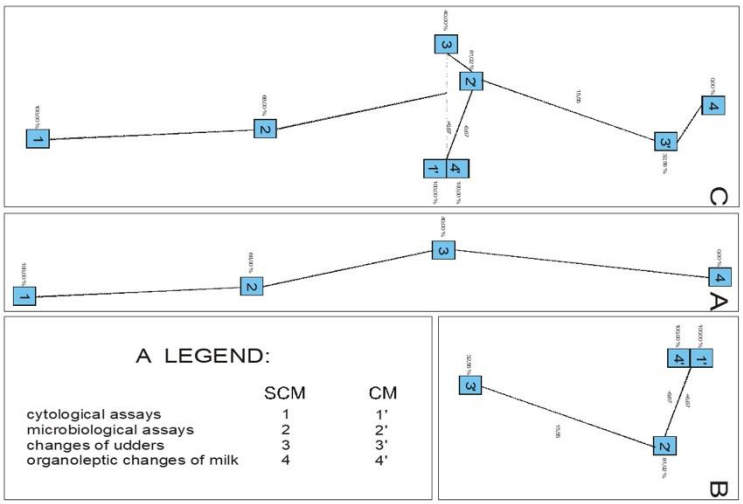


Figure 6. Dendrites of the mathematical relationship between SCM and CM cases in the herd examined in 1985

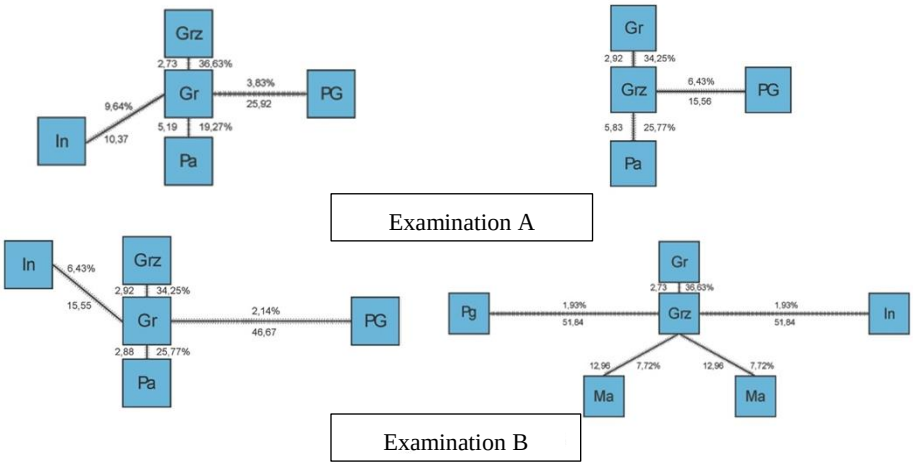


Figure 7. Dendrites for the coexistence of pathogens in the herd examined in 1985, with staphylococci placed at the centre of the graph and with yeast-like fungi placed at the centre of the graph

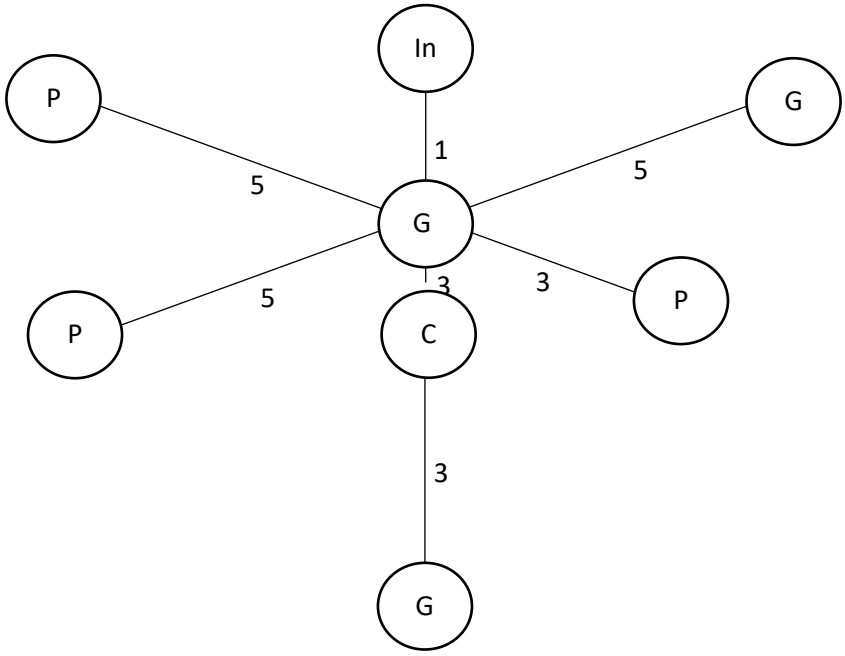
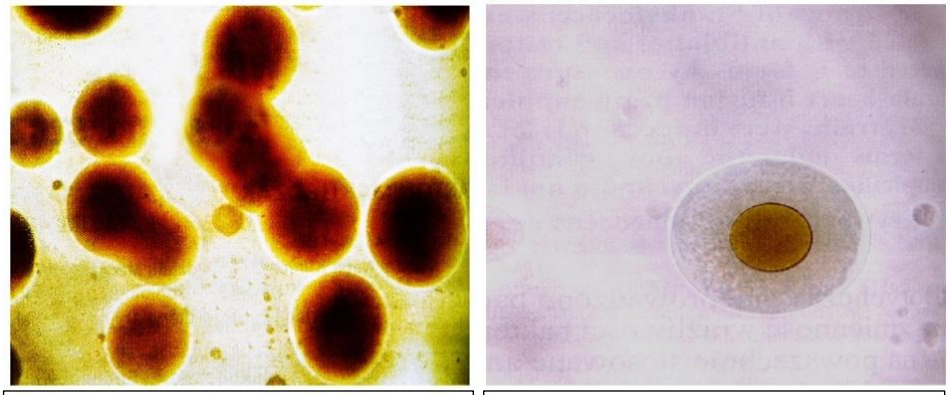


Figure 8. Dendrite for 2021 for the coexistence of pathogens in pairs at the same inflammatory site during mixed infections of bovine udder parenchyma

The photo on the left in Fig. 9 shows vegetative forms of *Staphylococcus aureus* on blood agar. L-forms of *S. aureus* (without cell walls) will not grow on this substrate because they cannot rebuild their cell walls on it. The photo on the right shows L-forms of *S. aureus* grown by Jakubczak et al. (2002) on mycoplasma culture medium, i.e. BHI with the addition of blood.. The photographs in Figure 10 present five phases of biofilm formation: (1) initial adhesion of bacteria to the surface, (2) permanent attachment to the surface, (3) initial maturation, (4) final stage of maturation, and (5) biofilm dispersion (possible expansion to new foci). Each of these stages was documented by photographing (a) with a confocal microscope. Row (b) contains microphotographs of cow milk samples stained by the H&E method. Row (c) contains two microphotographs of milk samples from cows with mastitis which document the confinement of lymphocytes in the bacterial matrix (of the biofilm).



Vegetative colonies of *S. aureus* on the surface of blood agras	L- forms of *S. aureus* grown on the surface of BHI solid medium

Figure 9. L and R forms of *Staphylococcus aureus*

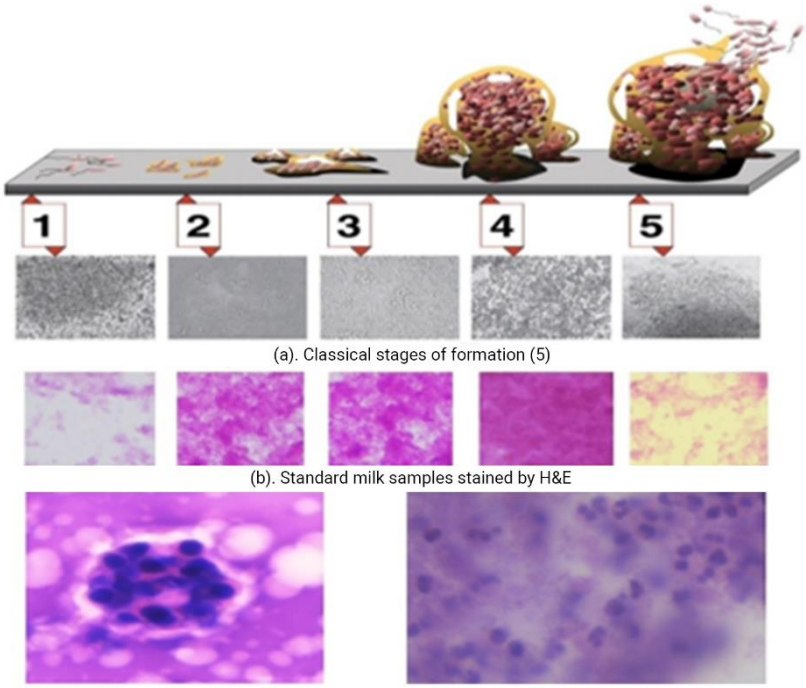


Figure 10. Stages of biofilm formation

DISCUSSION

For economic and sanitary reasons, cow udder diseases are among the most important contemporary problems in animal production, as cattle farmers and dairy producers incur substantial losses due to poor milk quality. Fig. 1, based on the paper by Zadoks (2002), graphically presents the results of 20 years of studies on mastitis aetiology in cattle and shows that pathogenic forms of mastitis decreased, while environmental and opportunistic infections increased. Analysis of this transformation indicates that more contagious cases began to dominate over milder cases; however, milk yield continued to increase. Fig. 11 presents examples of lactation curves according to Kirchgeßner (1997) for different values of milk productivity, since it increased milk yield seems to correlate with a decrease in immunity, as reported by Balbierz (1986). It must be emphasized that the mammary gland in cattle must perform two conflicting functions: providing essential antibodies to offspring while protecting itself from infections. This may result in less satisfactory performance of both roles, including perinatal immune deficits involving impaired function (margination) of neutrophils (Burton and Erskine, 2003; Rainard and Riollet, 2006).

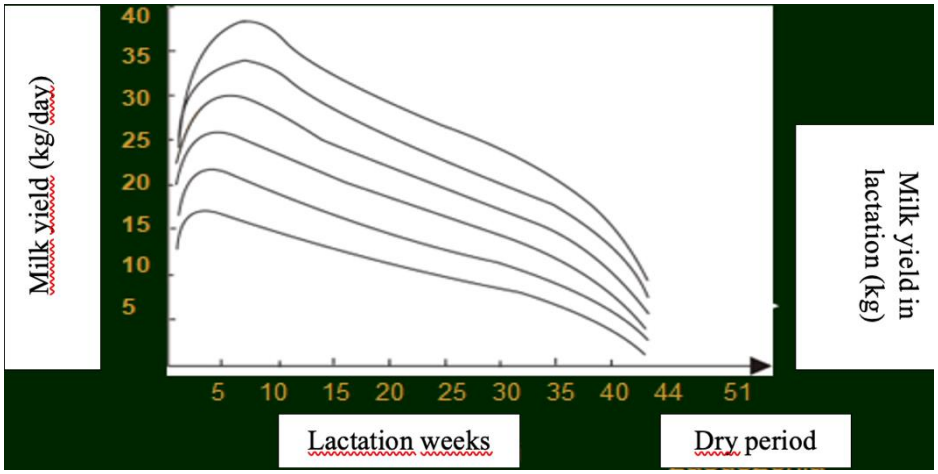


Figure 11. Standard lactation curves for cows with different milk yields (Krichgessner 1997)

It should be mentioned that in Fig. 1 we introduced a borderline date – the year 1990. This was based on research on the Experimental Farm in Swadzim (Poznań University of Live Sciences), which at that time was carrying out a mastitis control scheme (MCS) in a cattle herd with a milk production range of approx. 6000 kg of milk/year. Before 1990, when contagious forms of mastitis prevailed, there was an active MCS in one of the 49 Voivodeship Laboratories for Milk Hygiene and Mammary Gland Disease, which supervised the examination of the mammary glands of 60,000 cows on small milking farms and 60,000 cows in large-scale herds. A mastitis control scheme was implemented in a few of them, and there were some plans for the management of milk quality (Dudko, 1986; Dorko et al., 1999; Dudko, 2003a; Dudko, 2003b; Dahm and Strzelczyk, 2004; Dudko, et al. 2010). The recommendations of FIL-IDF Group A2 (1981) were diligently carried out, and a total retreat of *Streptococcus agalactiae* as a mastitis pathogen and the expansion of

staphylococci were observed. During the period when streptococci were predominant cause of mastitis, the cultures were uncontaminated by other contagions. The expansion of staphylococci was often associated with mixed infections. Mastitis pathogens were at this time subdivided into major (*S. agalactiae*, *S. dysgalactiae* and *S. uberis*) and minor pathogens (*Corynebacterium bovis* and coagulase-negative staphylococci), which at this time were considered saprophytes of the teat ducts and skin of the mammary glands. Coagulase-negative staphylococci (80–90%) were isolated more frequently than coagulase-positive staphylococci (e.g. *S. aureus*).

The economic transformation in Poland resulted in the elimination of the Piła Voivodeship as well as that of the above-mentioned Laboratory for Milk Hygiene and Mammary Gland Disease. Microbiological smears of milk samples were carried out in different laboratories. However, the costs of this operation together with the lack of veterinary consulting for mastitis caused a decrease in the number of laboratory analyses, and the mastitis control scheme was gradually withdrawn. The system was also damaged by the failure to implement proper management of milk quality, as was done in countries such as Switzerland (Rainard and Riollet, 2006). In countries in which food safety and health were guaranteed, the managing system for food supervision was also regulated by law. Such regulation resulted in a systematic decrease in the SCC in collected milk (in Switzerland for 10 consecutive years). At present, SCC values presented for Switzerland have been markedly lower than those obtained on Polish organic farms (Doherr et al. 2007). Malinowski (2006) also recommended reducing the SCC in milk.

The real cause of the deterioration in udder status in cows from organic farms was that antibiotics were only permitted to save the life of a cow, while their application for mastitis was prohibited. It should be noted, however, that when milk yield increased, the efficacy of antibiotics declined. A programme for eliminating *S. agalactiae* required only the use of suitable antibiotics in lactation and in the dry period. In herds with low milk production, it was sufficient to deliver antibiotics three times at 24-hour intervals. For staphylococcal therapy, it was essential to determine the actual pattern of bacterial sensitivity to the various antibiotics. Moreover, there was a necessity to repeat ineffective therapies, and in the case of persistent failure, to remove resistant cows from the herd. A further increase in milk yield required not only more frequent antibiotic administration but also intramuscular and intramammary injections. Later, a 20-year comparison of treatment with targeted antibiotic therapy to a group treated with propolis preparations (Dudko, 2003c; Dudko, 2003d; Dudko, 2008) indicated higher efficacy of propolis. However, it was Xue et al. (2016) who first clearly established that in biofilm situations, herbs and other natural drugs were more effective than antibiotics, since bacterial metabolism in such cases is latent, while antibiotic efficacy depends on blocking metabolizing pathogens.

After 1990 (when environmental and opportunistic infections prevailed), in addition to the lack of microbial analyses of milk samples, a serious problem for dairies was the fact that bulk tank milk from herds in which approximately 10% of cows suffered from clinical mastitis was still sold by dairies as ‘extra’ class (the highest quality class). This discouraged cattle owners from carrying out the MCS, which seemed not to pay off. Malinowski (2006) changed the methodology of the MCS from that recommended by the EU to that applied in the United States, recommended by the NMC (Hogan et al., 1999), as the US experts appeared more competent in these situations than Polish experts. However, due to the loss of contact with the FIL-IDF from Brussels, despite the fact that Poland was already in the European Community, common expert documents from the FIL-IDF

(1999; 2011) and NMC (Hogan et al., 1999) were overlooked. The recommendations of both institutions included new cut-off values for SCC in bulk milk for purchase (Dorko et al., 1999). Thus, the former classification of microbes into 'major' and 'minor' pathogens became outdated. Due to the lack of regular microbiological testing of milk samples, knowledge of the sources of infection in mastitis was obtained from experimental infection of cows. Schukken et al. (2011) compared results from intramammary infections (infusions) of the three pathogens which were then the most frequent causes of mastitis: *Escherichia coli*, *Streptococcus uberis* and *Staphylococcus aureus*. Transient infections with high numbers of bacteria were typical for *E. coli*, while infections with *S. aureus* were fairly persistent with a small number of bacteria. Suspensions of *S. uberis* were used to induce persistent (long-term) infections with a large number of colonies. White (2010) evaluated natural infections with *E. coli* and described substantial differences in the course of infections triggered by this pathogen. Based on mechanisms discriminating between transient and persistent infections, they constructed a mathematical model. At present, the acute, severe course of mastitis is known to be caused by virulent (verotoxin- and Shiga toxin-producing) *E. coli* strains (Kłossowska and Malinowski, 2007). Less virulent forms infect udders about two weeks prior to delivery and may induce CM or SCM immediately afterwards; infection may resolve after a month. Miller (1984) conducted an experiment which corrected current immunological knowledge and also proposed immunosuppressive drugs, whose efficacy was tested in experimental pyelonephritis in rats. The bovine mammary gland is a natural element of the genitourinary tract, from which nephrotoxic strains of *E. coli* may induce bovine mastitis, similar to urinary tract infections observed in women. There is a significant difference in the pathophysiology of these syndromes; in women, urinary tract infection usually ascends from the urethra to the uterus or kidney, while in cattle, it descends from the anus to the uterus and then to the mammary gland. This finding reminds us how infections with *Corynebacterium pyogenes* can spread from the uterus to the udder. This microbe is no longer believed to belong to the family *Corynebacteriaceae*, but belongs to the Actinomycetes. Therefore, its name was changed to *Arcanobacterium pyogenes* and then to *Trueperella pyogenes*.

Summing up the observations of the researchers cited, it must be underlined that they supported the hypothesis of Zadoks (2002) of the transformation of the population of pathogens in mastitis. This transformation was observed at different times in different countries. As in our study, in the herd examined in 1985, mixed infections and false-negative results were also documented by Klimaitė (2005) in Lithuania. Moreover, before 1990, fewer cows with healthy udders were observed in herds because older cows were kept in the herd, while after 2001, they were culled after three lactations. Within the Mastitis Control Programme, cows were examined monthly to identify and treat CM cases. Therefore, more cases were detected within this programme. It is difficult to provide adequate comparisons, since different methodologies prevailed at different times. Although scientific advancements may have costs, progress is unstoppable. In earlier times, microbiologists took greater care to choose the optimal media for bacterial growth in terms of sensitivity (FIL-IDF,1971; Hogan et al.,1999). In fact, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectrometry significantly extended the range of microbe identification. However, at the same time, we now have a problem with taxonomical divergence. Aware of these taxonomic hurdles, we compiled (Table 2) earlier data for easier comparison with currently classified pathogens. Thus in Tables 2 and 3 only groups of isolated and identified bacteria were compared, while a more precise taxonomical approach to the same strains can be recommended to those interested in their exact

genera and species (Kosecka-Strojek et al., 2021). A comparison of data obtained prior to 1990 (Table 2) with the present data (Table 3) revealed some similarities with respect to false-negative results and mixed infections. The significantly larger number of bacterial species recognized by the MALDI-TOF method together with the small number of infections caused by them diminished the statistical power of the data. Therefore, the Wrocław method was used, in which an approach with dendrites (graphic forms) substantially improved the analysis of the coexistence of several species in the same inflammatory focus. Further consequences of the increase in mixed infections included an increase in SCC, affecting milk quality, and the documentation of the biofilm phenomenon.

Phenomenon of negative cultures

This phenomenon often accompanies the use of antibiotics or disinfectants. It is also worth noting that disinfectants (e.g. during machine milking twice a day) are applied in cows more often than intramammary antibiotics. Some authors have suggested that negative cultures may in fact indicate a syndrome of aseptic inflammation, in which the parenchyma of the mammary gland is mechanically irritated by inefficient milking machines (Malinowski et al., 2011). Such potential inflammatory foci can quickly be colonized by true pathogens. Nicklas et al. (1980) reported that the typical symptoms of yeast-related mastitis may be induced by intramammary inoculations of aqueous phenolic extracts from dead yeast. Therefore, even if potential mastitis pathogens can be eliminated with antibiotics, the clinical symptoms would not necessarily completely vanish. In our study, both SCM and CM cases were revealed (earlier and at present); however, their rate is currently 3.5 times as high as prior to 1990. The most likely explanation was revealed by experiments conducted by Jakubczak et al. (2002), in which after the addition of antibiotics, typical cell wall-deficient (L-forms) *S. aureus* colonies (Fig. 10) developed after incubation in BHI medium. This possibility has been confirmed by Ovens et al. (1988) and other authors. In our study (in which bacteria were cultured in buffered blood agar supplemented with aesculin), small colonies resembling fried eggs developed. This means that only live forms of bacteria can multiply and be identified in routine broth, while their cell-wall-deficient forms cannot grow in *in vitro* conditions. Such growth may be impossible even in liquefied bases, which have been commercially produced for blood sample cultures and contain resins for neutralization of inhibitory substances (Neu and Goldreyer, 1968; Owens, 1988; Xue et al., 2016). Bacteria without cell walls include both protoplasts and sphaeroplasts, which lose their cell walls due to lysozyme activity (Xue et al., 2016). Both proto- and sphaeroplasts should die in classical bacteriological broth, since their walls cannot be restored in these media. Although adequate solid media can be used for the multiplication of cell wall-deficient L-forms, in which their cell wall is restored, in such conditions they may return to vegetative stages. Other authors maintain that the cell wall-deficient forms may be created not only from *Staphylococci*, *Streptococci*, *and Enterococci* strains (considered the genera of major mastitis pathogens), but also from gram-negative rods (Neu and Goldreyer, 1968; Calderon et al., 1971; White, 2010). Interest in the viability and proper multiplication of pathogens during *in vitro* culture has grown for at least three reasons: (i) the conviction that environmental microbes do not multiply *in vitro*, (ii) speculation that periodically some of them may not grow (viable but culturable – VBNC), and (iii) the dynamic development of molecular tests (Dahm and Strzelczyk, 2004).

Controversial mixed infections in mastitis aetiology

Although there is a precisely described protocol for sterile collection of samples (FIL-IDF, 1971), some laboratories argue that mixed infections have been detected due to improper sample collection.

It is difficult to state that all samples are always properly collected; however, research is mostly conducted by professionals. Moreover, the phenomenon of mixed infections is described in both human (Nowakowska et al., 2004) and veterinary medicine (Pejsak et al., 1995). The coexistence of infection by several pathogens at the same inflammatory site was revealed in the herd examined in 1985 (Table 2). Similar conclusions were drawn in 2005 by Klimaitè (2005) in Lithuania, in an analysis of only SCM cases. In both studies, joint bacteriological and mycological tests were performed, and the proportions of mixed bacterial–fungal infections were comparably high. False-negative cultures occurred in both studies, of which the most frequent were bacterial–fungal infections. In cowsheds where cows suffered from yeast infections of the udder, yeast-like fungi could be isolated from many surfaces (even from the ceiling); however, the main source of infections was found to be straw. In the recent part of the study (Table 3), infections of several bacterial species were recognized, and rarely bacteria together with fungi (yeast or mould-like forms). However, fungi *Trichoderma* sp., are often found in straw on organic farms, were successfully isolated and identified. This fungus is an antagonist of many phytopathogens, including yeast- and mould-like fungi (Thapa et al., 2020), which may explain why in 2021 cases of udder mycosis were very rare.

Analysis of the coexistence of several pathogenic species at the same inflammatory site

The 'Wrocław method' has been verified in life sciences (e.g. anthropology and botany, etc.) for comparing different plant populations originating in different biosystems (resemblance/similarity analysis). Inverse similarity is defined by the Steinhaus distance, which in the dendrite diagram allows us to evaluate the probability of the coexistence of microbial species in particular pairs. It could therefore be possible to distinguish transient infections from persistent ones, and in ecology, synergism from antagonism. For example, antagonism between coagulase-positive staphylococci and *E. coli* rods could be detected. Another advantage of this method is that it can be applied even in situations with a small number of cases, which usually makes statistical analysis impossible. Lower values are normally nonsignificant.

There is literature available on biofilm-forming bacteria, which are frequently mentioned in cases of *S. aureus*, CNS, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterococcus faecalis* (Owens, 1988; Jakubczak et al., 2002; Weiner, 2011). Less attention has been focused on biofilms of yeast of the genus *Candida*, but such biofilms are known to have formed both in living organisms and in their immediate vicinity. Dorko et al. (1999) drew attention to the marked tropism of yeast for prosthetic and plastic medical devices (including tube syringes and teat cups, which could be soaked before and after milking). In addition, biofilm was observed in colonies on milking equipment and vessels in contact with milk. Human medicine exposes biofilm pathogens involved in many diseases, including gynaecological disorders. For almost 30 years, the authors of this study have been using their fingers to wipe a nasal mucus-like substance from teat cups in milking machines that are insufficiently clean, but they only began putting on rubber gloves to do so when it became known that the substance was biofilm matrix. This is the best way to demonstrate the presence of bacteria to farmers. During the 2021 study, in cowsheds in which the milking collectors were not thoroughly cleaned, swabs were taken from their surface, which was covered with a dense biofilm matrix. Following incubation, the presence of *Pseudomonas aeruginosa* biofilm was confirmed.

CONCLUSIONS

I. The authors of this paper fully support Ruth Zadoks's (2002) hypothesis that mastitis pathogen populations have transformed over the years. The main advantage of her work was that she used the same methodology for 20 years. However, the smaller number of false-negative results in Poland before 1990 indicates that that the old method more accurately diagnosed infectious forms of mastitis. On the other hand, newer methods are more effective when there are more environmental and opportunistic infections. In addition, the false-negative results before 1990 were mainly associated with cases caused by *S. aureus* and some other staphylococci, whereas currently (2021) they may involve many other pathogens as well.

II. The milk yield of cows has also increased over the years, which usually results in the deterioration of both the systemic immunity of animals and the local immunity of the udder, especially given that the biofilm phenomenon documented in this work suggests that we are currently dealing with immunopathology of the mammary glands of cows rather than with normal local immunity.

III. The studies before and after 1990 were compared. Mixed infections occurred in both periods. However, previously only two pathogens coexisted at the focus of infection, causing a mixed fungal and bacterial infection of the udder. Currently, multiple bacterial infections have been diagnosed (2–6 bacteria). The fact that there are now more cows in herds with healthy udders is due to their younger age (<3 lactations). However, the significantly higher number and percentage of clinical forms of mastitis in these herds are indicative of the health status of udders in 2021.

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