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Effect of production season on technological suitability, fatty acids profile and cholesterol content in milk of cows maintained in free-stall barns and fed according to the TMR system*

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The study included 1146 milk samples (526 – spring-summer season and 620 – autumn--winter season) taken from 274 cows maintained in five farms with an intensive husbandry technology (free-stall barns and TMR feeding). Following parameters were determined: content of casein and non-fat dry matter, protein to fat ratio, active and potential acidity, thermal stability, rennet coagulation time, state of milk fat dispersion, share of fatty acids and cholesterol content. Significantly (p≤0.01) higher daily milk yield were obtained in the spring-summer season (25.8 kg) compared to the autumn--winter season (23.3 kg). Milk collected in the spring-summer season was characterised by significantly (p≤0.01) higher active acidity (pH 6.64), shorter rennet coagulation time (an average of 4:21 min), lower thermal stability (an average of 2:40 min) and higher share of small fat globules (65.97%). Moreover, it contained significantly more cholesterol (22.71 mg/100 ml). Generally, with year-round TMR feeding system of cows, the production season had no significant effect on share of fatty acids in milk, with the exception of UFA and MUFA, and PUFA/SFA and PUFA/SFA proportion (p≤0.05).

KEY WORDS: milk / technological suitability / fatty acids / cholesterol / production season / TMR feeding system

The Polish dairy sector is still undergoing considerable changes. The population of dairy cows has been decreasing systematically, although not as dramatically as in the 1990s. According to the data given by the Central Statistical Office GUS [18] in 2011 it was 2,626 thousand, while in 2005 – 2,795 thousand. The number of milk suppliers has also been decreasing systematically from 168,000 in the quota year of 2010/2011 to 156,000 in 2011/2012 [23], whereas the number of cows on individual farms is increasing.

*Synopsis of PhD dissertation

Now farms keeping from 20 to 100 cows dominate among milk producers. Milk yield per cow is increasing systematically, in 2012 reaching 7396 kg milk (an increase by 2017 kg from 2000) and it is now comparable to that in the EU leading milk producers [19, 20, 21].

These changes result from the accelerating concentration of production connected with breeding and technological progress. A growing number of dairy farms no longer apply traditional cow management systems, i.e. stanchion barns and feeding strictly divided into the summer season (with pasture grazing) and the winter season. In contrast, intensive milk production systems are being introduced on many farms, such as loose barns, in which feeding is based on complete feed and it is uniform through the year (TMR or PMR). This reduces labour intensity and time consumption of animal handling and thus increases efficiency of commercial milk production [24].

The aim of this study was to assess the effect of the production season as a major factor in the traditional system, which influences processability, the fatty acid profile and cholesterol content in milk obtained from cows managed in intensive production technologies.

Material and methods

The experimental material comprised 1146 milk samples collected from 274 cows kept in 5 loose barns and fed TMR throughout the year, i.e. 526 samples collected in the spring--summer season (May-August) and 620 in the autumn-winter season (November-March). Twice a year (in the spring-summer and the autumn-winter seasons) milk samples were collected from individual cows from the entire milk volume to 250 ml plastic bottles. Samples from cows with udder diseases were discarded. Milk was transported in cooler bags with gel packs to the laboratory of the Department of Commodity Science and Processing of Animal Raw Materials, the University of Life Sciences in Lublin [4].

The following parameters were determined in each milk sample:

– casein content, according to AOAC [3];

– solids-not-fat content using an Infrared Milk Analyzer by Bentley (the protein to fat ratio was also calculated);

– active acidity (pH) using a Pioneer 65 pH-meter by Radiometer Analytical;

– potential acidity (^{o}SH) by titration according to PN-86/A-86122;

– heat stability at 140^{o}C in an oil bath by TEWES-BIS according to White and Davies [8];

– rennet-induced milk coagulation time using the method proposed by Schern (the moment when the first casein flakes are formed) [8];

– the proportion of fat globules in individual size fractions, i.e. <6 μm, 7-10 μm and >10 μm (by microscopy in x1000 magnification in preparations stained with Sudan III) [8].

Moreover, somatic cell count (SCC) was determined using a Somacount 150 apparatus by Bentley in order to eliminate milk samples with SCC over 400 thousand/ml.

Additionally, the fatty acid profile and cholesterol content were determined in a representative number of 193 milk samples.

The fatty acid composition was assayed by gas chromatography using a Varian GC 3900 apparatus equipped with a flame ionization detector (FID) and a CP 7420 capillary column of 100 m. Analyses were run under variable temperature conditions. Initial temperature of the column oven was 50ºC, while the final temperature was 260ºC, whereas the operating temperature for the sample injector and detector was 270ºC. Hydrogen flow rate was 28 ml/min, air flow rate was 300 ml/min and make-up flow rate was 30 ml/min. The volume of the dosed sample was 1 µl, at a split ratio of 50. The percentage content of fatty acids was calculated using a Star GC Workstion Version 5.5 programme based on retention times of reference standards of fatty acid methyl esters. Milk samples were prepared for these analyses according to the AOAC standards [1, 2].

The following groups were detected in the analyses of the fatty acid profile:

– saturated fatty acids (SFA), including short- and medium-length fatty acids (SFAsmc), including C4:0 to C14:0 acids and long-chain saturated fatty acids (SFAlc) from C15:0 to C22:0;

– unsaturated fatty acids (UFA), including monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA).

Additionally, ratios of these acids, i.e. SFA/UFA, MUFA/SFA and PUFA/SFA, were calculated.

Cholesterol content was determined using the methodology developed by the Institute of Animal Science in Balice with the original modifications.

Data concerning daily yields of analysed cows came from breeding records kept by the Polish Federation of Cattle Breeders and Dairy Farmers.

The results were analysed statistically using the StatSoft Inc. STATISTICA ver. 6 programme. Significance of differences between means was determined using Tukey's test for varying populations at p(alfa)=0.05 and p=0.01.

Pearson's linear correlation coefficients were calculated for selected indexes of nutritive value and processability of milk. Significance of obtained correlations was determined at p=0.05; p=0.01 and p=0.001.

Results and discussion

Significantly higher ($p \leq 0.01$) daily milk yield was recorded in the spring-summer season (25.8 kg) in comparison to the autumn-winter season (23.3 kg).

It results from data contained in Table 1 that the production season had a significant effect ($p \leq 0.01$) on active acidity, heat stability and rennet-induced milk coagulation as well as the percentage shares of fat globules in individual size fractions. Milk collected in the spring-summer season was characterised by a higher active acidity pH (by 0.02) and a significantly shorter rennet-induced clotting time (on average by 0:29 min) and a lower heat stability (on average by 0:52 min).

Similar results were reported by Barłowska and Litwińczuk [5]. Cows of three breeds evaluated in their study in the spring-summer season produced milk which was thermally

Table 1
Selected technological suitability indicators of milk, taking production season into account

Specification		Production season	
		spring-summer	autumn-winter
n		526	620
Acidity			
pH	x	6.64^{A}	6.68^{B}
	SD	0.08	0.09
°SH	x	7.33	6.98
	SD	0.95	0.85
Non-fat dry matter (%)	x	9.02	9.13
	SD	0.52	0.52
Casein (%)	x	2.83	2.90
	SD	0.39	0.42
Protein-to-fat ratio	x	0.80	0.83
	SD	0.11	0.28
Clotting time (min)	x	4:21^{A}	5:29^{B}
	SD	2:26	2:36
Heat stability (min)	x	2:40^{A}	3:23^{B}
	SD	1:21	1:18
Fat globules (%)			
small (<6 µm)	x	65.97^{B}	57.89^{A}
	SD	8.24	10.98
medium (6-10 µm)	x	26.46^{A}	30.57^{B}
	SD	5.15	5.77
large (>10 µm)	x	8.80^{A}	10.60^{B}
	SD	3.56	4.17

A, B – differences significant at p≤0.01

stable for 3:15 min, while in the autumn-winter season it was thermally stable for 5:48 min. Similarly as in this this study, rennet clotting time was also shorter in the spring and summer months, amounting to 6:37 min, while in the autumn and winter milk coagulated under the influence of this enzyme after as long as 7:48 min. De Marchi et al. [12] also showed a shorter coagulation time in milk of cows in the summer months. Similarly as in this study, Brodziak et al. [10] reported a higher share of casein in milk in the autumn-winter season, statistically confirmed in Black-and-White Holstein-Friesian (p≤0.05) and Jersey cows (p≤0.01), by 0.08 and by 0.27 percentage points (pp), respectively.

Milk collected in the spring-summer season was characterised by a higher (p≤0.01) percentage share of small fat globules (at a lower share of medium-sized and large globules) in comparison to the autumn and winter months. Barłowska and Litwińczuk [5] showed a total share of small fat globules lower by 7.41 pp in milk of Black-and-White Holstein-Friesian, Simmental and White-back cows in the autumn-winter season in relation to the spring-summer season and the share of medium and large globules higher by 1.13 pp and 5.84 pp in those breeds in the autumn and winter.

Table 2
Fatty acid profile and cholesterol content of milk, taking production season into account

Production season	n		Fatty acid (%)							The ratio between the fatty acids			Cholesterol (mg/100 ml)
			SFA	SFAsmc	SFAlc	UFA	MUFA	PUFA	CLA	SFA/UFA	MUFA/SFA	PUFA/SFA	
Spring-summer	91	x	70.89	23.98	47.58	29.01[b]	25.99	3.02[b]	0.31	2.63	0.37[b]	0.04[b]	22.71[B]
		SD	4.89	3.88	4.21	3.01	4.49	0.60	0.08	0.36	0.09	0.01	13.11
Autumn-winter	102	x	71.69	24.11	46.90	27.62[a]	24.83	2.79[a]	0.29	2.53	0.34[a]	0.03[a]	18.69[A]
		SD	3.20	3.64	3.63	3.01	2.79	0.68	0.06	0.56	0.05	0.01	8.91

a, b – differences significant at $p \leq 0.05$; A, B – differences significant at $p \leq 0.01$

Table 2 presents percentage shares for individual groups of fatty acids, their ratios and cholesterol content in milk of evaluated cows depending on the season. Generally no significant differences were shown between the spring-summer and autumn-winter seasons in the shares of individual fatty acid families, except for UFA and PUFA, the MUFA/SFA and PUFA/SFA ratios ($p \le 0.05$) and cholesterol content ($p \le 0.01$). In the case of UFA and PUFA higher concentrations of these acids were recorded in milk in the spring-summer season (by 1.39 pp for UFA and 0.23 pp for PUFA, respectively). Similarly, statistically significant ($p \le 0.05$) seasonal dependencies were observed for the MUFA/SFA and PUFA/SFA ratios to the advantage of the spring-summer season.

These results are confirmed by findings presented by other authors [11, 13, 17]. Barłowska et al. [6] also showed this regularity for PUFA and the PUFA:UFA ratio. Also Lipiński et al. [15] reported a higher content of unsaturated fatty acids in milk in the spring-summer season. A lack of significant differences in the fatty acid profile between the production seasons is probably a consequence of the uniform supply of nutrients with the TMR feed ration, which composition is as a rule constant and not subject to seasonal changes.

Analysis of seasonal differences in cholesterol content in milk of investigated cows (Table 2) showed a significantly higher ($p \le 0.01$) level of this component in the spring-summer season (22.71 mg/100 ml) in comparison to the autumn-winter season (18.69 mg/100 ml). Comparable results (amounting to 22.12 mg/100 ml for the spring-summer season and 19.06 mg/100 ml for the autumn-winter season) were recorded by Krzyżewski et al. [14] and Strzałkowska et al. [22], who analysed a population of Holstein-Firesian cows fed TMR. This is probably connected with the significantly higher content of small fat globules in the spring-summer season, shown in this study (Table 1), which determine the concentration of cholesterol in milk.

Linear correlation coefficients were calculated in order to establish interdependencies between daily milk yields, contents of basic nutrients and indexes related with milk processability. In a large number of cases significant dependencies ($p \le 0.001$) were obtained for individual characteristics (Tables 3 and 4). The highest values of correlation coefficients were recorded in the spring-summer season between solids-not-fat and fat contents in milk (r=0.91***), as well as contents of protein (r=0.83***), casein (r=0.38***) and lactose (r=0.21***) and the share of large (r=0.40***) and medium fat globules (r=0.26***). We also need to stress high positive correlations (at $p \le 0.001$) in the spring--summer months between contents of solids-not-fat and crude protein (r=0.84***), fat (r=0.57***) and casein (r=0.33***). In the spring and summer high negative correlations were also observed between the value of the protein/fat ratio and fat content (r=–0.55***) and the share of small fat globules and the share of medium (r=–0.89***) and large fat globules (r=–0.75***). In turn, even higher correlations were obtained in the autumn--winter season for solids and fat (r=0.92***), as well as crude protein (r=0.85***) and

Table 3

The correlation coefficients between daily milk yield and nutritional value and technological suitability of milk in the spring-summer season

Variable	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15	16.
1. pH	–	–0.31 ***	0.41 ***	0.01	–0.13	–0.20 ***	–0.31 ***	0.29 ***	–0.06	–0.08	–0.15 ***	–0.10*	–0,10 *	–0.14 ***	–0.19 ***	0.11 **
2. ºSH		–	–0.22 ***	–0.06	0.13	0.02	0.06	–0.01	0.24 ***	0.23 ***	0.31 ***	0.04	0,15 ***	0.32 ***	0.35 ***	–0.10*
3. Clotting time (min)			–	–0.001	–0.08	–0.08	–0.11	0.11	0.06	–0.01	–0.10*	–0.11**	–0,14 ***	–0.08 *	–0.16 ***	0.0004
4. Heat stability (min)				–	0.05	–0.21 ***	–0.02	0.09	0.05	–0.14 ***	–0.12 **	0.02	0.07	–0.13 **	–0.07	0.12 **
5. Density (g/ml)					–	0.26	0.18	–0.22	0.001	0.34 **	–0.03	–0.28 *	0.19	0.24	0.04	–0.03
6. Large fat globules (%)						–	0.41 ***	–0.75 ***	0.27 ***	0.40 ***	0.37 ***	–0.10	–0.03	0.40 ***	0.28 ***	–0.21 ***
7. Medium fat globules (%)							–	–0.89 ***	0.31 ***	0.23 ***	0.34 ***	0.05	–0.09	0.26 ***	0.22 ***	–0.13 *
8. Small fat globules (%)								–	–0.35 ***	–0.35 ***	–0.42 ***	0.005	0.09	–0.37 ***	–0.29 ***	0.18 **
9. Casein (%)									–	0.35 ***	0.49 ***	0.04	–0.18 ***	0.38 ***	0.33 ***	–0.02
10. Fat (%)										–	0.66 ***	–0.55 ***	–0.02	0.91 ***	0.57 ***	–0.27 ***
11. Protein (%)											–	0.23 ***	–0.07	0.83 ***	0.84 ***	–0.18 ***
12. Protein/fat												–	–0.03	–0.26 ***	0.19 ***	0.15 ***
13. Lactose (%)													–	0.21***	0.47***	-0.01
14. Dry matter (%)														–	0.84 ***	–0.26 ***
15. Non-fat dry matter (%)															–	–0.17 ***
16. Daily milk yield (kg)																–

* – values significant at p≤0.05; ** – values significant at p≤0.01; *** – values significant at p≤0.001

Table 4

The correlation coefficients between daily milk yield and nutritional value and technological suitability of milk in the autumn-winter season

Variable	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.
1. pH	–	–0.29***	0.19***	0.23***	–0.17*	0.14*	–0.47***	0.32***	–0.08*	–0.30***	–0.26***	0.01	–0.05	–0.32***	–0.26***	–0.06
2. ºSH		–	–0.13***	–0.02	0.16*	0.001	0.28***	–0.24***	0.30***	0.29***	0.26***	–0.01	0.13***	0.33***	0.30***	–0.006
3. Clotting time (min)			–	0.05	–0.10	–0.05	0.05	–0.03	–0.08*	–0.03	–0.07	–0.02	–0.02	–0.06	–0.07*	0.03
4. Heat stability (min)				–	0.02	–0.06	–0.32***	0.28***	–0.19***	–0.33***	–0.38***	–0.009	0.06	–0.37***	–0.32***	–0.09*
5. Density (g/ml)					–	–0.0001	–0.05	0.03	0.29***	–0.10	–0.04	0.11	0.02	–0.08	–0.03	–0.14
6. Large fat globules (%)						–	0.04	–0.49***	0.03	0.02	0.05	0.04	–0.03	0.02	0.03	0.06
7. Medium fat globules (%)							–	–0.87***	0.27***	0.50***	0.40***	–0.11*	0.13*	0.52***	0.42***	–0.05
8. Small fat globules (%)								–	–0.23***	–0.43***	–0.34***	0.09	–0.12*	–0.45***	–0.37***	0.009
9. Casein (%)									–	0.42***	0.60***	0.07	–0.003	0.53***	0.54***	–0.31***
10. Fat (%)										–	0.65***	–0.42***	–0.08*	0.92***	0.56***	–0.03
11. Protein (%)											–	0.13***	–0.03	0.85***	0.89***	0.004
12. Protein/fat												–	0.04	–0.21***	0.14***	0.03
13. Lactose (%)													–	0.14***	0.41***	0.01
14. Dry matter (%)														–	0.83***	–0.01
15. Non-fat dry matter (%)															–	0.01
16. Daily milk yield (kg)																–

* – values significant at p≤0.05; ** – values significant at p≤0.01; *** – values significant at p≤0.001

casein (r=0.53***), while they were lower for lactose (r=0.14***). In the autumn-winter season correlation coefficients were also high for solids-not-fat and protein (r=0.89***), fat (r=0.56***) and casein (r=0.54***). Rather high negative correlations were found for milk collected in the autumn and winter for the relationships of heat stability with milk solids (r=–0.37***) and solids-not-fat (r=–0.32***) as well as the share of small fat globules in relation to large (r=–0.49***) and medium fat globules (r=–0.87***). A definitely greater effect on the value of the protein-fat ratio was found for the content of fat (r=–0.42***) rather than protein (r=0.13***). To a certain extent the results of this study are confirmed by the findings reported by Bohdanowicz-Zazula et al. [9], Litwińczuk et al. [16] and Barłowska et al. [7] in relation to the correlations for the basic chemical components of tested milk.

Summing up it may be stated that in the evaluated intensive milk production system the season (summer vs. winter) had a significant effect on most analysed indexes defining its processability. Milk collected in the spring-summer season was characterised by a significantly higher acidity, shorter rennet-induced coagulation time, lower heat stability and a higher share of small fat globules. Milk from that period also contained significantly more cholesterol. At the TMR feeding system used throughout the year the production season generally had no significant effect on the share of fatty acids in milk, except for UFA and PUFA, and the MUFA/SFA and PUFA/SFA ratios ($p \leq 0.05$).

REFERENCES

1. AOAC, 2000 – Official Methods of Analysis of the AOAC 969.22. Methyl esters of fatty acids in oils and fatts. 17th Ed. Arlington-Virginia USA.
2. AOAC, 2000 – Official Methods of Analysis of the AOAC 969.33. Fatty acids on oils and fatty. 17th Ed. Arlington-Virginia USA.
3. AOAC, 2000 – Official Methods of Analysis. Casein Nitrogen Content of Milk. 998.06. AOAC International, Chapter 32, 52.
4. AOAC, 2000 – Official Methods of Analysis. Collection of Milk Laboratory Sample. 925.20. AOAC International, Chapter 33, 4.
5. BARŁOWSKA J., LITWIŃCZUK Z., 2006 – Technological usefulness of milk from two local breeds maintained in the regions with great grassland share. ***Archiv fűr Tierzucht*** 49, 207-213.
6. BARŁOWSKA J., GRODZICKI T., TOPYŁA B., LITWIŃCZUK Z., 2009 – Physicochemical properties of milk fat from 3 breeds of cows during summer and winter feeding. ***Archiv fűr Tierzucht*** 52, 4, 356-363.
7. BARŁOWSKA J., WOLANCIUK A., KRÓL J., JAROSIŃSKA A., 2010 – Przydatność technologiczna mleka trzech ras krów żywionych systemem TMR. ***Roczniki Naukowe Polskiego Towarzystwa Zootechnicznego*** 6 (2), 97-105.

8. BARŁOWSKA J., 2011 – Mleko. W: Metody oceny towaroznawczej surowców i produktów zwierzęcych (red. Z. Litwińczuk). Wyd. UP w Lublinie.
9. BOHDANOWICZ-ZAZULA M., NOWOPOLSKA-SZCZYGLEWSKA A., SYNOWIEC M., PAWELSKA M., 2003 – Zmienność składu i parametrów technologicznych mleka krów żywionych w systemie TMR w zależności od pory roku, okresu laktacji i poziomu wydajności. ***Zeszyty Naukowe Przeglądu Hodowlanego*** 69, 197-204.
10. BRODZIAK A., LITWIŃCZUK A., TOPYŁA B., WOLANCIUK A., 2012 – Wpływ interakcji sezonu produkcji z rasą i systemem żywienia krów na wydajność i właściwości fizykochemiczne mleka. ***Roczniki Naukowe Polskiego Towarzystwa Zootechnicznego*** 8 (1), 19-27.
11. BRZÓSKA F., 2004 – Effect of copper inhibitors in diet on cow's yield milk composition and cholesterol level in milk and blood plasma. ***Annals of Animal Science*** 4 (1), 43-55.
12. DE MARCHI M., DAL ZOTTO R., CASSANDRO M., BITTANTE G., 2007 – Milk coagulation ability of five dairy cattle breeds. ***Journal of Dairy Science*** 90 (8), 3986-3992.
13. ELGERSMA A., ELLEN G., VAN DER HORST H., BOER H., DEKKER P.R., TAMMINGA S., 2004 – Quick changes in milk fat composition from cows after transition from fresh grass to a silage diet. ***Animal Feed Science and Technology*** 117, 13-27.
14. KRZYŻEWSKI J., STRZAŁKOWSKA N., JÓŹWIK A., BAGNICKA E., RYNIEWICZ Z., 2003 – Effect of nutrition and season on cholesterol level in milk of Holstein-Fresian cows. ***Annals of Animal Science*** 2, 45-49.
15. LIPIŃSKI K., STASIEWICZ M., RAFAŁOWSKI R., KALINIEWICZ J., PURWIN C., 2012 – Wpływ sezonu produkcji mleka na profil kwasów tłuszczowych tłuszczu mlekowego. ***Żywność. Nauka. Technologia. Jakość*** 1 (80), 72-80.
16. LITWIŃCZUK A., BARŁOWSKA J., KRÓL J., SAWICKA W., 2006 – Porównanie składu chemicznego i zawartości mocznika w mleku krów czarno-białych i simentalskich z okresu żywienia letniego i zimowego. ***Annales UMCS***, sec. EE, 10, 67-72.
17. LOCK A.L., GARNSWORTHY P.C., 2003 – Season variation in milk conjugated linoleic acid and Δ9 desaturase activity in dairy cows. ***Livestock Production Science*** 79, 47-59.
18. Mały Rocznik Statystyczny Polski 2012 – Główny Urząd Statystyczny, Warszawa.
19. OLKOWSKA O., 2012 – Raport. Rynek mleka [http://www.pfhb.pl/raport_rynek_mleka_styczen_2012.pdf].
20. POLSKA FEDERACJA HODOWCÓW BYDŁA I PRODUCENTÓW MLEKA, 2006 – Ocena i Hodowla Bydła Mlecznego. Dane za rok 2005, Warszawa.
21. POLSKA FEDERACJA HODOWCÓW BYDŁA I PRODUCENTÓW MLEKA, 2013 – Ocena i Hodowla Bydła Mlecznego. Dane za rok 2012, Warszawa.
22. STRZAŁKOWSKA N., JÓŹWIK A., BAGNICKA E., KRZYŻEWSKI J., COOPER R. G., HORBAŃCZUK J.O., 2010 – Factors affecting the cholesterol content of milk of cows fed conserved feeds in a TMR system throughout the year. ***Mljekarstvo*** 60 (4), 273-279.

23. SYCH-WINIAREK J., 2012 – Przewidywane tendencje na rynku mleka. ***Biuletyn Informacyjny ARR*** 2, 1-8.
24. ŻUK J., ORZECHOWSKI R., 2009 – Wpływ systemu chowu na pracochłonność i optymalną skalę produkcji mleka w gospodarstwach rolniczych. ***Roczniki Nauk Rolniczych***, seria G, 96 (4), 277-289.