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Original Article
Özgün Araştırma

Investigation of the effects of yogurt cultures on polycyclic aromatic hydrocarbons

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Abstract: The effect of cultures used in yogurt production on PAHs was investigated. PAH-free milk divided into 4 groups; (1) traditional village yogurt culture, (2) culture used with commercial yogurt, (3) combination culture of *Lactobacillus delbrueckki* ssp. *bulgaricus, L. acidophilus, Streptococcus thermophilus, Bifidobacterium animalis* ssp. *lactis, B. infantis* M-63, *B. bifidum* BGN4 and GOS, (4) combination of *L. delbrueckki* ssp. *bulgaricus, L. acidophilus*, *B. lactis* and *S. thermophilus*. Each group was divided into 4 subgroup and first group was kept as control while the other groups were treated with 50, 100, 200 µg kg^{-1} of a mixture 4 PAH [Benzo(a)pyrene, chrysene, benzo(b)fluoranthene, benzo(a)anthracene] respectively. Whereas the highest reduction was seen in 3rd group at 200 µg kg^{-1} with 21.7%, the lowest was seen in the 1st group at 200 µg kg^{-1} with 8.05%. It was concluded that yogurt cultures can inhibit PAHs in milk at a very low level and cannot completely degrade them.

Keywords: inhibition, polycyclic aromatic hydrocarbons, milk, yogurt

Polisiklik aromatik hidrokarbonlara yogurt kültürlerinin etkileri

Özet: Yoğurt üretiminde kullanılan kültürlerin PAH'lar üzerindeki etkisi araştırıldı. PAH içermeyen sütler 4 gruba ayrıldı; (1) geleneksel köy yoğurdu kültürü, (2) ticari yogurt kullanılan kültür, (3) *Lactobacillus delbrueckki* ssp. *bulgaricus, L. acidophilus, Streptococcus thermophilus, Bifidobacterium animalis* ssp. *lactis, B. infantis* M-63, *B. bifidum* BGN4 ve GOS, (4) *L. delbrueckki* ssp. *bulgaricus, L. acidophilus, B. lactis* ve *S. thermophilus* kombinasyonu. Her grup 4 alt gruba ayrıldı ve ilk grup kontrol olarak tutulurken, diğer gruplar sırasıyla 50, 100, 200 µg kg^{-1} 4 PAH [Benzo(a)piren, krizen, benzo(b)floranten, benzo(a)antrasen] karışımı ile muamele edildi. En yüksek azalma %21.7 ile 3. grupta 200 µg kg^{-1}'da görülürken, en düşük azalma %8.05 ile 1. grupta 200 µg kg^{-1}'da görüldü. Yoğurt kültürlerinin sütteki PAH'ları çok düşük düzeyde inhibe edebildiği ve tamamen parçalayamadığı sonucuna varıldı.

Anahtar kelimeler: inhibisyon, polisiklik aromatik hidrokarbonlar, süt, yoğurt

Introduction

Although human life has become easier with the development of industrialization, environmental problems have also started to emerge. People can be exposed to substances harmful to health by breathing contaminated air or consuming contaminated water and food. The most common group of environmental pollutants are polycyclic aromatic hydrocarbons (PAHs). PAHs are hydrophobic organic compounds consisting of 2 or more benzene rings, and are produced by defective combustion of organic compounds of natural or anthropogenic origin. Most PAHs, which are endocrine disrupting compounds, have mutagenic, carcinogenic and genotoxic properties (Shoaei et al., 2023).

Human exposure to environmental pollutants such as PAHs is mostly through food consumption (Jafarabadi et al., 2020). PAH compounds are found in air, water, soil, and can contaminate food during processing and cooking. Humans can get PAHs mainly from overconsumption of meat cooked over an open fire and also from cereals and vegetables. It has been shown that PAHs can be formed from food, especially when meat and fish are cooked over an open fire (Shoaei et al., 2023).

The function of milk, one of the most important human foods, is to maintain immunity. Milk from animals is used for human consumption in various parts of the world. Most of the major components of milk are proteins, lactose, fat and minerals (Pinotti et al., 2020). Since PAHs have a lipophilic structure, they can accumulate in the food chain, especially in foods with higher fat content. Animal-derived foods such as milk and dairy products can be major sourc-

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es of PAHs (Amirdivani et al., 2019). Pollution of milk with PAHs depends on environmental factors such as the origin of exposure, lactation period of the animal, animal health, and reproductive system. It has been shown that PAHs can be detected in milk of milking animals if they consume feed containing PAHs (Chay Rincón et al., 2019). In a study from Italy, 8 PAH compounds were found in raw milk, pasteurized milk, half-fat, and whole sterile milk. Total PAH concentration in milk was determined as 5.428 ng/g in raw milk, 5.941 ng/g in semi-skimmed sterile milk, 6.519 ng/g in pasteurized milk and 7.753 ng/g in whole sterile milk (Naccari et al., 2011). According to the European Union and Turkish Food Codex, the maximum residue limit for infant milk and follow-on milk was determined as 1 μg kg^{-1} for benzo(a)pyrene and the sum of 4 PAHs (benzo(a) pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene) separately (Commission Regulation 2011; Turkish Food Codex 2011). Thus, different physical, chemical and biological methods are being evolved to eliminate or reduce PAHs in foodstuffs to acceptable levels. The most remarkable method is the biodegradation/bioreduction of PAHs using microorganisms such as probiotics and lactic acid bacteria (Chiocchetti et al. 2019; Yousefi et al. 2019; Shoukat 2020; Cuevas-González et al. 2022; Yousefi et al. 2022).

Yogurt obtained from milk is rich in protein, minerals, and vitamins and is one of the best-known probiotic-containing foods (Pop et al. 2022). According to the Turkish Food Codex (2022), yogurt is a fermented milk product in which *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which are starter cultures specific to the fermentation of yogurt, are used together, which is mixed with its clot after incubation and obtained in unbroken or broken form and which contains a sufficient amount of live and active starter bacteria at the last consumption date. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* are two lactic acid bacteria traditionally used to produce yogurt from milk by converting glucose into pyruvic acid and then lactic acid (Battisti et al., 2015). A study conducted in Turkey showed that PAH residues were found in yogurts (Kaçmaz 2019). However, there is very limited research on this subject.

In one study, it was reported that there was a slight decrease in the concentration of PAHs during yogurt making by yogurt cultures (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) and that PAHs could be removed by this method. Although it was stated that LABs may be affected by PAHs at the beginning of yogurt incubation, microorganisms quickly adapt to the presence of such PAHs and can continue their growth (Abou-Arab et al. 2010). Another study was notified that LAB strains can bind to carcinogenic compounds such as PAHs that are commonly formed in foods and reduce their concentrations in foods. Thus, it was stated that the antimutagenic activity of probiotics may also be effective in PAH compounds (Sevim and Kızıl 2019). Thus, we aimed to investigate whether PAH4 would be degraded using commonly used yogurt cultures.

Materials and Methods

Reagents

All solvents used in the study were HPLC pure. PAH analytical standards [benzo(a)pyrene, benzo(a) anthracene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene] were ≥95% pure and purchased from Dr. Ehrenstorfer (Augsburg, Germany). Anhydrous magnesium sulfate (MgSO4), acetonitrile and n-hexane were obtained from Sigma Aldrich (Steinheim, Germany) and primary-secondary amine (PSA) and C18 SPE adsorbents were obtained from Agilent (Santa Clara, USA). Stock standard solutions and internal standard were dissolved in acetonitrile and stored at -20°C. A glass Pasteur pipette for SPE was prepared with 0.5 g C18, 0.5 g PSA and 0.1 MgSO4.

Yogurt cultures

Four different cultures were used to make yogurt. The first one was the yogurt culture traditionally used in villages, the culture taken from yogurt made by large companies and sold ready-made, and 2 cultures whose content is known and sold only as yogurt cultures. One of them contained *Lactobacillus delbrueckki* subsp. *bulgaricus, Lactobacillus acidophilus, Streptococcus thermophilus, Bifidobacterium animalis* ssp. *lactis, Bifidobacterium infantis* M-63, *Bifidobacterium bifidum* BGN4 and Galacto-oligosaccharide (GOS). The other contained *Lactobacillus delbrueckki* ssp. *bulgaricus, Lactobacillus acidophilus, Bifidobacterium lactics* and *Streptococcus thermophilus*. The content of the first two is unknown.

Trial plan

For the experimental studies, 4 groups of yogurts were used. The first group was traditional village yeast, the second group was yeast used in commercial yogurts and the other 2 groups were yogurt cultures with known ingredients. Each group of yogurts was divided into 4 subgroups, one of which was the control group, and each of them received equivalent amounts of 50, 100 and 200 µg kg^{-1} PAHs from 4 PAH compounds (Table 1).

Table 1. Trial plan and yogurt groups

Group/ Subgroups	Added PAH concentration (µg kg^{-1})				Total PAH
	Benzo(a)pyrene	Benzo(a)anthracene	Benzo(b)fluoranthene	Chrysene	
1a	No	No	No	No	No
1b	12.5	12.5	12.5	12.5	50
1c	25	25	25	25	100
1d	50	50	50	50	200
2a	No	No	No	No	No
2b	12.5	12.5	12.5	12.5	50
2c	25	25	25	25	100
2d	50	50	50	50	200
3a	No	No	No	No	No
3b	12.5	12.5	12.5	12.5	50
3c	25	25	25	25	100
3d	50	50	50	50	200
4a	No	No	No	No	No
4b	12.5	12.5	12.5	12.5	50
4c	25	25	25	25	100
4d	50	50	50	50	200

Freshly milked and analyzed PAH-free raw cow milk was used for yogurt. The yogurts were made using the traditional yogurt making method. Before yogurt making, the pH of the milk was measured by pH meter (HI981034, Hanna, USA), dry matter by refractometer (Loyka, Turkey) and acidity by acidometer. Then 1200 mL of raw cow milk was added to 4 separate pots for yogurt making. To the milk samples in 3 pots, 25, 50, 100 µL of the solution prepared with equivalent amounts of 4 PAH compounds (each PAH compound was prepared at a concentration of 600 µg L^{-1}) were added and mixed and kept for 30 mins to ensure homogeneous distribution. PAHs were not added to one pot. While the milk was boiling, its temperature was continuously measured with a thermometer and it was allowed to drop to 40-45 °C after boiling. The milk in each pot was divided into 4 equal parts (approximately 300 ml) and divided into 4 different jars and 4 different starter cultures (traditional yogurt culture, commercial yogurt culture and 2 yogurt cultures with known content) were added to each sample and mixed. Thus, a total of 16 jars of samples were prepared. These yogurts were stored at room temperature (+24°C) for 24 hours.

Analysis of polycyclic aromatic hydrocarbons

PAH analyses in yogurt samples were performed by Gas Chromatography-Mass Spectrometry (GC-MS). For the extraction of yogurts, the method previously developed in the Pharmacology and Toxicology laboratory of the Faculty of Veterinary Medicine, Ankara University was modified, validated, and used (Kuzukiran et al., 2021).

Results

The pH value of cow's milk was determined as 6.68 with a pH meter. Thus, it was seen that the milk used was fresh and recently milked. The soluble dry matter (brix) value of milk was determined as 10% by refractometer. Acidity determination was calculated in terms of lactic acid and found to be 0.153%. Thus, it was determined that the microbial content of milk was low.

In the method validation study, correlation coefficients (r2) were between 0.993-0.997, LOD values for all analytes were 0.11-0.15 μg kg^{-1} and LOQ values were 0.33-0.45 μg kg^{-1}. The RSD% values calculated for intermediate precision and reproducibility were all <10% and the mean retrieval was in the range of 90% to 97% (Table 2). Thus, the analytical method used was found to be able to measure PAH compounds in yogurt at very low levels and was considered enough to achieve the aims of the study.

Table 2. Validation data of target polycyclic aromatic hydrocarbons in yogurts

PAH compound	Linearity (μg kg^{-1})	Correlation coefficient (r^2)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Mean Recovery (%)	Repeatability (RSD%)	Intermediate precision (RSD%)
Benzo(a)anthracene	1-100	0.994	0.11	0.33	97.0±6.3	8.3	7.6
Chrysene	1-100	0.993	0.12	0.36	93.0±6.7	2.1	3.2
Benzo(b)fluoranthene	1-100	0.996	0.15	0.45	90.5±7.2	6.7	4.8
Benzo(a)pyrene	1-100	0.997	0.12	0.36	91.2±7.2	9.9	3.6

LOD: Limit of detection, LOQ: Limit of measurement, RSD: Relative standard deviation

The changes in PAH concentrations measured before and after yogurt making are shown in Table 3. It is seen that the PAH concentrations added to milk in all groups were degraded after contact with yogurt culture.

The reduction rates of benzo(a)pyrene, benzo(a) anthracene, benzo(b)fluoranthene and chrysene at concentrations of 12.5, 25 and 50 μg kg^{-1} in yogurts made with traditional village yogurt culture (group 1) are shown in Figure 1. It is seen that there is a decrease in all concentration ratios compared to the beginning. The highest decrease was seen in chrysene at 50 μg/kg concentration with 12.60% and the lowest decrease was seen in benzo(a)pyrene at the same concentration with 3.12%.

PAH degradation rates in yogurts made with different cultures after adding 50, 100 and 200 μg kg^{-1} PAHs to milk are shown in Figure 2. The total reduction was highest in group 3 (containing probiotics and prebiotics) at a concentration of 200 μg kg^{-1} with 21.7%. The lowest total degradation rate was in group 1 (traditional yogurt culture) at a concentration of 200 μg kg^{-1} with 8.05%.

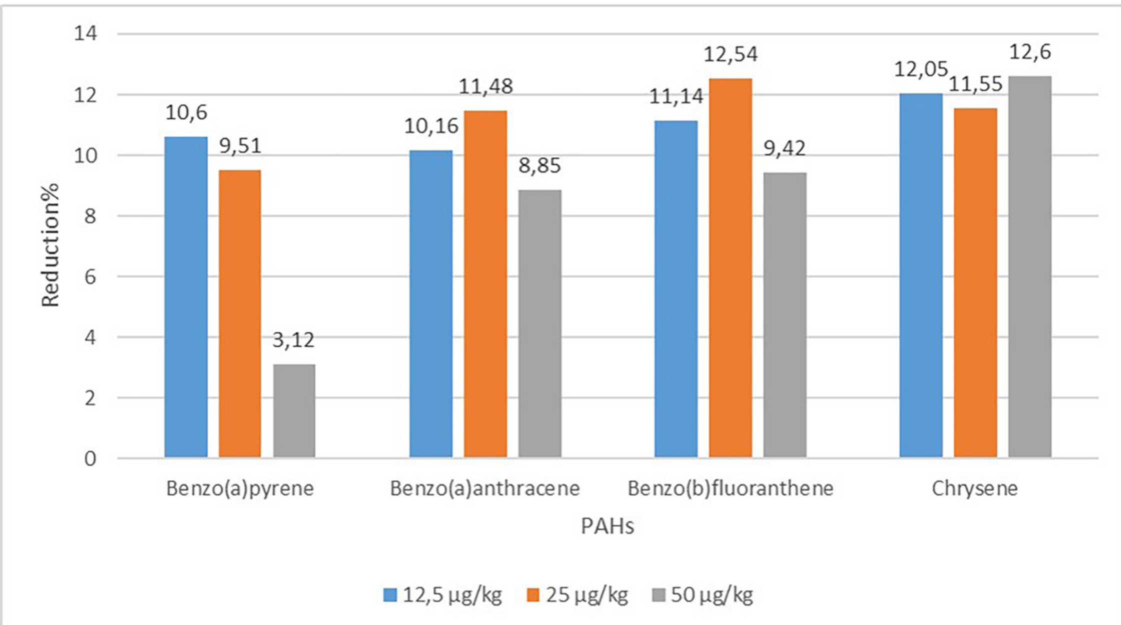

Figure 1. Yogurts made with traditional yogurt culture (group 1). Degradation levels of PAHs in yogurts made after adding 12.5, 25, 50 μg kg^{-1} PAHs to milk.

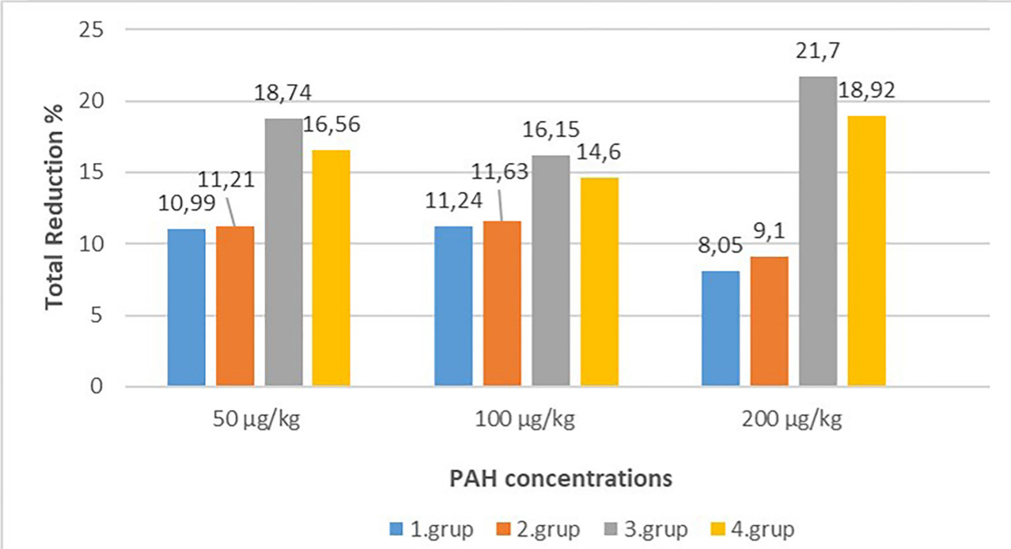

Figure 2. PAH degradation rates in yogurts made with different cultures after adding 50, 100 and 200 µg kg^{-1} PAH to milk.

The PAH concentrations measured in yogurts made after adding 12.5 µg kg^{-1} of each PAH compound to the milk are shown in Figure 3. The highest decrease in benzo(a)pyrene compound was in group 3 (containing probiotics and prebiotics), while the lowest decrease was in group 1 (yogurt made with traditional village yogurt culture). Similar to benzo(a)anthracene and chrysene, benzo(a)pyrene showed the highest and lowest decreases in the same groups. The highest decrease in benzo(b)fluoranthene was observed in group 3, while the lowest decrease was observed in group 2 (yogurt made with culture obtained from commercial yogurts).

The concentrations of PAH compounds added to milk at concentrations of 50, 100 and 200 µg kg^{-1} are shown in Figure 4. In these three concentrations, the highest decline was detected in group 3 (including probiotics and prebiotics) and the lowest decline was detected in group 1 (made with traditional village yogurt culture).

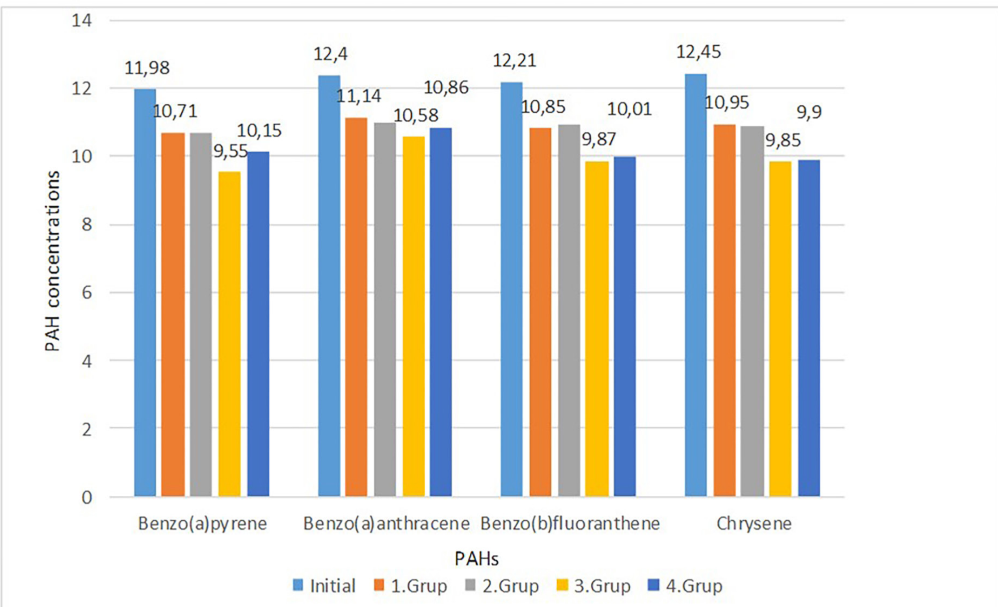

Figure 3. PAH concentrations measured in yogurts made after adding 12.5 µg kg^{-1} PAH to milk.

Table 3. PAH concentrations measured before and after yogurt making

PAH compound	Initial Concentration (μg kg^{-1})	1.Group		2.Group		3.Group		4. Group	
		Concentration (μg kg^{-1})	Reduction (%)	Concentration (μg kg^{-1})	Reduction (%)	Concentration (μg kg^{-1})	Reduction (%)	Concentration (μg kg^{-1})	Reduction (%)
Benzo(a)pyrene	11.98	10.71	10.6	10.68	10.85	9.55	20.28	10.15	15.27
Benzo(a)anthracene	12.40	11.14	10.16	11.01	11.21	10.58	14.68	10.86	12.42
Benzo(b)fluoranthene	12.21	10.85	11.14	10.96	10.24	9.87	19.16	10.01	18.02
Chrysene	12.45	10.95	12.05	10.89	12.53	9.85	20.88	9.90	20.48
Total	**49.04**	**43.65**	**10.99**	**43.54**	**11.21**	**39.85**	**18.74**	**40.92**	**16.56**
Benzo(a)pyrene	24.82	22.46	9.51	22.10	10.96	20.85	15.99	21.56	13.13
Benzo(a)anthracene	23.87	21.13	11.48	21.57	9.63	19.89	16.67	20.42	14.45
Benzo(b)fluoranthene	23.61	20.65	12.54	20.31	13.98	19.89	15.76	20.01	15.25
Chrysene	22.59	19.98	11.55	19.87	12.04	18.93	16.20	19.05	15.67
Total	**94.89**	**84.22**	**11.24**	**83.85**	**11.63**	**79.56**	**16.15**	**81.04**	**14.60**
Benzo(a)pyrene	46.82	45.36	3.12	44.98	3.93	36.75	21.51	37.85	19.16
Benzo(a)anthracene	49.25	44.89	8.85	43.96	10.74	39.45	19.92	41.28	16.18
Benzo(b)fluoranthene	45.98	41.65	9.42	42.89	6.72	36.94	19.66	38.35	16.59
Chrysene	47.85	41.82	12.60	40.78	14.77	35.56	25.68	36.49	23.74
Total	**189.9**	**173.72**	**8.05**	**172.61**	**9.10**	**148.70**	**21.70**	**153.97**	**18.92**

Group 1: Yogurt made with village culture, Group 2: Yogurt made with commercial yogurt culture, Group 3: Yogurt prepared with *Lactobacillus delbrueckki* ssp. *bulgaricus, Lactobacillus acidophilus, Streptococcus thermophilus, Bifidobacterium animalis* ssp. *lactis, Bifidobacterium infantis* M-63, *Bifidobacterium bifidum* BGN4 and Galacto-oligosaccharide (GOS), Group 4: Yogurt prepared with *Lactobacillus delbrueckki* ssp. *bulgaricus, Lactobacillus acidophilus, Bifidobacterium lactis* and *Streptococcus thermophilus*

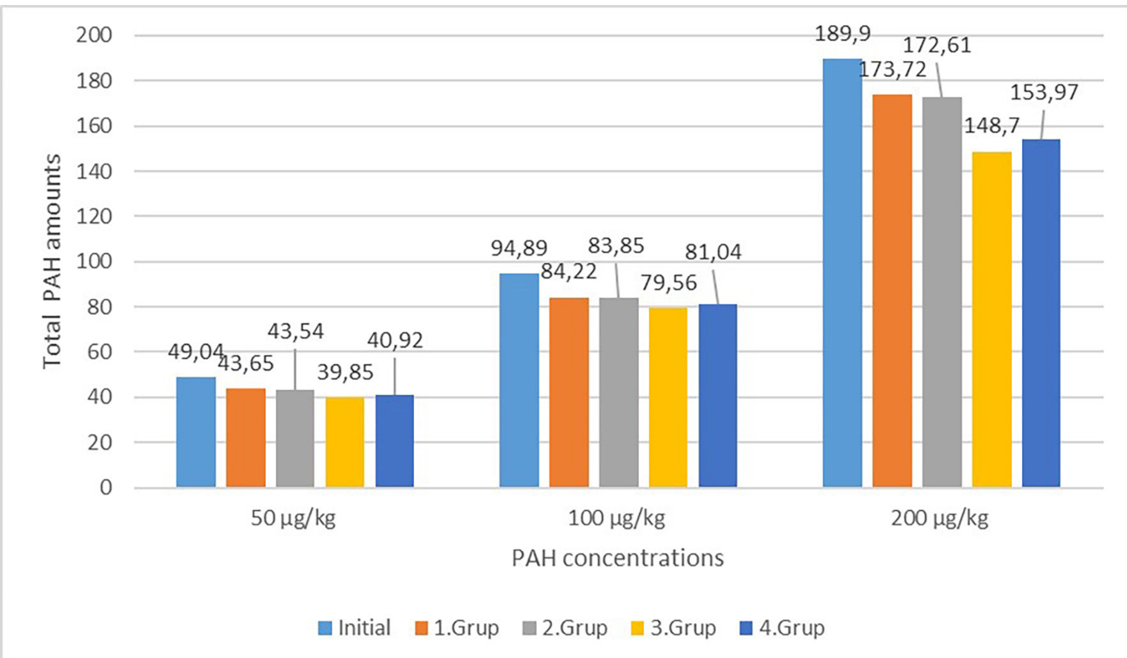

Figure 4. Concentrations of PAH compounds added to milk at concentrations of 50, 100 and 200 µg kg^{-1} measured in yogurts made with different yogurt cultures

Discussion and Conclusion

The pH value of milk gives a lot of information about product yield and quality. The pH value of freshly milked cow's milk should be between 6.6 and 6.8, but there may be deviations from this value. If the pH is lower than 6.5, it is suspected to be colostrum because colostrum milk has a lower pH than normal milk and it is stated that this value is 6. At the end of the lactation process, the pH value of milk generally increases slightly. If the pH is higher than 6.8, mastitis or the addition of a neutralizing agent to the milk is suspected. In milk with mastitis, pH can increase and reach up to 7.5 (Barone et al., 2021). Thus, it was seen that the milk used was fresh and recently milked.

With the refractometer, water addition tricks, dry matter of skim milk, lactose content of milk serum, iodine number and refractive index of milk fat can be determined. The value read by the refractometer is the non-fat dry matter value (AOAC, 1990). According to the Turkish Food Codex communiqué on drinking milks (2019), the fat-free dry matter for cow's milk is reported to be at least 8%. If the fat-free dry matter value is lower than 8%, it is an indication that water is added to the milk (Gai et al. 2021). Since the brix value of the milk was read as 10% by refractometer, it was understood that it complied with the Turkish Food Codex.

The acidity of milk gives information about whether it is fresh or normal. An acidity of more than 0.2% in terms of lactic acid indicates that microbial growth is high and thus the quality of raw milk is insufficient. The variability of lactic acid values in milk can be caused by the lactation process, diseases such as foot and mouth disease, mastitis, smallpox and mammary tuberculosis, and the addition of neutralizing agents to milk (Gai et al. 2021). According to the Turkish Food Codex communiqué on drinking milk (2019), the acidity value in cow's milk should be 0.135-0.2%. Thus, it was determined that the microbial content of milk was low.

According to Table 1, in all four figures drawn, the degradation of PAHs was highest in the 3rd group. However, the 3rd group (including probiotics and prebiotics) and the 4th group (including probiotics) were close to each other and degraded PAHs more than the other groups. The reason why these two groups degrade PAHs more than the other groups is thought to be because they have more cultured bacteria than the other groups, especially the prebiotic in Group 3. Group 3, unlike group 4, contained *Bifidobacterium infantis* M-63, *Bifidobacterium bifidum* BGN4, and GOS as prebiotic in yogurt culture. As it is known, prebiotics support the growth of probiotic bacteria as well as their positive effects on the human health (Ballini et al., 2023). According to these findings, it is estimated that the reason why the 3rd group biodegrades PAHs more than the 4th group is due to the prebiotics in yogurt cultures.

In one study, it was shown that at the end of 72 hours of incubation in the presence of lactic acid bacteria, PAHs could be degraded by 46.6%-91.5%, but the same microorganisms could biodegrade PAHs by 3.46% during yogurt making. According to the authors, the low levels of PAHs degradation by yogurt cultures may be due to the protein affinity and/or adsorption ability of these compounds on the fat globule. Furthermore, the bacterial cell is a high-protein material and therefore can adsorb PAHs that may interfere with cellular metabolism (Abou-Arab et al., 2010). Although one study reported that LAB bacteria reduced the concentration of aflatoxins during yogurt making (Wochner et al., 2019), another study reported that LAB bacteria reduced the binding ability of aflatoxins in the presence of milk (Tajik et al., 2020). Accordingly, it should be stated that micronutrients in milk may have a protective effect on PAHs and also that the persistence of PAHs depends on various factors such as the type of bacteria, interaction between bacteria, bacterial concentration, bacteria composition of the medium and bacterial growth conditions such as heat and pH.

In the study, it was investigated that four different yogurt cultures can inhibit PAH4 (benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene) compounds in milk. When the total reduction data were examined according to 4 PAH concentrations according to the analyses performed in two replicates; the highest reduction was seen in the 3rd group (containing probiotic and prebiotic) at 200 μg kg^{-1} with 21.7% and the lowest was seen in the 1st group (made with culture obtained from traditional village yogurt) at 200 μg/kg with 8.05%. In 3 groups, the reason why the decrease data were higher than the other groups are thought to be due to the probiotic and prebiotic microorganisms in yogurt cultures. Thus, it was concluded that the yogurt cultures used can inhibit the PAHs in milk at a very low level and cannot completely degrade them. It was concluded that PAHs should be below the maximum residue limits in the milk used for yogurt making and PAH residue analysis should be performed in milk.

Ethics committee for the use of experimental animals and other ethical committee decisions and permissions: No need.

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Authors contribution: AF, SYC, and OK conceived and designed the study. SYC and OK executed the experiment and analyzed the yogurt samples. SYC analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents, and approved the final version.

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