UNIVERSITY OF DEBRECEN
CENTRE FOR AGRICULTURAL SCIENCES AND ENGINEERING
FACULTY OF AGRICULTURAL SCIENCE
INSTITUTE OF FOOD PROCESSING, QUALITY CONTROL AND MICROBIOLOGY

DOCTORAL SCHOOL OF ANIMAL BREEDING SCIENCES

Leader of Doctoral School:

Dr. András Kovács
professor

Supervisors:

Dr. András Szabó
associate professor

Dr. Béla Béri
associate professor

„THESIS OF DOCTORAL (Ph.D.) DISSERTATION"

EFFECT OF KEEPING AND MILKING TECHNOLOGY ON MICROBIOLOGICAL QUALITY OF RAW COW MILK

Author:

Ferenc Árpád Peles
Ph.D. candidate

Debrecen
2008

1. INTRODUCTION

Goat, sheep, and cattle are economic animal species with diverse utilization. Cattle are the most significant, since 25-30% of the output value of the Hungarian animal husbandry is derived from their production.

Their role in public catering, export and utilization as industrial raw material gives national economic importance to cattle breeding. Cow milk and the products made from it (e.g. butter, yoghurt, cheese, curd cheese etc.) are essential in human nutrition.

The original function of milk is feeding the newborn. Cattle produce more milk than the calf needs, and this extra milk has long been used in human nutrition – process products such as cheese, curd cheese, butter, yoghurt etc. – or for industrial purposes. At present, cow's milk has become the most important foodstuff of people. This is because of it's the favourable composition, in that it contains all nutritive compounds in suitable proportions and in a form that is easy to digest.

However, milk is easily contaminated during milking, handling and transport. It is an ideal medium for most bacteria, and that is the reason that it spoils quickly. As excellent and healthy as it is when fresh and clear, milk is dangerous for the consumer if it is contaminated. The proper, clean handling of milk is of sanitarian interest, but it also serves the farmers' interests, because contaminated milk may not be distributed, and is not suitable for producing good quality products.

The quality of the milk is influenced by its composition, nutritional physiology and enjoyment value which in turn are, influenced by its hygienic and microbial characteristics. The aim of raw milk inspection is to estimate these factors regularly and to reflect the quality in the state purchase price of the milk. This provides the economic incentive to farmers to improve and maintain good quality. In our country there has been raw milk inspection in a European sense since 1984. As a result of stricter requirements and the resulting state purchase price, the quality of milk has improved significantly in the last few years.

In Hungary, the level of milk production and quantity of milk product consumption still lag behind the desired level for optimum human nutrition important tools to improve it. Can be widening the range of products and producing high quality products with long storability. However, this can be achieved only by producing raw milk of exceptional quality.

Milk processing plants and dairy farms are interested in the production of basic material (raw milk) of a good quality. The importance of the quality of raw milk increased after Hungary joined the EU. The 853/2004/EK order and 68/2007 (VII.26.) FVM-EüM-SZMM order prescribes strict conditions for food hygiene and methods of handling and distribution of raw milk. On delivery of raw milk, the microbiological quality, especially total plate count of the milk is very important. Dairy farms have to produce raw milk of extra quality, because milk processing plants do not accept raw milk of other quality. Moreover milk quality significantly affects the income of farmers, as they get more money for better quality milk.

The composition of the milk – mainly fat and protein content – and the microbiological status significantly influence the economic value of milk and the quality of manufactured products. On delivery of milk, the general microbiological quality (total plate count, somatic cell count, *Staphylococcus aureus* count) of milk has to meet strict requirements, because these factors basically influence the industrial use of raw milk.

Milk with low numbers of microorganisms is a basic requirement for modern manufacturing processes, as well as for making products that will keep. Milk derived from mastitis cows plants can result in products with reduced consumer acceptability and nutritional value. The presence of fermentation inhibitors in milk is in contrast to the human-health requirements, spoils the industrial usage, and furthermore endangers the quality of the products and finally the consumption.

The coliform bacteria count of raw milk provides useful information about hygienic conditions of a dairy farm. Among coliform bacteria, *Escherichia coli* is a very good indicator of fecal contamination, and thus suitable for characterizing hygienic circumstances.

For the production of good quality milk, strict animal-health regulations, appropriate housing, milking and handling technology are necessary, as well as following the clearing-disinfection and other hygienic instructions.

Among the factors mentioned above, we focused primarily on studying hygienic and microbiological characteristics of the milk (total plate count, coliform count, *Escherichia coli* count, *Staphylococcus aureus* count, psychrotrophic bacteria count, yeast and mold count) and evaluation of the affects of these factors.

2. AIMS OF THE RESEARCH

In our study we set the following aims:

- to investigate the microbiological status of raw milk produced in dairy farms (total plate count, coliform count, *Escherichia coli* count, *Staphylococcus aureus* count, psychrotrophic bacteria count, yeast and mold count);
- to collect milk from cows suffering from mastitis and to collect samples from places of the farms and, where there was significant microbial contamination in the bulk tank milk, to identity sources of contamination;
- to examine the connection between housing-, milking technologies on the farm and microbiological quality of the milk;
- to evaluate hygienic circumstances of milking and milk handling of the farms, by examining coliform bacteria and *Escherichia coli* contamination;
- to quantify the occurrence of mastitis caused by *Staphylococcus aureus* and *Escherichia coli*;
- to examine the phenotypic (tellurite reduction, lecithinase activity, hemolysis, coagulase test, Clumping factor, antibiotic resistance) and genotypic (enterotoxin genes, pulsotype, phage type) characteristics of the *Staphylococcus aureus* strains occurring on dairy farms.

3. MATERIALS AND METHODS

Seven large (LF1 to LF7), four medium-sized (MF8 to MF11), and eleven small farms (SF12 to SF22) were involved in the study. Information on housing and milking circumstances were collected by questionnaires and personal visits. At the request of dairy farms, we did not publish the identification data (name, address, identification code). We considered the different farm sizes, housing and milking circumstances during the selection of farms. The examinations were carried out between 2005 and 2007 on several occasions. The farms were located in Hajdú-Bihar county, at distances of 15 km to 100 km from each other.
We used the annual milk yield to assign farm size. Accordingly:

- large farm (>1000 tons)
- medium-sized farm (100-1000 tons)
- small farm (<100 tons).

Main characteristics of the farms are summarized in **Table 1**. The examined large farms used loose cubicle (LF1, LF2, LF6 and LF7) or deep litter (LF3, LF4 and LF6) housing systems and milking parlours. Three large farms (LF3, LF4 and LF6) used disinfectant; two farms used water (LF2 and LF7) and two farms dry (LF1 and LF5) udder preparation. In the medium-sized farms, loose, deep litter (MF8) and tie-stall (MF9, MF10 and MF11) housing system all were used, as well as milking parlours, pipeline milking and bucket milking, and dry (teat dipping and wiping with paper towel) or disinfectant udder preparation were employed. Small farms used tie-stall housing system, bucket milking and udder preparation by water. Unfortunately, they did not use pre- or post-milking disinfection. In the large and medium-size farms, mainly Holstein Friesian, and in the small farms, Hungarian Red Pied breeds were found.

We performed the bacteriological examinations at the laboratory of the University of Debrecen, Centre of Agricultural Sciences, Faculty of Agriculture, Department of Agricultural Microbiology and the microbiological laboratory of the Animal Health and Food Control Station of Hajdú-Bihar county, furthermore, at the University of Veterinary Medicine Vienna, Institute for Milk Hygiene, Milk Technology and Food Science.

For all farms, we examined the total plate count, coliform count, *Escherichia coli* count, *Staphylococcus aureus* count, psychrotrophic bacteria count, as well as, yeast and mold count of the bulk tank milk samples.

Table 1.: Management characteristics of the farms

Farm	Size	Housing form	Milking form	Udder preparation	Pre-milking disinfect.	Post-milking disinfect.
LF1	Large	Loose cubicle	Milking parlour	Dry	+	+
LF2	Large	Loose cubicle	Milking parlour	Water	-	+
LF3	Large	Deep litter	Milking parlour	Disinfectant	+	-
LF4	Large	Deep litter	Milking parlour	Disinfectant	+	+
LF5	Large	Deep litter	Milking parlour	Dry	+	+
LF6	Large	Loose cubicle	Milking parlour	Disinfectant	+	+
LF7	Large	Loose cubicle	Milking parlour	Water	-	+
MF8	Medium	Deep litter	Milking parlour	Dry	+	+
MF9	Medium	Tie-stall	Bucket milking	Disinfectant	+	+
MF10	Medium	Tie-stall	Pipeline milking	Dry	+	+
MF11	Medium	Tie-stall	Pipeline milking	Dry	+	+
SF12	Small	Tie-stall	Bucket milking	Water	-	-
SF13	Small	Tie-stall	Bucket milking	Water	-	-
SF14	Small	Tie-stall	Bucket milking	Water	-	-
SF15	Small	Tie-stall	Bucket milking	Water	-	-
SF16	Small	Tie-stall	Bucket milking	Water	-	-
SF17	Small	Tie-stall	Bucket milking	Water	-	-
SF18	Small	Tie-stall	Bucket milking	Water	-	-
SF19	Small	Tie-stall	Bucket milking	Water	-	-
SF20	Small	Tie-stall	Bucket milking	Water	-	-
SF21	Small	Tie-stall	Bucket milking	Water	-	-
SF22	Small	Tie-stall	Bucket milking	Water	-	-

On some farms (LF1, LF3, LF4, SF21 and SF22) in which there was significant microbial contamination in the bulk tank milk we collected samples from the environment in order to find the sources of contamination. For environmental examination, we used swap samplers which were dipped into sterile peptone water. Before the beginning of milking procedure, we collected samples from the surfaces of the teat cups, milk tanks, milk tank pipes, milking buckets, milk-can, as well as from the hands of dairy men, wall of the milk room and from the surface of the teat after udder preparation. We took the samples from a 20 cm^2 surface. We determined the contamination of the surfaces by the bacteria count on 1 cm^2 surface (CFU/ cm^2).

In the case of three farms (LF3, LF4 and LF5) milk samples (10 ml) were collected from the udder quarter of clinical and sub clinical mastitis cows into sterile milk sampling pots. We examined the occurrence rate of *Escherichia coli*, and *Staphylococcus aureus* in these milk samples. We examined 211 (LF3), 343 (LF4) and 331 milk samples (LF5). Furthermore the owners of farms LF1, LF2 and LF7 put the results of mastitis examinations results (2005-2007) at my disposal. Between 2005 and 2007 in the LF1 farm 59 samples, in the LF2 farm 431 samples, and in the LF7 farm 157 milk samples were examined.

According to requirements of the **MSZ ISO 6610 (1993)** standard to examine the **total plate count** of the bulk tank milk and environmental samples, were used TGE-agar and aerobic incubation at 30^{o}C for 72±3 hours. We evaluated the total plate count of bulk tank milk samples by the 853/2004/EK order.

For the determination of **psychrotrophic bacteria count** used TGE-agar media, plates were incubated at 5-7^{o}C for 7 days in refrigerator.

For the examination of coliform bacteria count of bulk tank milk, udder quarter milk and environmental samples, we used violet-red-bile-lactose-agar (VRBL-agar) media and aerobic incubation at 30^{o}C for 24±2 hours, according to the instruction of the **MSZ ISO 5541-1 (1994)** standard.

According to requirements of the **MSZ EN ISO 6888-1 (2000)** standard, we examined the ***Staphylococcus aureus* count** of the bulk tank milk and environmental samples using Baird-Parker agar supplemented with Egg Yolk Tellurite Emulsion, with verification by coagulase probe. The plates were incubated under aerobic conditions at 37°C for 48±2 hours.

For the examination of the occurrence of *Staphylococcus aureus* in udder quarter milk samples and environmental samples we used Columbia blood agar and Baird-Parker agar, and coagulase probe for the verification.

According to the instruction of the **ISO 11866-1 (1997)** national standard to examine the **presumed *Escherichia coli* count** of the bulk tank milk samples, we used MPN (Most Probable Number) methods.

For the examination of *Escherichia coli* count of udder quarter milk and environmental samples, we used Colinstant agar media. The plates were incubated under aerobic conditions at 37°C for 24±2 hours.

According to the instruction of the **MSZ ISO 6611 (1993)** standard to examine the **yeast and molds count** of the bulk tank milk and environmental samples, we used oxytetracyclin-glucose-yeast-agar and aerobic incubation at 25^{o}C for 4 days.

We collected *Staphylococcus aureus* strains from the bulk tank milk of fourteen farms (LF2, LF3, LF4, LF5, LF7, MF8, MF9, SF12, SF16, SF17, SF18, SF19, SF20 and SF21) and from udder quarter milk of mastitis cows on two farms (LF4 and LF5). Fifty *St. aureus* isolates from bulk tank milk and nine isolates from udder quarter milk isolates were selected for epidemiological examinations. We performed the epidemiological examinations at the University of Veterinary Medicine Vienna, Institute for Milk Hygiene, Milk Technology and Food Science. We determined the phenotypic (tellurite reduction, lecithinase activity, hemolysis, coagulase test, Clumping factor, antibiotic resistance) and genotypic (enterotoxin genes, pulsotype, phage type) characteristics of all *Staphylococcus aureus* strains.

For the examination of phenotypic characteristics of the *St. aureus* strains we used Baird-Parker agar, Columbia blood agar and rabbit plasma. The tellurite reduction and lecithinase activity were examined on Baird-Parker agar, the examination of the hemolysis on Columbia blood agar. For the examination of coagulase test and Clumping factor, rabbit plasma was used.

Antimicrobial drug susceptibility testing of the isolates was performed on Mueller-Hinton agar by the disk diffusion method in accordance with Clinical Laboratory Standards Institute guidelines. The antimicrobial agents tested included penicillin (10 U/disk), methicillin (5 µg/disk), cefoxitin (30 µg/disk), lincomycin (15 µg/disk), tetracycline (30 µg/disk), erythromycin (15 µg/disk), and sulfamethoxazole/trimethoprim (23.75/1.25 µg/disk). *Staphylococcus aureus* ATCC 25923 was the control strain in every test run.

We examined the occurrence of nine ***St. aureus* enterotoxin genes** (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*) and the TSST-1 gene (*tst*) using **multiplex PCR assay** (**Table 2**). DNA

was amplified by 30 cycles of 95°C for 60 s, 55°C for 60 s and 72°C for 60 s with a final extension at 72°C for 10 min. The amplification was performed in a GeneAmp PCR System 9700 (Perkin-Elmer, Wellesley, MA) using Platinum *Taq* DNA polymerase (Invitrogen, Lofer, Austria). PCR products were resolved by 1.5% agarose gel electrophoresis and visualized by UV transillumination.

Table 2.: Primers for amplification of genes encoding Staphylococcal enterotoxins

Gene	Primer	Primer sequence (5´ to 3´)	Amplification size, bp
sea	GSEAR-1 GSEAR-2	GGT TAT CAA TGT GCG GGT GG CGG CAC TTT TTT CTC TTC GG	102
seb	GSEBR-1 GSEBR-2	GTA TGG TGG TGT AAC TGA GC CCA AAT AGT GAC GAG TTA GG	164
sec	GSECR-1 GSECR-2	AGA TGA AGT AGT TGA TGT GTA TGG CAC ACT TTT AGA ATC AAC CG	451
sed	GSEDR-1 GSEDR-2	CCA ATA ATA GGA GAA AAT AAA AG ATT GGT ATT TTT TTT CGT TC	278
see	GSEER-1 GSEER-2	AGG TTT TTT CAG AGG TCA TCC CTT TTT TTT CTT CGG TCA ATC	209
seg	SEG1 SEG2	TGC TAT CGA CAC ACT ACA ACC CCA GAT TCA AAT GCA GAA CC	704
seh	SEH1 SEH2	CGA AAG CAG AAG ATT TAC ACG GAC CTT TAC TTA TTT CGC TGT C	495
sei	SEI1 SEI2	GAC AAC AAA ACT GTC GAA ACT G CCA TAT TCT TTG CCT TTA CCA G	630
sej	SEJ-FW SEJ-RV	CAT CAG AAC TGT TGT TCC GCT AG CTG AAT TTT ACC ATC AAA GGT AC	142
tst	TSSTR-1 TSSTR-2	ACC CCT GTT CCC TTA TCA TC TTT TCA GTA TTT GTA ACG CC	326

Macrorestriction analysis of chromosomal DNA from *S. aureus* isolates was performed using the restriction enzyme *Sma*I (New England BioLabs, Beverly, MA) followed by **pulsed-field gel electrophoresis (PFGE)**. *Salmonella enterica* subsp. *enterica* serotype Braenderup H9812 digested with *Xba*I was used as a molecular size marker. DNA restriction fragments were analyzed by using the Molecular Analyst Fingerprinting II software package, version 3.0 (Bio-Rad). Similarity coefficients were calculated and dendrograms were constructed using the Dice coefficient and the unweighted pair group method with arithmetic averages, respectively, with an optimization value of 0.5% and a position tolerance of 1%. The cluster cutoff value was set at 86% similarity. Isolates with indistinguishable banding patterns (i.e., 100% similarity) were assigned to the same pulsotype; those with similarities

ranging from 86% to 99% were designated as subtypes, and those under 86% were classified as a different pulsotype. Main types were designated by capital letters and subtypes by capital letters followed by Arabic numerals.

St. aureus strains which could not be differentiated with serological and biochemical methods were discriminated using virulent phages. Bacteria plated on agar media were susceptible for the formation of lytic plaques by phages. **Bacteriophage typing** was performed with phages of the international basic set for typing *St. aureus* strains from human sources (group I: 29, 52, 52A, 79, and 80; group II: 55 and 116; group III: 6, 42E, 47, 53, 54, 75, 84, and 85; group V: 96; miscellaneous: 81, 95, 187 and 812) and with those accepted as the international basic set for typing *S. aureus* strains from bovine sources (group I: 80; group II: 116; group IV: 42D, 102, 107, 108, 111, and 117; miscellaneous: 78, 118, and 119). When a culture was negative at the routine test dilution (RTD), it was retested at 100-fold RTD.

Because of the performance of the statistical calculations, we converted the CFU/cm^3 results to decimal logarithmic values (log_{10}). We calculated mean, standard deviation; and minimum and maximum values from the data.

For the statistical analysis of the impact of each factor on total plate count, in the case of two levels for a factor, we used **t-test** or nonparametric **Mann-Whitney test**. In the case of three levels, we used **analysis of variance (ANOVA)** and **Tukey-test** or nonparametric **Kruskal-Wallis test** and comparative **Dunn's test**. Significance was evaluated at the $P<0.05$ level.

First we examined the factors which influenced the total plate count by **binary logistic regression.** Then, we used **loglinear model** for the analysis of cell frequency of the multi-dimensional table.

All statistical analyses were performed using SPSS v.13.0 and GraphPad Prism 3.02 statistical programs.

4. MAIN RESULTS OF THE THESIS

4.1. Microbiological quality of the bulk tank milk, udder quarter milk and environmental samples

Differences among farm sizes, housing types and milking procedures in microbiological characteristics of bulk tank milk were similar for total plate count, coliform count, yeast and molds count, and psychrotrophic bacteria count (**Table 3**). Bacterial contamination estimates generally were significantly higher in small farms, and in farms that used tie-stall housing, bucket milking, udder preparation with water, and which did not use pre- or post-milking disinfection. The explanation is that total plate count, coliform count, yeast and molds count, and psychrotrophic bacteria count correlated with each other and describe general contamination and hygienic condition.

Besides cooling, the milking procedure and the type of udder preparation had the largest effect on the **total plate count**, with milking procedure having a greater effect than type of udder preparation. Statistical analysis shows that in medium and small farms, the combination of pipeline milking – tie stall housing – disinfectant preparation of the udder and in large farms, the combination of milking parlour – loose cubicle housing system – dry preparation of the udder were most beneficial for low total plate count.

The presumed *E. coli* count did not differ significantly among housing forms, milking forms, udder preparation, and pre- and post-milking disinfection. Values were, however, significantly smaller in medium-sized farms, which suggests lower fresh fecal contamination. The results show that, if the udder of the cows is highly contaminated or rather the udder preparation is insufficient, and then the bulk tank milk can be easily contaminated by *E. coli* in any size farm.

Staphylococcus aureus counts of bulk tank milk were significantly smaller in farms which used loose cubicle housing system, pipeline milking and post-milking disinfection.

On the basis of the results we can say that even in the case of extra quality bulk tank milk (total plate count fewer than 100 thousand CFU/cm^3), high coliform, presumably *E. coli*, and yeast and mold counts can occur. The evaluation of microbiological quality of bulk tank milk only by total plate count is not adequate in every case.

Table 3.: Microorganism counts in bulk tank milk for different management factors

Factors	Types	Microorganisms count, Log_{10} CFU/cm^3 mean ± standard deviation						
		Total plate	Coliform	Pres. *E. coli*	*St. aureus*	Yeast	Molds	Psychrotrophic
Farm size	Large	4,49±0,51 [b]	2,53±0,80 [b]	1,65±0,73 [a]	1,78±1,25 [a]	2,33±1,43 [b]	0,97±0,69 [b]	4,39±0,51 [b]
	Medium	4,07±0,53 [b]	1,77±1,18 [b]	1,09±1,05 [b]	1,05±1,07 [a]	2,24±1,48 [b]	1,03±0,67 [b]	3,78±0,72 [b]
	Small	6,09±0,74 [a]	4,53±0,92 [a]	1,81±1,11 [a]	1,75±1,58 [a]	3,58±0,97 [a]	2,28±1,16 [a]	5,76±0,63 [a]
Housing type	Loose cubicle	4,49±0,60 [b]	2,64±0,90 [b]	1,62±0,81 [a]	0,94±1,12 [b]	2,58±1,43 [b]	1,00±0,79 [b]	4,71±0,39 [b]
	Deep litter	4,39±0,42 [b]	2,26±0,76 [b]	1,48±0,70 [a]	2,42±0,77 [a]	2,09±1,43 [b]	0,90±0,52 [b]	3,87±0,36 [b]
	Tie-stall	5,55±1,23 [a]	3,88±1,59 [a]	1,67±1,13 [a]	1,55±1,54 [a]	3,19±1,29 [a]	1,99±1,17 [a]	5,12±1,16 [b]
Milking procedure	Milking parlour	4,43±0,50 [b]	2,43±0,84 [b]	1,55±0,75 [a]	1,75±1,19 [a]	2,33±1,43 [b]	0,95±0,67 [b]	4,29±0,55 [b]
	Pipeline milking	3,79±0,57 [b]	1,07±1,13 [b]	1,00±1,75 [a]	0,00±0,00 [b]	1,80±1,19 [b]	1,04±0,69 [b]	3,61±1,09 [b]
	Bucket milking	5,95±0,95 [a]	4,40±1,01 [a]	1,77±1,12 [a]	1,74±1,53 [a]	3,44±1,15 [a]	2,14±1,17 [a]	5,55±0,80 [a]
Udder preparation	Dry	4,17±0,56 [b]	1,86±1,11 [b]	1,26±0,97 [a]	1,56±0,99 [a]	2,17±1,37 [b]	0,92±0,64 [b]	3,90±0,68 [b]
	Disinfectant	4,50±0,35 [b]	2,61±0,73 [b]	1,60±0,85 [a]	2,26±1,09 [a]	2,39±1,57 [b]	1,00±0,65 [b]	4,22±0,64 [b]
	Water	5,68±1,02 [a]	4,13±1,26 [a]	1,77±1,01 [a]	1,42±1,58 [a]	3,25±1,21 [a]	2,01±1,19 [a]	5,49±0,72 [a]
Pre-milking disinfection	Yes	4,32±0,50 [b]	2,23±1,00 [b]	1,43±0,92 [a]	1,91±1,09 [a]	2,29±1,47 [b]	0,96±0,64 [b]	4,05±0,64 [b]
	No	5,68±1,02 [a]	4,13±1,26 [a]	1,77±1,01 [a]	1,42±1,58 [a]	3,25±1,21 [a]	2,01±1,19 [a]	5,49±0,72 [a]
Post-milking disinfection	Yes	4,32±0,57 [b]	2,30±1,01 [b]	1,51±0,89 [a]	1,35±1,20 [b]	2,40±1,46 [b]	1,01±0,69 [b]	4,20±0,66 [b]
	No	5,80±0,92 [a]	4,21±1,25 [a]	1,74±1,07 [a]	1,92±1,50 [a]	3,26±1,22 [a]	2,06±1,21 [a]	5,48±0,94 [a]

[a, b] The means with different superscripts differ at P<0,05

During the examination of the environmental samples, we found that contamination with mesophylic aerobic germs was larger in small farms than in large farms. Results suggest that the contamination of equipment used during the milking and handling of milk significantly influence the total plate count of bulk tank milk.

The contamination of the environmental samples by **coliform bacteria** on small farms was larger than on large farms. Milking equipment contaminated by coliform bacteria and inefficiently cleaned teat surfaces can contribute substantially to the contamination of the bulk tank milk. In small farms the high coliform counts of bulk tank milk were primarily caused by the contamination of the milking and milk handling equipment (teat cup, milking bucket, milk cooler tank and milk-can).

During the examination of the ***Escherichia coli*** contamination of environmental samples, we found that samples taken from the teat surfaces were contaminated in all five farms (LF1, LF3, LF4, SF21, SF22), creating a possible source of contamination of bulk tank milk. In addition we could demonstrate *E. coli* on the surface of the dairy man's hand, teat cup and milking bucket. In the SF22, contamination of teat surface and equipment could also contribute to the high *E. coli* count of bulk tank milk.

During the examination of the udder quarter milk samples derived from mastitic cows, the results show that, in some farms (LF2, LF3, LF4 and LF7) in which the occurrence of mastitis caused by *E. coli* was relatively high, there would be risk of contamination of the bulk tank milk if farmers could not separate the ill cows at each milking.

If farmers conscientiously check the first few milk squirts every time, then the contamination of the bulk tank milk by mastitic cow's milk can be avoided. In this case there would be no contamination, in spite of the high occurrence rate of *E. coli*.

These high values may correlate with hygienic conditions of housing type (inadequate littering) and the state of health. On the basis of the results we conclude that in the case of bulk tank milk of small farms the high *E. coli* values may primarily be caused by the contaminated equipment and inadequate hygienic circumstances.

During the examination of ***Staphylococcus aureus*** contamination of the environmental samples we did not find prominently high counts which could cause the contamination of bulk tank milk. Out of the environmental samples examined, small bacteria numbers could be detected only on the surface of teats. During the examination of the udder quarter milk

samples derived from mastitic cows we found that, in farms where subclinical mastitis caused by *St. aureus* often occurred the *St. aureus* count of bulk tank milk was generally higher.

During the examination of contamination of environmental samples by **yeast and molds** we found that more samples were contaminated in small farms than in large farms. In large farms, yeast and molds occurred only sporadically (milk tank, milk tank pipe), while in small farms they were detected in almost every sample (from the surface of teat cup, milk tank, milk tank pipe, milking bucket, milk-can and teat after udder preparation). We detected molds on all five farms on the surface of ready for milking teats. The results show that contamination with yeast and molds of bulk tank commonly observed milk in small farms was caused by contamination of the milking and milk handling equipment.

On the basis of microbiological results, analysis of answers on our questionnaires, as well as by checking milking procedures in small farms, the following mistakes occurred, which could contribute to the poor milk quality:

- they use water for washing of inadequate temperature or cleanliness;
- they do not change the water used for teat washing;
- they often wash the teats of all cows with the same bucket water and rag;
- the drying of teat is often ignored or they use the same rag in the case of all cows;
- the milking of the first few milk squirts is not collected in a test cup for examination;
- the examination of first few milk squirts is cancelled (so the selection and separation of mastitis cows cannot be done);
- the pre- and post-milking disinfection of teats is cancelled;
- the washing of milking and milk handling equipment is inadequate;
- the technological status of milking machine and teat cups is inadequate (teat cups are changed more rarely than the prescribed 6 months or 2500 working hours);
- they cool the raw milk in milk coolers which are in bad status (often breaking down);
- they do not in every case cool down the morning milk before they transport it to the milk collecting center.

4.2. The main characteristics of the *Staphylococcus aureus* isolates which were collected from milk samples

During the examination of the phenotypic characteristics of *Staphylococcus aureus* strains, we found that all 59 strains were coagulase and Clumping factor positive. During the tellurite reduction, the colony color of two types (grey, black) occurred in fifty-fifty proportion. Most of the isolates showed strong lecithinase activity (38 isolates) and α-β hemolysis (31 isolates). All isolates were uniformly susceptible to methicillin, cefoxitin, lincomycin, tetracycline, erythromycin, and sulfamethoxazole/trimethoprim. Forty-one isolates were also susceptible to penicillin, whereas 18 isolates (30.5%) were resistant (**Table 4**). The prevalence rates of penicillin resistance were 20.0% and 88.9% among the *St. aureus* strains isolated from bulk tank milk and mastitic quarter milk, respectively. These values are in agreement with the published values.

During the examination of the occurrence of Staphylococcal enterotoxin (SE) genes 16 (27.1%) of the 59 *St. aureus* isolates were positive (**Table 4**). Fifteen of them carried just one gene and one strain carried two genes (*seg* and *sei*). These occurrence rates are considerably lower than those obtained by other authors who reported that approximately 55% to 80% of the *St. aureus* strains isolated from mastitic or bulk milk samples harbored at least one of the enterotoxin genes studied. *Seb, sea* and *sec* genes were the most commonly detected SE genotypes in our study. Classical SE genes (*sea* to *sed*) were detected only in strains isolated from bulk tank milk, whereas the only strain (no. 26) harboring newly described SE genes (*seg* and *sei*) originated from mastitic quarter milk. It should also be noted that none of our isolates possessed the *see*, *seh, sej*, or *tst* genes. On 15 out of 20 farms tested no enterotoxigenic staphylococci were recovered from either bulk tank milk or mastitic quarter milk.

Pulsed-field gel electrophoresis (PFGE) analysis of *Staphylococcus aureus* isolates showed 22 distinct pulsotypes, including 14 main types and 8 subtypes, at a similarity level of 86% (**Figure 1 and Table 4**). Isolates from bulk tank milk were divided into 13 main types (A to D and F to N) and 7 subtypes (A1, A2, C1, F1, G1, J1, and L1). The *St. aureus* strains originating from quarter milk belonged to 3 main types (D, E, and F) and 1 subtype (D1). On each of the farms tested, only one or two main types were observed, indicating a lack of genetic diversity among *St. aureus* isolates within farms. There were only two pulsotypes (D

and N) which occurred on more than one farm. The PFGE patterns revealed genetic relationship between the strains recovered from udder quarter milk and bulk tank milk on both farms (i.e., LF4 and LF5) where *St. aureus* isolates of quarter milk origin were available. A plausible explanation for this finding is that on farms having a high number of cows with subclinical or clinical mastitis, as was the case on LF4 and LF5, *St. aureus* from infected udders may contaminate bulk milk and, as a result, raw milk products. The main sources of contamination of bulk tank milk by *St. aureus* are the infected udder quarters. We can avoid the contamination by the separation of mastitic cows' milk.

The results in **Table 4** also indicate that the *sea*, *seb*, *sec*, *sed*, and *seg*/*sei* genes were harbored by pulsotypes C, K/F1, I/C1, B, and F, respectively; and the penicillin resistant isolates belonged to 7 pulsotypes, including main types D, F, I, and K and subtypes A2, D1, and F1.

Out of the 59 *St. aureus* strains tested, 8 (13.5%) were non-typeable by the phages. Most (62.7%) of the 51 typeable strains were recognized by phages in group III or IV of the international basic set (**Table 4**).

We could classify the isolates into ten phage groups (**Table 5**). On the basis of the occurrence of the lysis-causing phages, we could classify the isolates into additional subgroups. By the phage typing, we could distinguish most of the isolates of different pulsotype and subtype. One third of the isolates belonged to the III-IV and III-IV-81 phage groups. Based on the results of phage typing, the majority of isolates were found to be of human-bovine biotypes.

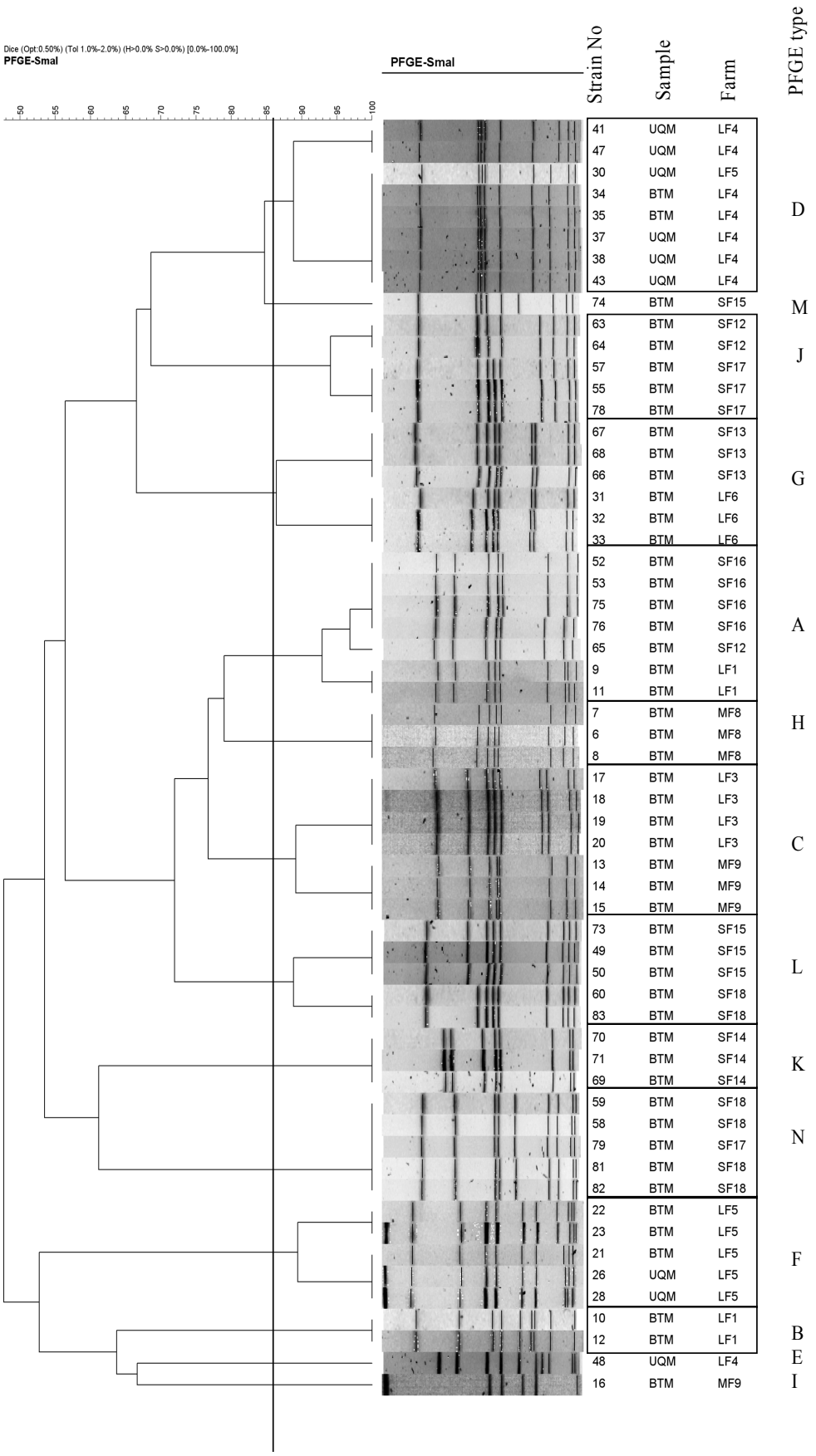

Figure 1.: Dendrogram showing the level of similarity between *St. aureus* isolates

Table 4.: Main characteristics of *Staphylococcus aureus* isolates

Farms	Pulso-type	Total no. of isolates	Origin of isolates	Phenotypic properties						Enterotoxin gene	Phage group
				Tellurite reduction	Lecithinase activity	Hemolysis	Clump. factor	Coag. test	Antibiogram pattern		
LF2	A	2	BTM	Gray	Strong	α-β	+	+	S	None	NT
	B	2	BTM	Black	Weak	α-β	+	+	S	*sed*	NT
LF3	C	4	BTM	Black	Strong	Weak	+	+	S	*sea*	III-IV
LF4	D	5	BTM, UQM	Black	None	α	+	+	R (Pen)	None	III-IV-812
	D1	2	BTM	Black	None	α	+	+	R (Pen)	None	III-81
	E	1	BTM	Gray	None	α-β	+	+	S	None	78
LF5	D	1	BTM	Black	Weak	α	+	+	R (Pen)	None	III-IV-812
	F	3	BTM, UQM	Black	Strong	Weak	+	+	R (Pen)	*seg/sei* (1), None (2)	II
	F1	2	BTM	Gray	Strong	Weak	+	+	R (Pen)	*seb (3)*	II
LF6	G	3	BTM	Gray	Strong	β	+	+	S	None	III-IV
MF8	H	3	BTM	Gray	Strong	α-β	+	+	S	None	III-IV
MF9	C1	3	BTM	Black	Strong	α-β (2), W (1)	+	+	S	*sec*	NT
	I	1	BTM	Gray	Strong	Weak	+	+	R (Pen)	*sec*	NT
SF12	K	3	BTM	Gray	Strong	α-β	+	+	R (Pen)	*seb*	81
SF16	A2	1	BTM	Black	Strong	α-β	+	+	R (Pen)	None	III-IV-81
	J	2	BTM	Black	None	α-β	+	+	S	None	IV
SF17	G1	3	BTM	Black	Strong	α-β	+	+	S	None	III-IV-81
SF18	L	3	BTM	Gray	Strong	α-β	+	+	S	None	III-81
	M	1	BTM	Black	None	α-β	+	+	S	None	IV
SF19	A1	4	BTM	Gray	Weak	Weak	+	+	S	None	III-IV-81
SF20	J1	3	BTM	Black	None	α-β	+	+	S	None	III-IV-81
	N	1	BTM	Gray	Strong	α-β	+	+	S	None	III-IV-81
SF21	L1	2	BTM	Gray	Strong	Weak	+	+	S	None	III
	N	4	BTM	Gray	Strong	α-β	+	+	S	None	III-IV-81-118

Clump.: Clumping, Coag.: Coagulase, BTM: bulk tank milk, UQM: udder quarter milk, S: Susceptible to penicillin, R (Pen): Resistant to penicillin

Table 5: The distribution of *St. aureus* isolates belonging to different phage groups

Phage groups	**Number of isolates**
II	5
III	2
IV	3
78	1
81	3
III-81	5
III-IV	10
III-IV-81	12
III-IV-812	6
III-IV-81-118	4
Non-typeable	8

5. NEW SCIENTIFIC RESULTS

The following new scientific conclusions can be drawn from the results:

1. The total plate count, coliform count, yeast- and molds count, and psychrotrophic bacteria count of bulk tank milk samples were significantly higher in small farms, and in farms which used tie-stall housing, bucket milking, udder preparation with water, and which did not use pre- and post-milking disinfection. Out of the examined factors, milking procedure and the type of udder preparation had the largest effect on the total plate count. In the medium and small farms that were examined, the combination of pipeline milking – tie stall housing system – disinfectant preparation of the udder resulted in the most favorable milk quality. In large farms, the combination of milking parlour - loose cubicle housing system - dry preparation of the udder was the most appropriate as shown by the total plate count.
2. The milk derived from subclinical mastitic cows largely influence the *Staphylococcus aureus* count of bulk tank milk, than the contamination of milking and milk handling equipment by *St. aureus*.
3. The examined *St. aureus* strains were all coagulase positive. 30.5% of the isolates were resistant to penicillin. The prevalence rates of penicillin resistance were 20.0% and 88.9% among the *St. aureus* strains isolated from bulk tank milk and mastitic quarter milk, respectively.
4. 27.1% of the *St. aureus* isolates carried Staphylococcal enterotoxin (SE) gene. *Seb, sea* and *sec* genes were the most commonly detected SE genotypes. During the pulsed-field gel electrophoresis (PFGE) analysis isolates were categorized into 14 main types and 8 subtypes, at a similarity level of 86%. On each of the farms tested only one or two main types were observed, indicating a lack of genetic diversity among *St. aureus* isolates within farms.
5. During the phage typing of the *St. aureus* strains, most of the isolates (62.7%) were recognized by phages in group III or IV of the international basic set. We could classify the isolates into ten phage groups. One third of the isolates belonged to the III-IV and III-IV-81 phage groups.

6. PRACTICAL UTILITY OF THE RESULTS

➢ During our investigations we found that in farms which use tie-stall housing forms, bucket milking, udder preparation with water, and which do not use pre- and post-milking disinfection the microbiological quality of bulk tank milk is generally less favorable. These results place emphasis on the importance of pre- and post-milking disinfection. In our opinion, although in large farms technical and environmental factors of milk production are more favorable, under adequate circumstances, and by meeting the hygienic requirements, small farms using tie stall housing system and bucket milking may produce good quality milk.

➢ We also state that the milking procedure and the type of udder preparation had larger effects on the total plate count than other factor that were examined. In medium and small farms, the combination of pipeline milking – tie stall housing system – disinfectant preparation of the udder; and in large farms the combination of milking parlour – loose cubicle housing system – dry preparation of the udder produced milk of highest quality, as shown by the total plate count.

➢ During the examination of microbiological variables (total plate count, coliform count, *Escherichia coli* count, *Staphylococcus aureus* count, psychrotrophic bacteria count, yeast and mold count) of the bulk tank milk samples we found that, in the case of extra quality bulk tank milk (total plate count fewer than 100 thousand CFU/cm^3) high coliform count, presumed *E. coli* count, and yeast and molds count can occur. The judgment of the microbiological quality of bulk tank milk by the total plate count only is not reliable in every case. Therefore it would be useful from time to time to examine other microbiological parameters during the acceptance of raw milk.

➢ On the basis of the examination of bulk tank and udder quarter milk samples we would like to attract the attention of dairy farms to the regular monitoring of the milking cows (with test tray), and furthermore to the practical advantage of taking samples from mastitis cows to carry out bacteriological and resistance examination. Analysis of regular bacteriological and resistance examination results allow the selection and conscious medication of mastitic cows. In view of the above information, farms can guard against pathogens and hereby the conscious therapy is also more effective.

➢ The results of the examination of environmental samples emphasize to dairy farms, how important it is to ensure the hygienic circumstances during housing, milking and milk handling (regular littering and manure removal, furthermore the washing and flushing of milking and milk handling equipments) in the aspect of microbiological quality of raw milk.

➢ Small farms only rarely receive feedback about the microbiological quality of raw milk which they produce and transport to the milk collecting center. Our research work partially alleviates that deficiency; together with large and medium-sized farms also examined, small farms could get feedback about the microbiological quality of raw milk they had produced. Because we returned the results of the examinations to farms on each occasion, furthermore at the personal meetings there was possibility to discuss the reasons which may cause higher contamination values. Presenting the results of environmental investigations, we attract the attention of farmers to what kind of hygienic deficiencies may occur in farms, furthermore what kind of changes should be made to produce better quality milk.

➢ The information which was acquired during the examination of the phenotypic (tellurite reduction, lecithinase activity, hemolysis, coagulase test, Clumping factor, antibiotic resistance) and genotypic (enterotoxin genes, pulsotype, phage type) characteristics of the *Staphylococcus aureus* strains collected from dairy farms (bulk tank and udder quarter milk) contribute to knowledge of *St. aureus* strains (antibiotic resistance, pulsotype etc.) which occur in dairy farms, the more effective protection against them, and the effective therapy.

7. PUBLICATIONS RELATED TO THE THESIS

Reviewed scientific publications:

F. Peles, P. Keresztúri, A. Iglói, A. Szabó (2006): The effect of environmental factors on the microbiological quality of bulk tank milk. Cereal Research Communications. 2006. 34. 1. 755-758 p. (IF: 1,037)

Peles Ferenc, Szabó András, Béri Béla, Keresztúri Péter (2006): A nyerstej-minták feltételezetten *Escherichia coli* számának vizsgálata néhány tejtermelő gazdaságban. Agrártudományi Közlemények. Különszám. 2006. 21. 31-37 p.

Ferenc Peles, Martin Wagner, Petra Rieck, Péter Keresztúri, Béla Béri, András Szabó (2006): Occurrence of enterotoxin-producing *Staphylococcus aureus* on several dairy farms of Hajdú-Bihar county. Acta Microbiologica et Immunologica Hungarica. 2006. 53. 3. 330 p.

Ferenc Peles, Martin Wagner, Ingeborg Hein, Petra Rieck, Klaus Gutser, Péter Keresztúri, Béla Béri, András Szabó (2006): Characterization of *Staphylococcus aureus* isolates recovered from milk samples. Acta Microbiologica et Immunologica Hungarica. 2006. 53. 3. 329 p.

Peles Ferenc, Martin Wagner, Keresztúri Péter, Béri Béla, Szabó András (2007): *Staphylococcus aureus* törzsek főbb jellemzői és előfordulásuk két Hajdú-Bihar megyei tejtermelő gazdaságban. Agrártudományi Közlemények. Különszám. 2007. 26. 34-39 p.

F. Peles, M. Wagner, L. Varga, I. Hein, P. Rieck, K. Gutser, P. Keresztúri, G. Kardos, I. Turcsányi, B. Béri, A. Szabó (2007): Characterization of *Staphylococcus aureus* strains isolated from bovine milk in Hungary. International Journal of Food Microbiology. 2007. 118. 2. 186-193 p. (IF: 2,608)

Peles Ferenc, Kovács Sándor, Béri Béla, Szabó András (2007): A nyers tej összcsíraszámát befolyásoló tényezők összehasonlító vizsgálata néhány Hajdú-Bihar megyei tejtermelő gazdaságban. Állattenyésztés és Takarmányozás. 2007. 56. 4. 333-342 p.

Ferenc Peles, Sándor Kovács, Béla Béri, András Szabó (2007): Comparative analysis of factors influencing total plate count of raw milk in some dairy farms in Hungary. Acta Microbiologica et Immunologica Hungarica. 2007. 54. Suppl. 1. 99-100 p.

Conference proceedings in Hungarian and foreign language:

Peles F., Iglói A., Keresztúri P., Szabó A. (2004): A nyers tej mikrobiológiai minőségének az alakulása Hajdú-Bihar megye eltérő méretű gazdaságaiban. A Magyar Mikrobiológiai Társaság 2004. évi Nagygyűlése és a X. Fermentációs Kollokvium. Keszthely. 2004. október 7-9.

Peles F., Keresztúri P., Iglói A., Szabó A. (2005): Néhány Hajdú-Bihar megyei tejtermelő gazdaság értékelése a tartás és a tejminőség szempontjából. XI. Ifjúsági Tudományos Fórum. Keszthely. 2005. március 24.

Peles F., Keresztúri P. (2005): A tartásmód hatása a nyers tej mikrobiológiai állapotára. Tavaszi Szél Konferencia. Debrecen. 2005. május 5-8.

Peles F., Keresztúri P., Iglói A., Béri B., Szabó A. (2006): A *Staphylococcus aureus* jelentősége és előfordulása néhány Hajdú-Bihar megyei tejtermelő gazdaságban. XXXI. Óvári Tudományos Nap. 2006. október 5.

Peles F., Wagner M., Keresztúri P., Béri B., Szabó A. (2006): *Staphylococcus aureus* törzsek főbb jellemzői és előfordulásuk a tejtermelő gazdaságokban. A jövő tudósai, a vidék jövője doktoranduszok konferenciája. Debrecen, 2006. november 23.

Peles F., Keresztúri P., Szigeti Zs., Béri B., Szabó A. (2007): Az elegytej kóliform számának a vizsgálata néhány Hajdú-Bihar megyei tejtermelő gazdaságban. XLIX. Georgikon Napok. Keszthely, 2007. szeptember 20-21.