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Original Article
Özgün Araştırma

Taxifolin attenuates cisplatin-induced kidney damage in rats via suppressing p53 and iNOS

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Abstract:Cisplatin (CP) is a platinum-based anticancer drug used to treat many different solid tumors. Although CP has strong anticancer properties, its clinical use is limited due to side effects such as ototoxicity, neurotoxicity, myelosuppression and nephrotoxicity. Taxifolin (Tax) is reported to exhibit various possess effects such as anti-inflammatory, antioxidant, antimicrobial, antiviral and anticancer. In this study, we aimed to investigate the possible effects of Tax on CP-induced nephrotoxicity. This study consisted of Control (C), Taxifolin (Tax), Cisplatin (CP) and Cisplatin + Taxifolin (CP + Tax) groups, and there were 6 rats in each group. CP was administered to rats intraperitoneally (i.p.) in a single dose of 7 mg/kg, and Tax was administered orally at a dose of 50 mg/kg for 7 consecutive days. Histopathologically, significant changes such as tubular epithelial degeneration and necrosis, tubular dilatation, inflammatory cell infiltrates, hyaline cast, and glomerular atrophy were detected in the CP group. It was seen that the CP+Tax group significantly reduced histopathological changes ($p<0.001$). In addition, immunohistochemically, the expressions of inducible nitric oxide synthase (iNOS) and p53 were highly irregular in the CP group relative to the control groups ($p<0.001$). Taxifolin treatment (CP+Tax group) significantly decreased the expressions of iNOS and p53 ($p<0.001$). Current findings revealed nephroprotective and ameliorative effects of Tax against CP-induced kidney toxicity.

Keywords: Cisplatin, histopathology, iNOS, taxifolin, p53.

Taksifolin, p53 ve iNOS'u baskılayarak sıçanlarda sisplatin kaynaklı böbrek hasarını hafifletir

Özet: Sisplatin (SP), birçok farklı solid tümörün tedavisinde kullanılan platin bazlı bir antikanser ilaçtır. Sisplatin güçlü antikanser özelliklere sahip olmasına rağmen ototoksisite, nörotoksisite, miyelosüpresyon ve nefrotoksisite gibi yan etkileri nedeniyle klinik kullanımı sınırlıdır. Taksifolin (Tak)'in antiinflamatuar, antioksidan, antimikrobiyal, antiviral ve antikanser gibi çeşitli etkilere sahip olduğu bildirilmektedir. Bu çalışmada Tak'in SP kaynaklı nefrotoksisite üzerindeki olası etkilerinin araştırılması amaçlandı. Bu çalışma Kontrol (K), Taksifolin (Tak), Sisplatin (SP) ve Sisplatin+Taksifolin (SP+Tak) gruplarından oluştu ve her grupta 6 sıçan yer aldı. SP, sıçanlara intraperitoneal (i.p.) olarak tek doz 7 mg/kg uygulandı ve Tak, oral olarak 50 mg/kg dozunda ardışık 7 gün uygulandı. Histopatolojik olarak SP grubunda tübüler epitelyal dejenerasyon ve nekroz, tübüler dilatasyon, yangı hücre infiltrasyonu, hiyalin silindir ve glomerüler atrofi gibi önemli değişiklikler tespit edildi. SP+Tak grubunun histopatolojik değişiklikleri anlamlı düzeyde azalttığı görüldü ($p<0,001$). Ayrıca immünohistokimyasal olarak indüklenebilir nitrik oksit sentaz (iNOS) ve p53 ekspresyonlarının SP grubunda kontrol gruplarına göre oldukça düzensiz olduğu görüldü ($p<0,001$). Taksifolin tedavisi (SP+Tak grubu) iNOS ve p53 ekspresyonlarını anlamlı düzeyde azalttı ($p<0.001$). Mevcut bulgular, Tak'in SP kaynaklı böbrek toksisitesine karşı nefroprotektif ve iyileştirici etkileri ortaya koydu.

Anahtar kelimeler: histopatoloji, iNOS, taksifolin, p53, sisplatin

Introduction

Cisplatin (CP) is known as a platinum-derived anticancer agent used in the treatment of many tumors such as brain, kidney, lung, head and neck tumors, testicles, ovaries and bladder (Dasari and Tchounwou, 2014). CP is also used as a combination therapy in the treatment of squamous cell carcinoma and osteosarcoma (Wagner et al., 2016; Le and Hanna, 2018). CP is one of the most potent and effective chemotherapeutics with well-known antitumor ef-

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fects (Dasari and Tchounwou, 2014). In cases of cancer, CP cross-links with purine bases on DNA and is subsequently considered to induce cell cycle arrest and apoptosis in cancer cells (Dasari and Tchounwou, 2014).

Although CP has strong anticancer properties, its clinical use is limited due to side effects such as ototoxicity, neurotoxicity, myelosuppression and nephrotoxicity (Skinner et al., 1998; Ben Ayed et al., 2020). CP-induced nephrotoxicity includes important processes such as inflammation, vascular damage, oxidative stress, endoplasmic reticulum (ER) stress, cellular uptake and accumulation, necrosis, and apoptosis (Ben Ayed et al., 2020; McSweeney et al., 2021; Kazak et al., 2022; Akcakavak et al., 2023). It is stated that inflammation and oxidative stress, in particular, play a key role in CP-induced acute kidney injury (Ben Ayed et al., 2020; Alanezi et al., 2022).

Taxifolin (Tax), 3,5,7,3,4-pentahydroxyflavanone, is primarily known as a compound derived from Douglas fir and Larix gemelini (Jain and Vaidya, 2023; Yang et al., 2023). Tax is also found in various plants such as camphor pine, black pine, safflower and olive oil (Liu et al., 2014). Tax is reported to exhibit various possess effects such as anti-inflammatory, antioxidant, antimicrobial, antiviral and anticancer (Yang et al., 2021; Jain and Vaidya, 2023; Ölmeztürk Karakurt et al., 2023; Yang et al., 2023).

Although nephrotoxicity is common in patients treated with CP, treatments to reduce and/or prevent nephrotoxicity are of great importance today in order to eliminate its harmful effects and enable it to demonstrate clinically strong anticancer properties. In recent years, the therapeutic and/or protective effects of many different agents have been evaluated in different studies to reduce and/or minimize CP-induced nephrotoxicity. Tax is reported to have a nephroprotective effect against renal toxicity caused by various nephrotoxic agents (Topal et al., 2023). There are limited studies evaluating the effects of Tax on CP-induced nephrotoxicity (Kara et al., 2019; Alanezi et al., 2022). In present study, the effects of Tax on CP-induced nephrotoxicity were assessed histopathologically and immunohistochemically.

Material and Methods

Animals

Present study, 24 male Wistar Albino rats, 2 months old, weighing 250-300 g, were utilized. Rats were housed in rooms with a temperature of 20-22°C, in standard plastic cages, with 12 hours of light and 12 hours of darkness, and were fed *ad libitum*. Cisplatin (Cipintu 100 mg/100 ml, Istanbul, Türkiye) administration to rats was performed intraperitoneally (i.p.) at a dose of 7 mg/kg, according to a previously reported study (Aldemir et al., 2014). Taxifolin (Evalar, Russia) solution was prepared in physiological saline at 50 mg/kg and was administered by oral gavage (Erhan et al., 2021; Ersoy et al., 2021).

The study was designed to include 6 rats in each group. **Control (C) group;** Rats were administered 0.5 ml/rat saline i.p. once on the first day and distilled water was conducted orally once a day for 7 days. **Taxifolin (Tax) group:** Rats were administered 0.5 ml/rat physiological saline i.p. once on the first day, and 50 mg/kg Tax was conducted orally once a day for 7 days. **Cisplatin (CP) group**: Rats were administered 7 mg/kg CP i.p. once on the first day of the study, and distilled water was conducted orally once a day for 7 days. **Cisplatin+ Taxifolin (CP+Tax) group:** Rats were administered 7 mg/kg CP i.p. once on the first day of the study, and 50 mg/kg Tax was conducted orally once a day for 7 days. On day 8^{th} of the study, all rats were sacrificed under i.p. xylazine (10 mg/kg) and ketamine (100 mg/kg) anesthesia. Necropsies were performed and kidney tissues were removed. Afterwards, they were placed in neutral formaldehyde solution for histopathological and immunohistochemical examination.

Histopathological examination

The kidney tissues were fixed in 10% neutral formaldehyde solution for 24-48 hours. Afterwards, paraffin blocks were obtained through routine tissue follow-up. Sections were taken from paraffin blocks onto ground slides, stained with Hematoxylin-Eosin (H-E) and examined under light microscopy. Histopathological scoring was evaluated semi quantitatively in 10 different areas at x20 magnification (0; none, 1; mild, 2; moderate, 3; severe) (Akcakavak et al., 2023).

Immunohistochemical examination

Sections were cut from paraffin blocks onto adhesive slides. Immunohistochemical staining was done according to a previously mentioned study (Akcakavak et al., 2023). IHC staining was performed with the Ultra vision detection system anti-polyvalent, HRP (Thermo Scientific, TP-60-HL, USA) kit, in accordance with the manufacturer recommendations. Anti-iNOS (Abcam, ab283655, 1/200 dilution) and Anti-p53 (Proteintech, 60283-2-Ig, 1/500 dilution)

were utilized as primers. 3,3 diaminobenzidine (DAB) was used as chromogen and counterstaining was done with Mayers-Hematoxylin. Immunohistochemical scoring was evaluated semi-quantitatively in 20 different areas at x20 magnification the average was taken (0; none, 1; mild, 2; moderate, 3; severe)(Akcakavak et al., 2023).

Statistical analysis

Evaluation of data between groups was done with SPSS (Inc., Chicago, USA 25.0) statistical program. Histopathological and immunohistochemical scores were evaluated with Kruskal wallis. Mann-Whitney U test was utilized to determine the difference among groups. The accepted importance limit was $p < 0.05$.

Results

Histopathological results

Histopathological scores between the groups are shown in table 1. Control and Tax groups were found to exhibit normal histological appearance. In the CP group, significant histopathological changes such as degeneration and necrosis in the tubular epithelium, tubular dilation, hyaline cast, inflammatory cell infiltration and glomerular atrophy were determined. It was found that the CP+Tax group reduced the relevant changes at a statistically significant level ($p<0.001$). In addition, it was determined that there were occasional bleeding foci in the CP group. In the CP+Tax group, bleeding foci were less frequent.

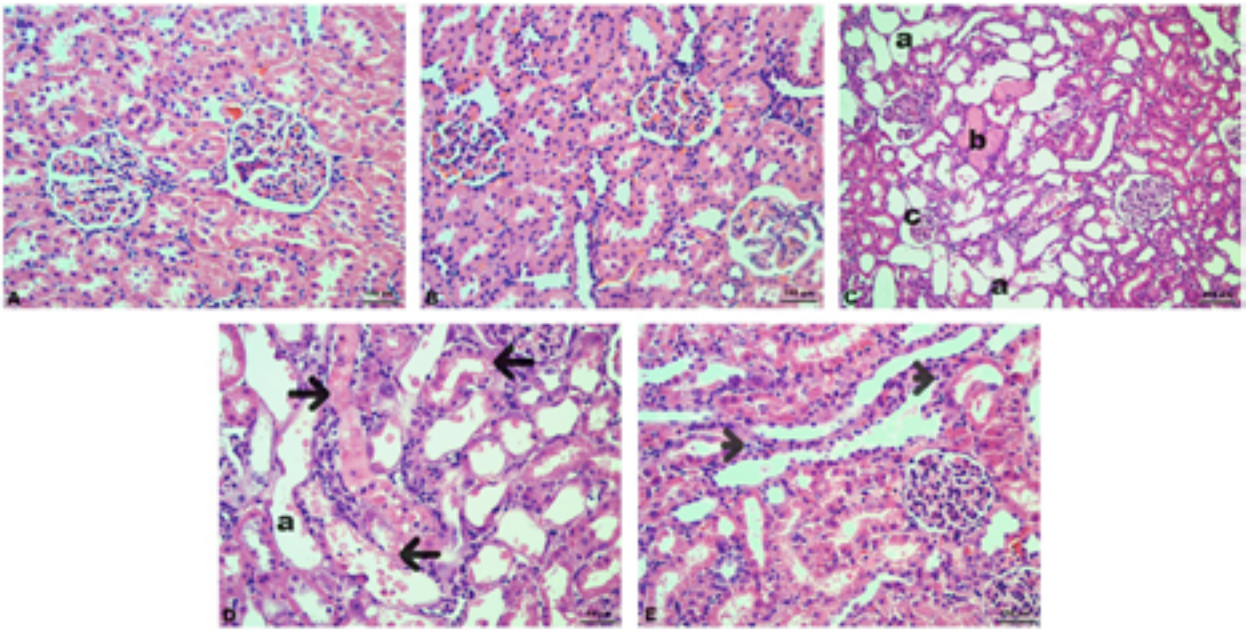

Figure 1. Histopathological evaluation of the effect of taxifolin on cisplatin-induced kidney damage, H-E, **A**; Control group, **B**; Tax group, **C-D**; CP group, **E**; CP+Tax group, necrosis of tubular epithelium (arrows), degeneration of tubular epithelium (arrowheads), tubular dilation (a), hyaline casts (b), glomerular atrophy (c).

Table 1. Histopathological scoring of the effects of taxifolin on cisplatin-induced renal injury.

Histopathological lesion	C	Tax	CP	CP+Tax
Degeneration of tubular epithelium	0.33±0.21^{c}	0.50±0.22^{c}	2.67±0.21^{a}	1.67±0.21^{b}
Necrosis of tubular epithelium	0.17±0.17^{c}	0.33±0.21^{c}	2.50±0.22^{a}	1.50±0.22^{b}
Inflammatory cell infiltration	0.50±0.22^{c}	0.67±0.21^{c}	2.17±0.17^{a}	1.33±0.21^{b}
Tubular dilation	0.50±0.22^{c}	0.67±0.21^{c}	2.17±0.30^{a}	1.33±0.21^{b}
Hyaline cast	0.33±0.21^{c}	0.33±0.21^{c}	2.50±0.22^{a}	1.50±0.22^{b}
Glomerular atrophy	0.50±0.22^{c}	0.67±0.21^{c}	2.17±0.17^{a}	1.33±0.21^{b}

$^{a-c}$ Letters in the same line indicate statistical significance ($p<0.001$). Group means were given as Mean ± SE (n;6). (C;Control, Tax;Taxifolin, CP;Cisplatin, CP+Tax; Cisplatin+Taxifolin)

Immunohistochemical results

The immunohistochemical scores between the groups are given in table 2. iNOS and p53 expressions were very mild or absent in the control groups. iNOS immunoreactive had luminar localized staining, and p53 had cytoplasmic and nuclear staining. Significant increases were determined in the relevant expressions (iNOS and p53) in the CP group ($p<0.001$). In the CP+Tax group, iNOS and p53 expression levels were found to be significantly reduced ($p<0.001$).

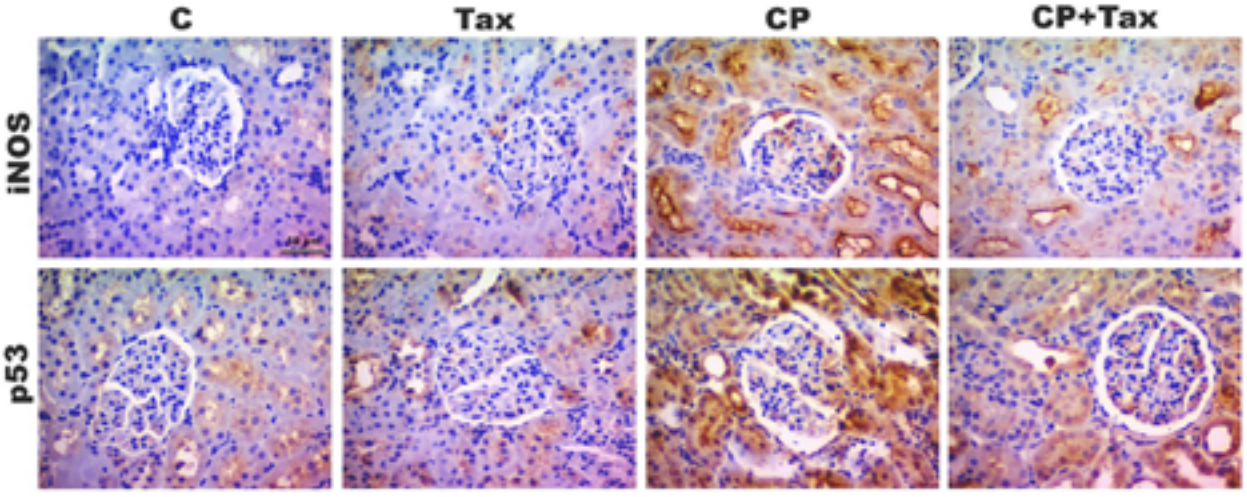

Figure 2. Immunohistochemical evaluation of the effect of taxifolin on iNOS and p53 expressions on cisplatin-induced kidney damage (C; Control, Tax; Taxifolin, CP; Cisplatin, CP+Tax; Cisplatin+Taxifolin groups, iNOS; inducible nitric oxide synthase).

Table 2. Immunohistochemical scoring of the effects of taxifolin on cisplatin-induced renal injury.

Primer Antibody	C	Tax	CP	CP+Tax
p53	0.33 ± 0.21^{c}	0.50 ± 0.22^{c}	2.67 ± 0.21^{a}	1.67 ± 0.21^{b}
iNOS	0.50 ± 0.22^{c}	0.67 ± 0.21^{c}	2.17 ± 0.17^{a}	1.33 ± 0.21^{b}

[a-c] Letters in the same line indicate statistical significance ($p<0.001$). Group means were given as Mean ± SE (n;6). (C;Control, Tax;Taxifolin, CP;Cisplatin, CP+Tax; Cisplatin+Taxifolin, iNOS; inducible nitric oxide synthase)

Discussion and Conclusion

Present study, we aimed to investigate the beneficial effects of Tax on CP-induced nephrotoxicity. Current findings showed that Tax had a nephroprotective/curative effect on kidney injury caused by CP treatment by reducing the expressions of iNOS and p53, improving histopathological changes.

Regarding the histopathological examination, the present study showed good evidence of nephrotoxicity after CP (7 mg/kg i.p.) injection. These changes were degeneration and necrosis of tubular epithelium, tubular dilation, hyaline casts, inflammatory cell infiltration, and glomerular atrophy. In addition, it was determined that there were occasional bleeding foci in the CP group. Researches on cisplatin-induced renal toxicity, it was reported that histopathologically, degeneration and necrosis of tubular epithelium, inflammatory cell infiltrations, hyaline cast, tubular dilatation and glomerular atrophy, edema and bleeding were detected (Kara et al., 2019; Alanezi et al., 2022; Kazak et al., 2022). Present study, histopathological findings were found to be compatible with the findings of previous studies.

In experimental toxicity studies induced by many different chemicals, taxifolin is reported to alleviate and/or improve histopathological changes and is attributed to its anitoxidative, anti-inflammatory and anti-apoptotic effects (Obeidat et al., 2022; Alanezi et al., 2022; Althunibat et al., 2023). The current study revealed that Tax significantly alleviated histopathological changes in CP-induced renal toxicity.

The mechanisms of cisplatin nephrotoxicity include many signals, such as oxidative damage and disruption of the inflammatory process in the kidney. In normal homeostasis, there is a balance between ROS production and the antioxidant defense system. CP may cause excessive ROS production and impairment of antioxidant defense systems,

leading to oxidative stress and mitochondrial dysfunction (Halliwell, 2006). In response to oxidative stress damage nuclear factor-kappa B (NF-kB) is activated, leading to ROS/RNS (reactive nitrogen species) stress imbalance and consequently increased cytokine release (Kurutas, 2015). It has also been reported to increase the synthesis of iNOS through the activation of NF-κB (Tuñón et al., 2003).

iNOS is a nitric oxide synthase (NOS) known to be the major producer of nitric oxide (NO). NO produced via iNOS is more important in inflammatory responses and diseases such as cancer (Vannini et al., 2015). It has been stated that iNOS expressions are upregulated in many cisplatin-induced renal toxicity studies (Chirino et al., 2008; Wang et al., 2018; Aladaileh et al., 2021). Pan et al. (2009) reported that in their cisplatin-induced renal toxicity study, canabidiol treatment resulted in the suppression of excessive iNOS expressions that occurred with cisplatin application and thus reduced renal tubular damage. Chirino et al. (2008) reported that selective iNOS inhibition attenuated cisplatin-induced nephrotoxicity. In a different study, it was reported that mesenchymal stem cells reduced cisplatin-induced nephrotoxicity via iNOS (Simovic Markovic et al., 2017). Increased levels of iNOS-mediated NO can cause apoptosis and DNA damage. Thus, stimulation of iNOS can result in tubular cytotoxicity and renal failure (Morsy et al., 2014; Akcakavak et al., 2024). It has been stated that inhibition of iNOS activity can decrease oxidative stress in renal tubular cells (Wu et al., 2007). Present study, iNOS expression was found to be significantly increased in the CP group relative to the control groups ($p<0.001$). Tax treatment reduced iNOS expressions and demonstrated nephroprotective effects against CP-induced renal toxicity. It was thought that this situation may be due to the antioxidant and anti-inflammatory effects of Tax (Jain and Vaidya, 2023; Ölmeztürk et al., 2023). A recent study reported that Taxifolin reduces oxidative stress and NF-kB cytokinin expressions in cisplatin-induced nephrotoxicity (Alanezi et al., 2022). Present study, NF-kB downregulation may have played a role in the decreased iNOS expressions.

p53 is a transcription factor that acts a central role in processes such as DNA repair, cell death, and cell cycle arrest, in response to various stress signals. The p53 gene is encoded by the TP53 gene locus, which is located on the short arm of human chromosome 17 (17p13.1) (Levine and Oren, 2009; Sabapathy and Lane, 2018). p53 tumor suppressor protein induces apoptosis in response to DNA damage and oncogene activation (Bassett et al., 2008). It has been reported that procedures aimed at p53 suppression reduce cisplatin-induced apoptosis and kidney damage (Molitoris et al., 2009; Zhang et al., 2020). Zhang et al. (2020) reported that Pioglitazone prevents cisplatin nephrotoxicity by suppressing the p53-mediated mitochondrial apoptotic pathway via SIRT1 activation. Wu et al. (2021) reported that Nicotinamide protects against cisplatin-induced tubular damage by suppressing the PARP1/p53 pathway. Research show that p53 can induce apoptosis due to oxidative stress, DNA damage and mitochondrial dysfunction in cisplatin-induced kidney toxicity. Indeed, DNA damage and the resulting DNA damage response are known to be the main trigger of p53 activation in the kidneys (Tang et al., 2019). It is also reported that it causes an increase in the expression of 8-hydroxy-2-deoxyguanosine (8-OHdG), which is the most important indicator of DNA damage in cisplatin-induced kidney toxicity studies (Geyikoglu et al., 2017; Mercantepe et al., 2018). Additionally, different studies have reported that taxifolin has reducing effects on 8-OhdG expressions (Unver et al., 2019; Okkay et al., 2022). In present study, an increase in p53 expression was detected in the CP group and was compatible with the findings of previous studies (Zhang et al., 2020; Wu et al., 2021). This situation was thought to cause p53 upregulation due to oxidative stress, DNA damage and mitochondrial dysfunction caused by cisplatin administration. Additionally, considering the findings of the study, it shows that the p53 gene plays an important role in the process of kidney damage caused by cisplatin. Taxifolin treatment (CP + Tax group) significantly reduced p53 expression and revealed that it had a tubular damage-reducing effect against CP-induced renal toxicity. It is possible to interpret that p53 downregulation may decrease apoptosis, especially in the CP + Tax group. Moreover, in a recent study, evidence that taxifolin reduced apoptosis in cisplatin-induced kidney toxicity further strengthened our opinion (Alanezi et al., 2022).

The current study shows that Tax given simultaneously with CP treatment acts a protective/curative role in alleviating CP-induced renal injury by suppressing histopathological changes and iNOS, p53 expressions. Thus, Tax may be a promising candidate for attenuating kidney damage in patients undergoing with CP chemotherapy.

Ethics committee for the use of experimental animals and other ethical committee decisions and permissions: Selcuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee approved the ethical compliance of the study (Approval No: 2024/056).

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Original Article
Özgün Araştırma

Differentiation of bovine Tuberculosis and Paratuberculosis infections with antemortem diagnostic methods

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Abstract: In this study, based on the results of tuberculin skin tests (Bovine and Avian PPD) used in the antemortem diagnosis and differentiation of Bovine Tuberculosis, the animals in the farms with suspected Tuberculosis were serologically examined to diagnose Paratuberculosis infection and fecal bacterioscopy was performed. In addition, it was aimed to obtain data that will contribute to the eradication studies of Bovine Tuberculosis disease by comparing the antemortem diagnostic methods of Bovine Tuberculosis disease, which is endemic in Türkiye and by determining the sensitivity and specificity values of the interferon gamma (IFN-γ) test. In this context, intradermal tuberculin test was applied to 423 cattle with suspected Tuberculosis in a total of 5 dairy cattle farms, one each from Çankırı, Çorum, Ankara, Eskişehir and Konya regions, and this test was determined as the gold standard method and the sensitivity and specificity of the IFN-γ test were determined as 86% and 97%, respectively. For the diagnosis of Paratuberculosis infection, antibody ELISA, fecal bacterioscopy and IFN-γ ELISA were performed on these animals and the prevalence of these tests were 10.4%, 5.44% and 4.96% respectively and 4 (0.95%) of the cattle were positive for each of the diagnostic methods for *Mycobacterium avium* spp. *paratuberculosis* (*Map*) infection. As a result, it was concluded that IFN-γ test, which gives similar results to intradermal tuberculin test results, can also be used in the antemortem diagnosis of Bovine Tuberculosis. Also, in the comparative intradermal tuberculin test for the diagnosis of Tuberculosis infection, avian PPD positive animals were found to play a decisive role in the detection of nonspecific reactions or Paratuberculosis infected animals, supported by other tests used for the diagnosis of Paratuberculosis.

Keywords: Cattle, ELISA, IFN-γ test, *Mycobacterium bovis*, Paratuberculosis

Sığır Tüberküloz ve Paratüberküloz enfeksiyonlarının antemortem tanı yöntemleriyle ayrımı

Özet: Bu araştırmada, Tüberküloz şüpheli işletmelerde bulunan hayvanlara, Sığır Tüberkülozunun antemortem tanı ve ayrımında kullanılan tüberkülin deri testleri (PPD bovine ve avian) sonuçlarından yola çıkılarak, Paratüberküloz enfeksiyonu teşhisi koymak için serolojik olarak incelendi ve fekal bakteriyoskopi yapıldı. Ayrıca Türkiye'de endemik olarak görülen Sığır Tüberküloz hastalığının antemortem tanı yöntemleri karşılaştırılıp, interferon gama (IFN-γ) testinin sensitivite ve spesifite değerleri belirlenerek Sığır Tüberküloz hastalığının eradikasyon çalışmalarına katkı sağlayacak verilerin elde edilmesi amaçlandı. Bu kapsamda Çankırı, Çorum, Ankara, Eskişehir ve Konya bölgelerinden birer adet olmak üzere toplam 5 süt sığırcılığı işletmesinde Tüberküloz şüphesi olan 423 sığıra intradermal tüberkülin testi uygulandı ve bu test altın standart metot olarak belirlenerek, IFN-γ testinin sensitivitesi % 86, spesifitesi % 97 olarak belirlendi. Paratüberküloz enfeksiyonunun tanısı için bu hayvanlara antikor ELISA, fekal bakteriyoskopi ve IFN-γ ELISA yapıldı ve bu testlerin prevalansları sırasıyla % 10,4, % 5,44 ve % 4,96 bulundu ve sığırlardan 4 (%0,95)'ü *Mycobacterium avium* spp. *paratuberculosis* (*Map*) enfeksiyonu tanı yöntemlerinin her birine pozitif sonuç verdi. Sonuç olarak, intradermal tüberkülin testi sonuçlarına benzer sonuçlar veren IFN-γ testinin Sığır Tüberkülozunun antemortem tanısında kullanılabileceği sonucuna varıldı. Ayrıca Tüberküloz enfeksiyonunun tanısı için yapılan karşılaştırmalı intradermal tüberkülin testinde PPD aviana pozitif reaksiyon veren hayvanların Paratüberküloz tanısı için kullanılan diğer testlerle desteklenerek nonspesifik reaksiyonların ortaya çıkarılmasında ya da Paratüberküloz enfekte hayvanların tespit edilmesinde belirleyici rol oynadığı görüldü.

Anahtar kelimeler: ELISA, IFN-γ test, *Mycobacterium bovis*, Paratüberküloz, Sığır

Introduction

Bovine Tuberculosis (bTB), caused by *Mycobacterium bovis,* is a chronic infectious disease of many domestic and wild animals, including cattle, buffalo, sheep, goats, badgers, pigs, deer, Australian-American opossums and humans (Menzies and Neill 2000; O'hagan et al. 2016). The zoonotic nature of

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the disease has a serious impact on human health worldwide. In addition to the significant economic costs caused by such diseases, the lack of accurate estimates of the true prevalence of disease, especially in developing countries, necessitates more effective detection and control measures (Hashem et al. 2022). Cattle are the main reservoir of bTB and eradication programs worldwide focus primarily on these domestic species (Mohamed 2020).

Diagnosis of the disease depends mainly on tests that measure the cellular immune response following infection, culture-based bacteriological examination and molecular-based Polymerase Chain Reaction (PCR) methods (Smith et al. 2021; Thomas et al. 2021). None of the tests currently available for the diagnosis of Bovine Tuberculosis allow perfect detection of *M. bovis* infection in cattle. The Tuberculin Skin Test (TST), which has a specificity of 99.5% in TB (Tuberculosis)-free bovine populations, is used as the primary antemortem diagnostic tool (Eisenberg et al. 2016). Bovine Tuberculosis is subject to an official eradication program based on a slaughter policy and using intradermal TST and Interferon Gamma (IFN-γ) diagnostic tests, mainly measuring cellular immunity. Intradermal TST is recognized by the OIE and the European Commission as the primary screening test for the diagnosis of TB in cattle (Çakır 2021). The IFN-γ test has a higher sensitivity and is as specific as the Comparative Intradermal Tuberculin Test (CITT), but some studies have reported that the IFN-γ test has lower specificity than the CITT (Proud et al. 2015).

Paratuberculosis (PTB) or Johne's Disease is a disease caused by *Map* that affects domestic and wild ruminants worldwide. PTB is a highly contagious disease characterized by a chronic progressive granulomatous enteritis that is endemic in many parts of the world and causes significant economic losses in livestock and related industries (Moyano et al. 2021). *Map* infection, showing a chronic diarhea that does not respond to treatment is the specific clinical sign of infection (Cruz-Estupınan et al. 2022). Infection in ruminants consists of different phases including early, subclinical and clinical phases, and symptoms such as weakness, muscle wasting, diarrhea, decrease in milk yield, which do not show clinical signs in the early or subclinical phase, but continue in the clinical period with the end of the subclinical period (Eamens et al. 2015; Whittington et al. 2017). The primary source of the disease is clinically sick and asymptomatic animals. The most common route of infection is ingestion of contaminated milk, colostrum or feces (Gilardoni et al. 2012; Lievaart-Peterson et al. 2019). The causes of economic losses due to PTB are mortality, early elimination of animals, increased susceptibility to other infectious diseases, decreased milk, meat and reproductive yields (Garcia and Shalloo 2015; Barratt et al. 2018; Camanes et al. 2018).

The methods used in the diagnosis of PTB are divided into two as direct and indirect. Direct diagnostic methods include histopathology, necropsy, culture, bacterioscopy and PCR, while indirect diagnostic methods include cellular (TST and IFN-γ) and humoral (ELISA) immune response tests (Eamens et al. 2015; Şababoğlu 2019; OIE 2021). The diagnosis of PTB by bacterioscopy involves microscopic examination of preparations of feces or intestinal mucosa stained with Ziehl-Neelsen (ZN) staining. If acid fast bacteria (AFB) are found in clusters (at least three or more), it may be a possible diagnosis of PTB. The skin test for Delayed Type Hypersensitivity (DTH) is based on the measurement of cell-mediated immunity. Since avian and johnin Prufiye Protein Derivative (PPD) skin tests that reveal DTH show similar sensitivity and specificity values, it has been stated that one can be used instead of the other (OIE 2021). Tests measuring IFN-γ level, one of the in vitro assays that detect cellular immune response, have recently been used in the diagnosis of Tuberculosis and Paratuberculosis in cattle. Since the production of IFN-γ cytokine is one of the earliest detectable immune responses in PTB diagnosis, it has been stated that the IFN-γ test, which detects infected animals in the subclinical period, is the best method among the diagnostic methods (Nielsen 2010; Vazquez 2013). ELISA (Enzim Linked Immunosorbent Assay), one of the indirect diagnostic methods of PTB infection, is frequently used and is the main diagnostic method for determining seroprevalence in countries with disease surveillance programs. The advantage of ELISA is its low cost, fast results and versatility (De Lacerda Roberto 2021). ELISA has the highest sensitivity and specificity among available tests to detect antibodies to *Map* in cattle (OIE 2021). ELISA is a serological test widely and conveniently used to detect antibodies in milk and serum during and after the subclinical stage of *Map* infection (Radostits 2007; Garvey 2018).

The aim of this study was to determine whether the animals were infected with PTB infection by using the methods used in the antemortem diagnosis of bTB. In addition, it was aimed to obtain data that will contribute to the eradication studies of Bovine Tuberculosis by comparing the antemortem diagnostic methods of bTB, which is endemic in Türkiye,

and determining the sensitivity and specificity values of IFN-γ test.

Materials and Methods

Sampled Animals

This study was carried out on 423 cattle from a total of 5 dairy cattle farms with suspected bTB between 2021-2023 in Çankırı, Çorum, Ankara, Eskişehir and Konya regions. The number of farms and animals in the regions are as follows; Çankırı 1 farm 144 cattle, Çorum 1 farm 51 cattle, Ankara 1 farm 31 cattle, Eskişehir 1 farm 47 cattle, Konya 1 farm 150 cattle. The age and gender distribution of cattle are presented in Table 1. To conduct this study, ethics committee approval was taken, which was 2021-13-106 number from Ankara University Animal Experimental Local Ethics Committee.

Table 1. Age and gender distribution of cattle used in the study.

	Age (Years)						Gender	
Animal	**0-1**	**1**	**2**	**3**	**4**	**≥5**	**Male**	**Female**
Number of cattle	11	76	96	65	53	122	70	353
Total	423						423	

Blood and Fecal Samples

Before the cattle were subjected to PPD skin test, 8 ml of venous blood was collected from each animal into vacuum tubes containing lithium heparin for IFN-γ test and vacuum tubes containing clotting activator for *Mycobacterium paratuberculosis* antibody test. Fecal samples were also collected from each animal for the diagnosis of PTB. Fresh fecal samples were collected from the rectum with plastic gloves and transferred to sterile plastic containers. These fecal samples from each cattle were delivered to the laboratory at +4°C (Paolicchi et al. 2003; Borum et al. 2014).

Tuberculin Skin Test (TST)

Avian and Bovine PPD with a protein content of 1 mg/ml (produced by Etlik Central Veterinary and Control and Research Institute, Türkiye) were used in tuberculin skin test**.** Cattle were subjected to CITT and the results were evaluated. This application and evaluation of the results were performed according to Office International Epizootica (OIE), Ministry of Agriculture and Forestry of the Republic of Türkiye Bovine Tuberculosis Regulations. PPD skin test was performed by intradermal injection in the middle third of the neck with a dose of 0.1 ml of avian PPD on the top and bovine PPD on the bottom with a distance of 12-13 cm between the two injections. Skin thickness of both injection sites was recorded 72 hours later by measuring the skin thickness again with calipers. The diagnosis of bTB infection or suspected PTB was determined by measuring and evaluating the skinfold thickness at the site of bovine PPD and avian PPD injection (Resmi Gazete 1978; Resmi Gazete 2009; OIE 2022).

Interferon Gamma (IFN-γ) Test

Blood samples collected in lithium heparinised tubes were brought to the laboratory at room temperature (22±3°C) within 12 hours and whole blood cultures were performed in 24-well cell culture plates. Blood samples from each animal were distributed into 3 wells of each animal in a 24-well tissue culture plate. 100 µl of nil control antigen (PBS) was added to the blood sample in the first well, 100 µl of bovine PPD antigen was added to the blood sample in the second well, 100 µl of avian PPD antigen was added to the blood sample in the third well. Then, the microplates were incubated at 37 °C in an incubator with 5% CO_2 for 16-24 hours. Then, plasma samples were harvested from the cultures and a commercial kit (Bovigam®, Prionics AG, Australia) was used to diagnose the cellular immune response resulting from Paratuberculosis and Tuberculosis infection based on the elevated IFN-γ levels. Sandwich ELISA was performed according to the protocol reported by the manufacturer. Within 5 minutes after the reaction was terminated, Optical Density (OD) was measured on an ELISA reader with a 450 nm microplate photometer. Samples tested for Tuberculosis infection according to the kit protocol;

Negative: OD Bovine PPD - OD Nil/ Phosphate Buffer Solution (PBS) antigen < 0.1 and OD Bovine PPD – OD Avian PPD < 0.1,

Positive: OD Bovine PPD - OD Nil/ Phosphate Buffer Solution (PBS) antigen ≥ 0.1 and OD Bovine PPD – OD Avian PPD ≥ 0.1 evaluated according to the criteria.

Evaluation of OD values in terms of *Map* infection; It was performed according to the calculation criteria reported by Vazquez et al. (2013). Consider-

ing this criterion, cattle were considered positive for Paratuberculosis infection when the avian PPD OD value of each sample was subtracted from the nil/ PBS OD value and the difference was equal to or greater than 0.05 and the avian PPD OD value was higher than the bovine PPD OD value (OD Avian > OD Bovine and OD Avian - OD Nil/ PBS ≥ 0.05).

Bacterioscopy

Approximately 40 grams of fecal samples from each cattle were homogenized with sterile distilled water, 2 direct smear preparations were prepared from three different areas of each feces and stained with ZN staining method for fecal bacterioscopy diagnosis. AFB were visualized as short-thick, red-pink coccobacilli on a blue background. At least 100 microscope fields were scanned and the results recorded. These results were evaluated according to the Acid Fast Bacteria scoring (AFB scorin) criteria as indicated in Table 2 (Fujiki 2001).

Table 2. Acid Fast Bacteria Scoring Criteria (AFB Scorin).

Assessment	Result
No acid-fast bacteria in the microscope field	Negative
1-9 acid-fast bacteria in the microscope field	Suspect
10-99 acid-fast bacteria in the microscope field	Positive

Mycobacterium paratuberculosis Antikor Testi

The samples taken into blood tubes containing clotting activator were centrifuged at 3000 rpm for 5 minutes and after the sera were obtained in the tubes, they were transferred to eppendorf tubes and stored in a deep freezer at -20°C until the time of testing**.** A commercial kit (IDEXX Paratuberculosis Screening, France) was used for ELISA to detect antibodies raised in Paratuberculosis infection. Indirect ELISA was performed according to the protocol reported by the manufacturer. OD was measured on an ELISA reader with a 450 nm microplate photometer. Results, individual sample interpretation; Sample/Positive (S/P), according to the value evaluated. S/P % ≥ 55 % positive, 45 % < S/P % < 55 % suspect, S/P % ≤ 45 % evaluated as negative.

Determining the Specificity and Sensitivity of a Diagnostic Method

The sensitivity of a diagnostic method is the positive values obtained by this method divided by the actual positive values. The specificity of a test is calculated by dividing the negative values found with this test by the true negatives (Erganiş 1993).

Results

Tuberculin Skin Test

A total of 423 cattle that performed intradermal bovine PPD and avian PPD tests were evaluated by measuring the skin thickening in the PPD treated area with calipers. As a result of this evaluation, 86 (20.33%) cattle were diagnosed with Tuberculosis due to positive results of PPD skin test, while 337 (79.67%) cattle gave negative results. Cattle found suspicious in the first PPD skin test application were added to the first application results by being found negative or positive according to the results of the second PPD skin test application performed 60 days later (Figure 1).

Figure 1. A. Avian (A) PPD positive reaction, B. Avian (A) PPD and Bovine (B) PPD positive reaction.

bTB IFN-γ ELISA

According to the results of this evaluation; 84 (19.86%) out of 423 cattle were positive for Tuberculosis. Since CITT was also applied to the same animals for the diagnosis of Tuberculosis, when we compared these two diagnostic methods, 12 cattle gave positive results to PPD skin test and negative results to IFN-γ test. On the other hand, 10 cattle gave positive results to IFN-γ test and negative results to PPD skin test (Table 3).In this study, when IFN-γ test was compared with tuberculin skin test in cattle herds with suspected Tuberculosis; the sensitivity of IFN-γ test was 86% and specificity was 97%.

Table 3. IFN-γ test and Intradermal Tuberculin test results.

Farm of No.	Number Animals	Intradermal Tuberculin Test		IFN-γ Test	
		Positive	Negative	Positive	Negative
1	144	34	110	**28**	**116**
2	51	31	20	**27**	**24**
3	31	11	20	**13**	**18**
4	47	0	47	**8**	**39**
5	150	10	140	**8**	**142**
Total	423	86	337	**84**	**339**

Antibody ELISA

ELISA was performed on blood sera obtained from 423 cattle for the diagnosis of Paratuberculosis infection and according to the ELISA results: In 423 bovine blood sera, 44 (10.4%) were positive, 10 (2.37%) were suspicious and 369 (87.23%) were negative for antibodies against *Map*. All 5 dairy cattle farms sampled were positive for Paratuberculosis (Table 4).

PTB IFN-γ ELISA

Out of a total of 423 cattle in 5 farms, 21 of them were positive for IFN-γ ELISA, while 402 of them were negative. (Table 4).

Bacterioscopy

According to the results of fecal examination by ZN staining method, 23 cattle were evaluated as positive, 19 cattle as suspicious and 381 cattle as negative, and they were scanned under a microscope at 100x objective for AFB (Table 4) (Figure 2).

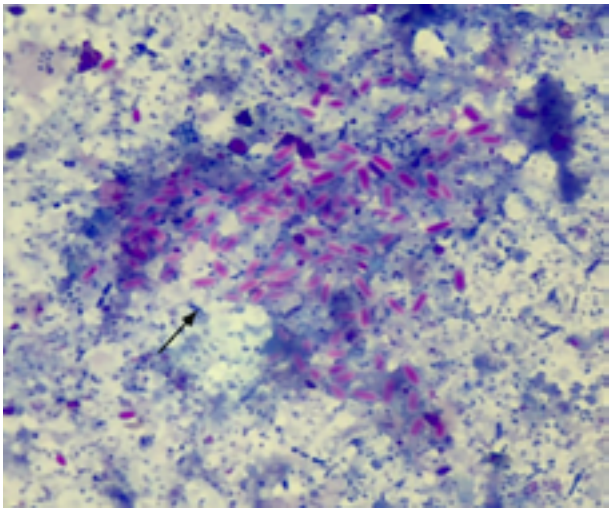

Figure 2. Microscopic appearance of AFBs in fecal samples by ZN staining method at 100x objective (Short, red-pink, thick and intertwined coccobacilli).

Table 4. Results of antemortem diagnosis of *Map* infection.

Farm No	Antibody ELISA			Fecal Bacterioscopy			IFN-γ ELISA		Number of Animals
	Positive %	Suspect %	Negative %	Positive %	Suspect %	Negative %	Positive %	Negative %	
1	15 (10.42%)	1 (0.7%)	128 (88.88%)	5 (3.47%)	7 (4.86%)	132 (91.67%)	8 (5.55%)	136 (94.45%)	144
2	7 (13.72%)	2 (3.93%)	42 (82.35%)	-	7 (13.73%)	44 (86.27%)	-	51 (100%)	51
3	7 (22.58%)	2 (6.45%)	22 (70.97%)	6 (19.35%)	-	25 (80.65%)	1 (3.23%)	30 (96.77%)	31
4	1 (2.13%)	-	46 (97.87%)	-	-	47 (100%)	3 (6.38%)	44 (93.62%)	47
5	14 (9.33%)	5 (3.33%)	131 (87.34%)	12 (8%)	5 (3.33%)	133 (88.67%)	9 (6%)	141 (94%)	150
Total									**423**

Intradermal Avian PPD

As a result of this evaluation, 14 cattle were suspected for PTB infection and these animals were subjected to antibody ELISA, fecal bacterioscopy and IFN-γ ELISA for PTB diagnosis (Table 5) (Figure 1).

Table 5. Results of PTB diagnostic tests of 14 cattle suspected for *Map* infection.

Cattle No	Antibody ELISA	Fecal Bacterioscopy	IFN-γ ELISA
13	-	-	-
78	-	+	-
83	-	+	+
33	-	-	-
B8	-	±	-
B9	-	±	-
B18	+	±	-
17	+	+	-
31	+	+	-
29	-	-	-
K14	-	-	-
101	-	-	-
133	+	+	+
82	+	+	+

Positive (+), Negative (-), Suspect (±)

PTB Comparison of Diagnostic Methods

Among the diagnostic tests, the highest rate of positivity in cattle was detected in antibody ELISA (10.4%). This was followed by fecal bacterioscopy with ZN staining (5.44%) and IFN-γ ELISA (4.96%), respectively. When evaluated as a herd (farm); antibody ELISA (100%) showed the highest positivity value and this rate was followed by IFN-γ ELISA (80%) and ZN staining (60%) methods, respectively. According to the results of antibody ELISA, fecal bacterioscopy and IFN-γ ELISA performed on 423 cattle in 5 dairy farms, positivity was obtained from one, two or all three of these tests. Antibody ELISA, fecal bacterioscopy and IFN-γ ELISA results were positive in 27 (6.39%), 7 (1.66%) and 10 (2.36%) animals, respectively. Antibody ELISA and fecal bacterioscopy were positive in 11 (2.6%), antibody ELISA and IFN-γ ELISA in 6 (1.42%), fecal bacterioscopy and IFN-γ ELISA in 5 (1.18%) animals (Table 6).

Table 6. Comparison of positive results of antemortem diagnostic methods of *Map* infection.

Farm No	Analysis	Antibody ELISA	Fecal Bacterioscopy	IFN-γ ELISA
1	Antibody ELISA	9	2	4
	Fecal Bacteriscopy	2	1	2
	IFN-γ ELISA	4	2	2
2	Antibody ELISA	7	0	0
	Fecal Bacterioscopy	0	0	0
	IFN-γ ELISA	0	0	0
3	Antibody ELISA	3	4	0
	Fecal Bacterioscopy	4	2	0
	IFN-γ ELISA	0	0	1
4	Antibody ELISA	1	0	0
	Fecal Bacterioscopy	0	0	0
	IFN-γ ELISA	0	0	3
5	Antibody ELISA	7	5	2
	Fecal Bacterioscopy	5	4	3
	IFN-γ ELISA	2	3	4
Total	Antibody ELISA	27	11	6
	Fecal Bacterioscopy	11	7	5
	IFN-γ ELISA	6	5	10

Discussion and Conclusion

In this study; IFN-γ test and CITT, one of the antemortem diagnostic methods of Tuberculosis infection were performed on 423 cattle in a total of 5 dairy cattle farms with suspected Tuberculosis in Çankırı, Çorum, Ankara, Eskişehir and Konya regions. While 84 cattle gave positive results to IFN-γ test, 86 cattle gave positive results to CITT. The prevalences of CITT and IFN-γ test were 20.33% and 19.86%, respectively. PPD skin test was used as the gold standard method to determine the sensitivity and specificity of the IFN-γ test, and its sensitivity and specificity were 86% and 97%, respectively. Lahuerta-Marin et al. (2015) reported the sensitivity and specificity of IFN-γ test to be 88-94% and 85-98%, respectively. Sayın (2010) reported the sensitivity and specificity values of IFN-γ test to be 91.5% and 93.4%, respectively. Öztürk et al. (2010b) used PPD skin test as the gold standard method for the diagnosis of Bovine Tuberculosis and detected that the sensitivity and specificity of IFN-γ test were 90% and 97%, respectively. Gormley et al. (2006) reported that the sensitivity value of the IFN-γ test (90-93%) was higher than the sensitivity value of the PPD skin test (70-75%) and the specificity value of the skin test was 99.9% and the specificity value of the IFN-γ test was 95%. Hashem et al. (2022) used PPD skin test as the gold standard method for the diagnosis of Bovine Tuberculosis in their study and reported that the sensitivity and specificity values of IFN-γ test were 98% and 71.4%, respectively. The sensitivity and specificity values obtained from the studies were consistent with the values in our study. When we compared both tests, 12 cattle gave positive results to PPD skin test and negative results to IFN-γ test. Ahir et al. (2016) and Praud et al. (2015) reported that some animals reacted positively to CITT but negatively to IFN-γ test in their studies. Alvarez et al. (2009) reported that IFN-γ test was applied as an auxiliary test to tuberculin tests to determine the maximum number of infected animals in the diagnosis of Bovine Tuberculosis but among the possible factors affecting the performance of tuberculosis diagnostic tests. Paratuberculosis, a common disease in Spain and other European countries, may be the cause of false positive reactions when coinfected with Tuberculosis infection and detected that while the sensitivity of IFN-γ test was 50% in Tuberculosis and Paratuberculosis infected herds, the sensitivity of IFN-γ test was 78% only in Tuberculosis infected herds. Therefore, since the prevalence of *Map* infection in 5 farms was 100% and *Map* infection was present in all farms, it was thought that the sensitivity of IFN-γ test may decrease.

To diagnose PTB infection, antibody ELISA, IFN-γ ELISA and fecal bacterioscopy with ZN staining were used in combination in this study. In addition, based on the CITT results used for the diagnosis of Tuberculosis, animals with a positive reaction to avian PPD were considered suspicious for Paratuberculosis and antibody ELISA, IFN-γ ELISA and fecal bacterioscopy were performed on these animals. It was observed that most of the animals that tested positive were crossbred and pure cattle breeds with good body condition. The chronic nature of *Map* infection and the long incubation period portray a healthy appearance for many years before infected animals show overt symptoms of the disease, which may explain the findings of this study (Mortier et al. 2015). Diagnosis of PTB by direct bacterioscopy from feces, different rates of *Map* scattering or regular or intermittent excretion of the agent will cause errors in diagnosis (Nielsen and Toft 2008; Gilardoni et al. 2012; Borum et al. 2014). Animals found ELISA positive may or may not be *Map* shedders; antibodies may be produced before or many years after fecal shedding of the agent begins (Nielsen 2010). According to the results of this study, 23 (5.44%) animals were positive by ZN staining method in feces, 44 (10.4%) animals were positive by ELISA and 11 (2.6%) of 423 cattle were positive by antibody ELISA and fecal bacterioscopy. The fact that the prevalence of antibody ELISA was higher than the prevalence of fecal bacterioscopy and that negative or suspicious cases in fecal bacterioscopy were positive in antibody ELISA was thought to be due to the fact that, as reported by other researchers, the scattering of *Map* with feces occurs in periods and this scattering is absent when the sample is taken (Nielsen and Toft 2008; Nielsen 2010; Gilardoni et al. 2012; Borum et al. 2014).

Although the presence of PTB infection is known throughout Türkiye, the number of studies investigating the infection and its prevalence is limited. Tütüncü et al. (2018) reported the seroprevalence of ELISA as 10% and herd prevalence as 46.7% in blood serum samples obtained from a total of 859 dairy cattle older than 2 years of age, consisting of Hostein-Friesian cattle breeds and showing signs of chronic diarrhea in 15 farms in Amasya and Samsun regions. Makav and Gökçe (2013) reported a seroprevalence of 3.5% by ELISA and a herd prevalence of 41.6% in their study conducted in Kars region. Öztürk et al. (2010a) found a prevalence of 6.2% in a study conducted by ELISA on dairy cattle

in Burdur region. Karatay et al. (2020) determined the prevalence as 4.25% by antibody ELISA performed on blood serum samples taken from a total of 400 cattle from 22 dairy farms in Ardahan region. In a study conducted by Yıldırım and Civelek (2013) in Uşak region with fecal samples, ZN staining, was applied and the prevalence was found to be 17%. Borum et al. (2014) in a study using 305 Holstein Fresian dairy cattle aged 4-8 years from farms in and around Afyon region, the prevalence of Paratuberculosis in dairy cattle was determined as 31.8% by ELISA in blood serum samples and 4.59% by ZN staining in fecal samples. Knowledge of the global distribution of PTB is important for establishing control programs. The prevalence of PTB has been reported from different countries, mainly bovine PTB. In a study conducted in the Boyoca region of Colombia, Cruz-Estupinan et al. (2022) reported that the seroprevalence of ELISA was 3.1% in blood serum samples obtained from 882 cattle of different breeds and age groups. In another study conducted with dairy cattle in Sudan, Elmagzoub et al. (2020) reported a seroprevalence of 6.3% by ELISA and a herd prevalence of 18.9% and Ozsvari et al. (2020) reported the seroprevalence as 5.5% by ELISA in a study conducted in Hungary. Weber et al. (2009) in the Netherlands in dairy cattle farms and AL Anbagi and Salman (2022) in Iraq in buffalo farms reported that the prevalence was 27% according to the results of fecal examination with ZN staining method. In this study, the prevalence according to fecal examination results was 5.44%, while antibody ELISA seroprevalence was 10.4% and herd prevalence was 100%. The PTB prevalence rates obtained in this study were similar to those reported globally and with the results obtained from studies conducted in different regions of Türkiye.

ELISA is more specific and sensitive in animals over 2 years of age (Öztürk et al. 2010a; Makav and Gökçe 2013; Borum et al. 2014). In Paratuberculosis infection, although the animal may acquire the causative agent at an early stage, it is usually not until after 2 years of age that it is shed in the feces and clinical signs appear. The animal goes through a long subclinical period and slowly spreads the agent into the environment This period is important for the spread of the agent into the environment (Nielsen and Toft 2008; Dieguez et al. 2009). In this study, 31 (70.46%) of the 44 cattle positive for antibody ELISA were over 2 years of age and 13 (29.54%) were under 2 years of age, and 19 (82.61%) of the 23 animals positive for fecal bacterioscopy results were over 2 years of age and 4 (17.39%) were under 2 years of age. The prevalence values obtained in this study showed that the age of the animals had an effect on the antibody ELISA and fecal bacterioscopy results.

Measuring IFN-γ release is a very important diagnostic method for the identification of *Map* infected animals in the early stage of infection (Stabel et al. 2007). IFN-γ production has been reported to be one of the earliest detectable immune responses in the diagnosis of PTB infection (Nielsen 2010). Hence, it has been stated that IFN-γ test is the best option for the detection of subclinically infected animals (Vazquez et al. 2013). However, the specificity of diagnostic tests based on IFN-γ values is low for cattle under 16 months of age, and cattle identified as IFN-γ positive need to be supplemented with ELISA or additional tests detecting *Map* agents in feces to assess the disease process within the infected herd (Corneli et al. 2021). In Iraq, AL Anbagi and Salman (2022) reported that the prevalence was 18% according to IFN-γ ELISA results in a study conducted in buffalo farms. Vazquez et al. (2013) detected *Map* in tissues by culture or real-time PCR in 36.1% of the positive cattle in their study with IFN-γ ELISA. Şababoğlu conducted a study on sheep in Burdur region of Türkiye for the diagnosis of *Map* infection by IFN-γ ELISA and found positivity in 33 (22%) of 150 sheep (Şababoğlu 2019). In the present study, the criterion reported by Vazquez et al. (2013), was used and positivity was detected in 21 (4.96%) of 423 cattle. Alvarez et al. (2009) reported that the presence of Tuberculosis and Paratuberculosis infections in the same herd may decrease the sensitivity of IFN-γ ELISA by approximately 20%. It was thought that the low prevalence of IFN-γ ELISA may be due to the fact that there were animals with positive results for both infections in 5 farms participating in this study. In addition, out of a total of 423 cattle in 5 farms, 21 cattle were positive for IFN-γ ELISA, while 402 cattle were negative. Since none of these 21 cattle showed clinical signs and only 5 (23.81%) cattle were found to be positive in IFN-γ ELISA and 16 (76.19%) were found to be negative according to the results of fecal examination, these animals were considered to be in the early or subclinical stage of infection as reported by other researchers (Stabel et al. 2007; Nielsen 2010; Vazquez et al. 2013).

In a study conducted by Vural et al. (1995) in cattle farms belonging to the General Directorate of Agricultural Enterprises under the Ministry of Agriculture and Forestry in Türkiye. In their study, the results of intradermal avian PPD and bovine PPD tests applied to 4923 cattle; 73 positive and 19 suspicious

reactions were detected against avian, while 52 positive reactions and 6 suspicious reactions were observed against intradermal PPD johnine applied to cattle with avian reactions. Although there is antigenic similarity between avian PPD and johnin PPD strains, it has been stated that a slight difference can be seen according to the reactions received. Therefore, PPD johnin was administered to animals that reacted to avian PPD to obtain more sensitive results. In the current study, animals with positive intradermal avian PPD reactions were considered suspicious for PTB infection and antibody ELISA, fecal bacterioscopy and IFN-γ ELISA were performed and 14 (3.31%) out of 423 cattle were suspected for PTB infection. Of these 14 cattle, 5 (35.71%) were positive for PTB infection by antibody ELISA, 6 (42.86%) by fecal bacterioscopy and 3 (21.43%) by IFN-γ ELISA. Among the 14 cattle suspected for PTB infection, while 2 (14.29%) cattle were positive for antibody ELISA, fecal bacterioscopy and IFN-γ ELISA, 2 (14.29%) cattle were positive for antibody ELISA and fecal bacterioscopy and 1 (7.14%) cattle was positive for fecal bacterioscopy and IFN-γ ELISA. According to these results, in PPD bovine and PPD avian intradermal tuberculin applications applied for the diagnosis of tuberculosis infection in tuberculosis suspected farms, it was thought that animals reacting to PPD avian may be infected with PTB or may be a reaction caused by atypical (bird type) AFBs found in cattle.

As a result of this study; the results of the IFN-γ test in the antemortem diagnosis of Bovine Tuberculosis are similar to the results of the CITT, and by applying both tests together, more Tuberculosis infected animals will be diagnosed. In a comparative intradermal tuberculin test for the diagnosis of Tuberculosis infection, animals with a positive reaction to intradermal avian PPD, supplemented by other tests used for the diagnosis of Paratuberculosis, seemed to play a significant role in the detection of nonspecific reactions or in the identification of PTB infected animals. In the diagnosis of *Map* infection in cattle, more infected animals can be diagnosed by combining different diagnostic methods.

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Original Article
Özgün Araştırma

Study on the labels of selected cat and dog foods from Türkiye

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Abstract: At first look, the label contains details regarding the contents and properties of cat and dog foods. Label information must be added in accordance with national and international legal requirements. The information competence of cat and dog food labelling in Türkiye has not before been analyzed. In this study, the labelling information for 215 chosen cat and dog foods (164 cats and 51 dogs) available in Türkiye has been analyzed through using Turkish national feed legislative and the Federation of the European Pet Food Industry recommendations. It is observed that all food labelling information is compliant with the Turkish feed legislation. In comparison, only 67.1% of cat and 78% of dog diets includes the metabolizable energy value in the package. 60.8% cat foods have website's have, whereas 81.7% dog foods have ones. It is also found that cat and dog food labels completely complied with the Turkish legislation rules. It is obvious that, more information may be valuable for the customers. Information about the recommended amount of food per day is unclear in numerous products, which is an area of improvement. In future studies, the competencies of daily nutrition advice information on labels can be evaluated.

Keywords: Customer enlightenment, FEDIAF, legislation, pet food, regulation.

Türkiye'den seçilmiş kedi ve köpek mamalarının etiketleri üzerine kesitsel bir çalışma

Özet: Etiket bilgileri, ilk bakışta evcil hayvan mamasının içeriği ve özelliklerine ilişkin ayrıntıları içerir. Etiket bilgileri ulusal ve uluslararası gerekliliklere uygun olarak yer almalıdır. Türkiye'de kedi ve köpek mamalarının etiket bilgilerinin yeterliliği daha önce değerlendirilmemiştir. Bu çalışmada, Türkiye'de satışta olan seçilmiş 215 evcil hayvan mamasının (Kedi=164, Köpek=51) etiket bilgileri, Türk Yem Mevzuatı ve Avrupa Evcil Hayvan Maması Endüstrisi Federasyonu tavsiyeleri kullanılarak incelenmiştir. Tüm mamaların etiket bilgilerinin Türk yem mevzuatına uygun olduğu tespit edilmiştir. Buna karşılık, kedi mamalarının sadece %67,1'i ve köpek mamalarının %78'i metabolize olabilir enerji değerini ambalaj üzerinde belirtmiştir. Kedi mamalarının %60,8'i, köpek mamalarının ise %81,7'si internet sitesine sahiptir. Kedi ve köpek maması etiketlerinin Türk yem mevzuat kurallarına tamamen uygun olduğu tespit edilmiştir. Daha fazla bilginin müşteirler için değerli olabileceği açıktır. Günlük önerilen beslenme miktarı birkaç üründe belirsizdir ve bu da iyileştirilmesi gereken bir alandır. Gelecekteki çalışmalarda, etiketlerdeki günlük beslenme önerileri bilgilerinin yeterlilikleri değerlendirilebilir.

Anahtar kelimeler: Evcil hayvan maması, FEDIAF, mevzuat, müşteri bilgilendirilmesi, yönetmelik.

Introduction

Cats and dogs are becoming increasingly popular companion animals in Türkiye. In 2023, the Federation of the European Pet Food Industry (FEDIAF) reported 1.386.000 pet dog and 4.660.000pet cat population in Türkiye (FEDIAF 2023). In recent years, parallel to the increased interest in having cats and dogs as companion animals, the cat and dog food industry has also grown tremendously. Domestic and imported commercial cat and dog foods are now easily accessible in the Turkish market. In the meantime, label information is a practical approach to understanding whether foods on the market meet the nutritional needs of cats and dogs. Many caregivers must rely on these labels for information regarding the nutritional adequacy and palatability of the product (Case 2011).Legislation and guidelines accepted by various bodies in each nation govern the ingredients and labelling of the foods. For example, in Türkiye, it is regulated with additional recommendations by the Ministry of Agriculture and Forestry (MoAF); in the USA, by the Association

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of American Feed Control Officials (AAFCO) and the US Food and Drug Administration (FDA); and in Europe and the United Kingdom, by FEDIAF and the Regulation of the European Parliament and Council (EC) (EC 767/2009; FEDIAF 2019; FDA 2022; AAFCO 2023). Moreover, Turkish feed legislation has been harmonized with the EU since 2011 (RG 2011). As stated in Annex-1 to the "Regulation on the Placing on the Market and Use of Feeds" set forth by the MoAF in Türkiye, "Provided that the methods of analysis are not changed, the labels for domestic and ornamental animal foods may contain the following statements: protein instead of crude protein, fat content instead of crude oils and fats, inorganic matter instead of crude ash" (RG 2011).

All commercial according to foods sold in Europe and the United Kingdom include a range of information on the label in compliance with the FEDIAF and the implementation requirements of the EC Regulation. According to FEDIAF recommendations, the daily intake amount should be arranged the pet's life stage, size, and lifestyle (FEDIAF 2020). In order to achieve this, informative label information plays a crucial role.

A few research has focused on these labels of cat and dog foods. In a Brazilian study, which analyzed the labels of 64 complete kibbles for dogs and cats, all of these labels were non-compliant with at least one of the national statutory standards. (De Souza et al. 2013). Nonetheless, there are standards defined at the national and international levels to assure food safety and customer information on the labels; however, previous studies have revealed that compliance with the regulations was insufficient. (De Souza et al. 2013; Gosper et al. 2016; Burdett et al. 2018).

There is a scarcity of research on the food labelling in Türkiye. These food's labels sold in Türkiye are assumed to be accurate and easy for the consumers to understand and being also in compliance with Turkish Feed legislation and international recommendations. The purpose of this study is to determine whether the labelling of selected the foods available in the Turkish market complies with the national regulations and the FEDIAF recommendations.

Material and Method

The study focused on the cat and dog foods manufactured in Türkiye (domestic) and imported foods. These foods were purchased from markets, veterinary clinics, and pet shops in the Marmara region between June 2023 and May 2024. A total of 215 food samples (64 cats and 51 dogs) were collected. Product name, assigned period, flavor, segment, origin (domestic-imported), website address, whether there is a nutritional recommendation, nutrients [crude protein (CP), crude fat (CF), crude ash (CA), crude fiber (CFib), carbohydrate (CHD), moisture (MOIST), metabolizable energy (ME) Kcal/Kg] data were recorded. Then, the foods were classified as adult, puppy, or sterile, as well as premium, market, or pet shop food, based on their availability in markets, pet shops, or veterinary clinics, as well as their labeling data. Both the presence of ME information on the website and conformity of the website's content information with the label have been controlled. In the classification of food ingredients, the first meat type is accepted as the primary ingredient. Foods containing chicken, turkey, and duck were classified as "poultry"; foods containing trout, anchovy, tuna, sardine, prawns, salmon, herring, anchovy, and cod as "fish"; and foods containing lamb, veal, pig, and beef as "red meat". Descriptive statistics (percentages, means, and standard deviations) were calculated and reported using Microsoft Excel. Differences between groups were assessed using the Chi square test, and if necessary, the Fisher's exact test was used, with a significance level of 0.05.

Results

In this study, a total of 148 dry and 16 wet cat foods and 43 dry and 8 wet dog foods have been examined. When the foods were classified according to age terms, a total of 132 adult foods [61.39% (132/215)], include 82.4% of the adult dog foods (42/51) and 54.90% of the adult cat foods (90/164); 18.13% of the puppy foods (39/215), contains 17.6% of the puppy foods (9/51) and 18.3% of the kitten foods (30/164); and 26.8% of the sterilized cat foods (44/164) have been determined. More detailed information can be found in Table 1.

All labels have complied with the mandatory packaging and content provisions of the Turkish regulations. The labels have contained no assertions that can deceive or mislead consumers. While 67.1% of cat foods (110/164) have lacked ME information on the labels, this incidence was 78.00% for dog foods (78/51). In line with this information, the rate of ME value on food website addresses was 62.2% for cat foods (102/164) and 82.4% for dog foods (42/51) (p=0.14). More information on the period, flavor, segment, origin, website, nutrition information, ME on the website, CP in label, CF in label, car-

bohydrate in label, ash in label, fiber in label, moisture in label, and ME in label can be found in Table 1.

Except for one Turkish-manufactured wet dog food, all the other food labels have included moisture values. CP values have been found to be lower than the FEDIAF suggested value in 1.90% of dog foods (1/51), and crude fat values have been lower than the FEDIAF suggested value in 7.8% of dog (4/51) and 1.21% of cat (2/164) foods (Table 2-3).

When all cat and dog foods have been analyzed, it was found that 81.7% (134/164) of cat foods and 60.8% (31/51) of dog foods have label information on their official websites (p=0.002). In terms of finding of nutritional information, 78.7% of cat food (129/164), 58.8% of dog food (30/51) (p=0.05), while 79.6% of imported (82/103) and 68.8% (77/112) of Turkish manufactured food (p=0.08) had feeding recommendation. 37.8% of cat (62/164) and 17.6% of dog (9/51) foods were founded to have ME information on their websites (p=0.01), while 49.5% of imported (51/103) and 17.9% of Turkish manufactured (20/112) foods had ME information on their websites (p<0.001).

Table 1. Characteristics of the imported and Turkish manufactured (TR) feeds included in the study.

Parameters		Cat				Dog			
		Imported		TR		Imported		TR	
		n	%	n	%	n	%	n	%
Period	Kitten	19	21.3	11	14.7	3	21.4	6	16.2
	Adult	43	48.3	47	62.7	11	78.6	31	83.8
	Sterilized	27	30.3	17	22.7				
Flavour	Fish	29	32.6	31	41.3	5	35.7	13	35.1
	Red meat	11	12.4	15	20.00	6	42.90	20	54.1
	Poultry	49	55.1	29	38.7	3	21.4	4	10.8
Segment	Market	6	6.7	14	18.7	2	14.3	2	5.4
	Petshop	38	42.7	40	53.3	1	7.1	22	59.5
	Premium	45	50.6	21	28.00	11	78.6	13	35.1
Origin	Imported	74	83.1	74	98.7	7	50.00	36	97.3
	TR	15	16.90	1	1.3	7	50.00	1	2.7
Website	No	15	16.90	15	20.00	9	64.3	11	29.7
	Yes	74	83.1	60	80.00	5	35.7	26	70.3
Nutrition information	No	13	14.6	22	29.3	8	57.1	13	35.1
	Yes	76	85.4	53	70.7	6	42.90	24	64.90
ME on the website	No	41	46.1	61	81.3	11	78.6	31	83.8
	Yes	48	53.90	14	18.7	3	21.4	6	16.2
CP in label	Yes	89	100	75	100	14	100	37	100
CF in label	Yes	89	100	75	100	14	100	37	100
CHD in label	No	89	100	75	100	14	100	37	100
Ash in label	Yes	89	100	75	100	14	100	37	100
Fiber in label	Yes	89	100	75	100	14	100	37	100
Moisture in label	No	51	57.3	60	80.00	7	50.00	27	75.00
	Yes	38	42.7	15	20.00	7	50.00	9	25.00
ME in label	No	50	56.2	60	80.00	9	64.3	30	83.3
	Yes	39	43.8	15	20.00	5	35.7	6	16.7

Abbreviations: ME: metabolizable energy, CP: crude protein, CF: crude fat, CHD: carbohydrate

Table 2. Nutritional label information of the imported and Turkish manufactured (TR) dry feeds included in the study.

Parameter	Cat				Dog			
	Imported		TR		Imported		TR	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Crude Protein (%)	36.69	4.95	32.45	3.96	23.54	8.48	24.61	4.61
Crude Fat (%)	15.41	4.01	13.69	3.39	13.51	5.34	12.58	3.55
Carbohydrate (%)	28.87	7.10	34.97	6.35	34.69	14.98	42.95	7.53
Crude Ash (%)	7.73	1.06	7.86	1.50	6.34	2.28	8.07	0.94
Crude Fiber (%)	3.68	2.20	3.23	0.97	2.99	1.64	3.43	0.88
Moisture (%)	7.93	1.13	7.92	0.40	18.93	28.04	8.36	0.76
ME (kcal/1000g)	3870.20	285.57	3984.27	170.67	2786.00	1736.54	3838.83	214.17

Abbreviations: SD: standard deviation, kcal: kilocalories, g: gram

Table 3. Nutritional label information of the imported and Turkish manufactured (TR) wet feeds included in the study.

Parameter	Cat			Dog		
	Imported		TR	Imported		TR
	Mean	SD	Mean	Mean	SD	Mean
Crude Protein (%)	11.56	1.88	18.00	9.03	1.91	7.00
Crude Fat (%)	3.13	1.47	1.90	5.23	2.20	3.00
Carbohydrate (%)	1.86	0.67	1.10	3.47	1.35	4.60
Crude Ash (%)	1.86	0.68	1.70	2.83	0.42	3.0
Crude Fiber (%)	0.40	0.27	0.30	0.30	0.17	0.40
Moisture (%)	81.19	2.91	77.00	79.14	2.73	82.0
ME kcal/1000g	1121.00			1140.00	8.49	

Abbreviations: SD: standard deviation, kcal: kilocalories, g: gram

Table 4. The study's food labels were evaluated in accordance with Turkish feed standards and FEDIAF recommendation

Subject	Article No. A: MoAF (RG 2011) B: FEDIAF (FEDIAF 2019)	Percentage
	Mandatory specific labeling	
Label information is presented clearly, visibly, and indelible.	A. 28155 RG/2011Section four Article 13 (1) (2) B. R. 767/2009, Article 14. 1) 2)	100% of cat and dog foods.
i. Food's intended function and target species ii. Shortest storage life iii. Approval, batch, or serial numbers iv. Net weight or volume v. Name and address of food operator vi. Contact phone number for further information	i. A. 28155 RG/2011 Section Four Article 16 (1) a) b) B. R. 767/2009, Art. 17.1(a), R. 767/2009 Annex II, Article 3b ii. A. 28155 RG/2011 Section Four Article 16 (1) ç) (1-2-3) B. R. 767/2009, Art. 17. 1(d), Annex II.2 R. 178/2002, Art. 15 (2) iii. A. 28155 RG/2011 Section Four Article 14 (1) c) ç) B. R. 767/2009, Art. 15 (d) section 4, Art. 3. (2) (r), Art. 15 (c), Art. 24, Annex V.2 iv. A. 28155 RG/2011 Section Four Article 14 (1) d) B. R. 767/2009, Art. 15 (e) v. A. 28155 RG/2011 Section Four Article 14 (1) b) B. R. 767/2009, Article. 15 (b) vi. A. 28155 RG/2011 Section Four Article 18 (1) B. R. 767/2009, Art. 19 (a) (b), Annex VII, I, 6	100% of cat and dog foods.

i. Declaration of analytical components ii. The name and weight percentage of the highlighted food ingredient. iii. Food ingredients, with their exact names in descending order of weight.	i. A. 28155 RG/2011 Annex-7 Section Two 1) B. R. 767/2009, Annex II.5 (Synonyms) ii. A. 28155 RG/2011 Section Four Article 16 d) 1) B. R. 767/2009, Art. 17.2 (a). iii. A. 28155 RG/2011 Section Four Article 16 3) d) B.R. 767/2009, Art. 17.1(e)	100% of cat and dog foods.
FEDIAF recommendations		
General labeling rules- General labelling rules and daily nutrition guidelines.	B. R. 767/2009, Annex II 4.	Cat food 78.8% (129/164) Dog food 58.8% (30/51)
Technical conditions- include moisture content declaration.	A. 28155 RG/2011 Annex-1 6. B. R. 767/2009, Art. 15 (g) & Annex I.6	Cat food 32.3% (53/164) Dog food 32.0% (16/51)
Labeling of nutrient components- energy and protein values declaration	A. 28155 RG/2011 Annex-7 Section Two 3) B. R. 767/2009, Annex VII.II.3 CEN standard 16967:2017	Cat food %32.9 (54/164) Dog food %22 (11/51)
Feed additive- Declaration for feed additives, including amino acids, vitamins, and trace minerals.	A. 28155 RG/2011 Annex 6 Section Two 2) B. R. 767/2009, Annex VII.I. Final section	%100 of cat and dog foods.

Abbreviations: R: Regulation, Art: article, CEN: European Committee for Standardization

Discussion

The nutritional adequacy of the food is vital for the companion animal lives. Labelling is one of the most significant aspects of understanding the food specifications. Because there has been no previous research on cat and dog food labelling rules in Türkiye, the findings of this study are intended to shed light on the current state of labels. In a prior Brazilian study, 12.5% of the label information was not viewable because it was in different positions of the packaging (De Souza et al. 2013). On the other hand, a similar study was previously conducted for human food packages in Türkiye. Dikmen and Pekcan (2013) founded that 13% of the human food packages had no label information. In this study all packages have visible labels. Label information must be visible and legible in order to provide customers with accurate information.

In the aforementioned investigation, 12.5% of the labels (8/64) were misclassified based on the type of foods required by the Brazilian Feed legislation (De Souza et al. 2013). In this investigation, all foods were accurately classified according to the Turkish legislation. Providing the right type of food is critical for the pet's appropriate and balanced nourishment. Storage conditions and consumption time are important for the safe consumption of the food. De Souza et al. (2013) reported in his study that all of the labels contained storage conditions, but in 17% of the samples, the storage period was not given in exact dates, but they provided as the consumption period. In this study, it was found that the storage conditions and expiration dates of all foods were in accordance with the regulation and FEDIAF guidelines.

The rate of ME values on the labels and website of both cat and dog foods is below 40%. It is thought that the most important reason for this may be that the declaration of ME value on the label is optional in national and international feed regulations and guidelines. However, it has been stated that to have ME information on the label plays a critical role in understanding calorie information and influencing cat and dog foods purchasing decisions (Schleicher et al. 2019).

There is no obligation for food companies to have a website. Previous studies did not evaluate the website information on the package. Therefore, it is not possible to compare this study's website information findings to the previous studies. However necessary information that is not on the labels can be published on the websites and quickly accessed via QR codes. It is thought that this is a feature that can be easily added to the packages by the feed manufacturers.

Very few studies has only reviewed the conformity of cat and dog food label information with leg-

islation (De Souza et al. 2013; Holda et al. 2014). Furthermore, there are certain studies where chemical analyses were performed and the results and label information compliance were reviewed with AAFCO, FEDIAF, and NRC recommendations (Carciofi et al. 2006; Akinrinmade and Akinrinde 2011; Gosper et al. 2016; Davies et al. 2017; Burdett et al. 2018; Stercova et al. 2022). Both studies indicated the necessity for a revision and audit of the label regulation of cat and dog foods.

De Souza et al. (2013) founded that 47% of the food had false words and graphics on the benefits of food or animal health confidence. Holda et al. (2014) examined the label compliance of feeds before (2011) and after (2013) the EU legislation came into force. It was reported that the rate of misinformation on food in 2013 decreased, but the rate of misleading information remained the same for dog food, while the rate for cat food increased from 22% to 29% and the assessment of the compliance of label information with the EU regulations revealed an unsatisfactory level of accuracy (Holda et al. 2014). In this study, no food labels were found to contain false information. Misleading labels might lead to misconceptions among owners. All feed packages have contained a clear and legible description of the mandatory labelling information in this study. In the study by De Souza et al. (2013) 37.5% of the food (17/64) did not meet this criteria.

The low moisture content of dry cat and dog foods is important as it inhibits the growth of most organisms (Case 2011). All dry foods with a moisture value above 7% in our study have a moisture value on the label in accordance with the relevant regulation. In one dog wet food did not have a moisture value on the label. It would be suggested to have moisture value in all foods.

In this study, it was observed that CP in one adult imported dry, in four adult domestic dog foods, and CF in one imported kitten and one domestic adult cat food to be below the recommended minimum reference value of FEDIAF (2020). Previously, Stercova et al. (2022) reported one dry dog food with a CP value below the FEDIAF minimum reference values.

Ingredients were determined to be insufficiently detailed. Few foods have detailed ingredient lists. Olivry and Muller (2018) found that mislabeling was extremely common in "limited" ingredient foods used in elimination diets for food allergy detection, which would be a challenge for animals on elimination diets. More detailed information is recommended, particularly for prescription foods. It is believed that presenting information in compliance with both Turkish and international norms will boost the worldwide competitiveness of Turkish feed products in the international level. Similarly, it is accepted that updating the current cat and dog food labelling system is important for the expansion of the cat and dog food sector in South Korea (Sung-Ho and So-Young 2023).

This study has some limitations. The study was conducted at a certain period and products. The results may not be representative of the entire Turkish market.

Conclusion

All cat and dog foods assessed met Turkish regulations. In addition, adhering to FEDIAF recommendations can boost Turkish feed producers' worldwide competitiveness. Consumers should be well-informed about the labelling of cat and dog foods. ME levels may be further investigated in future studies, as well as daily feeding recommendations. Stakeholders undertaking feed production research and legal authorities should provide good labelling practice guidelines for Turkish pet food manufacturers.

Ethics committee permission: Ethics committee approval is not required for this research.

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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 Galactooligosaccharide (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 cul-

tures 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%.

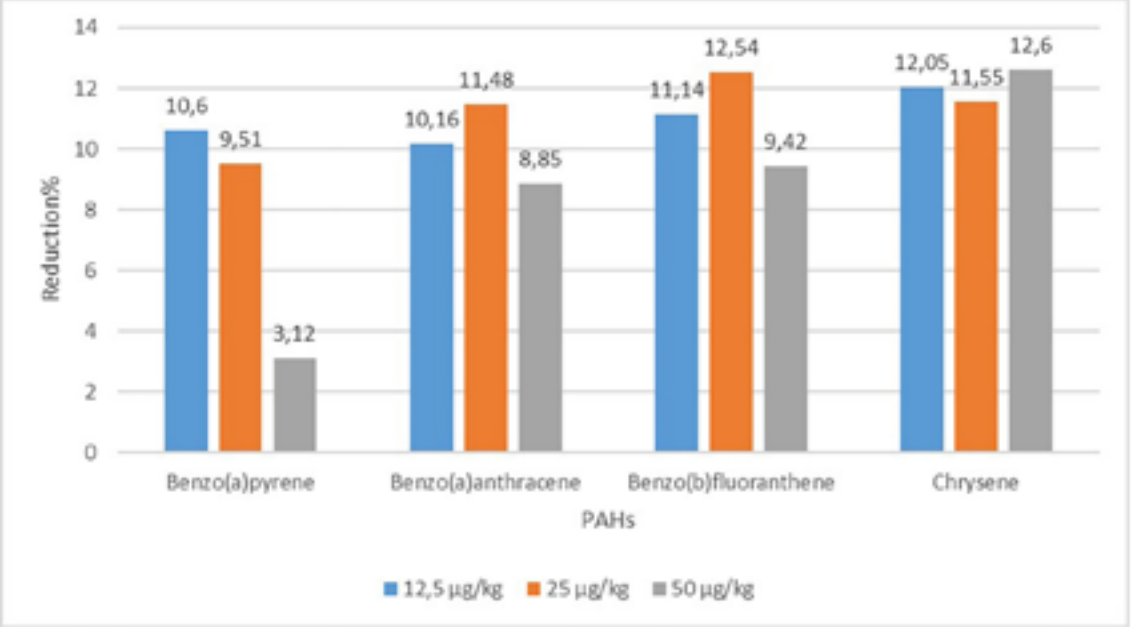

Figure 1. Yogurts made with traditional yogurt culture (group 1). Degradation levels of PAHs in yogurts made after adding 12.5, 25, 50 µg kg^{-1} PAHs to milk.

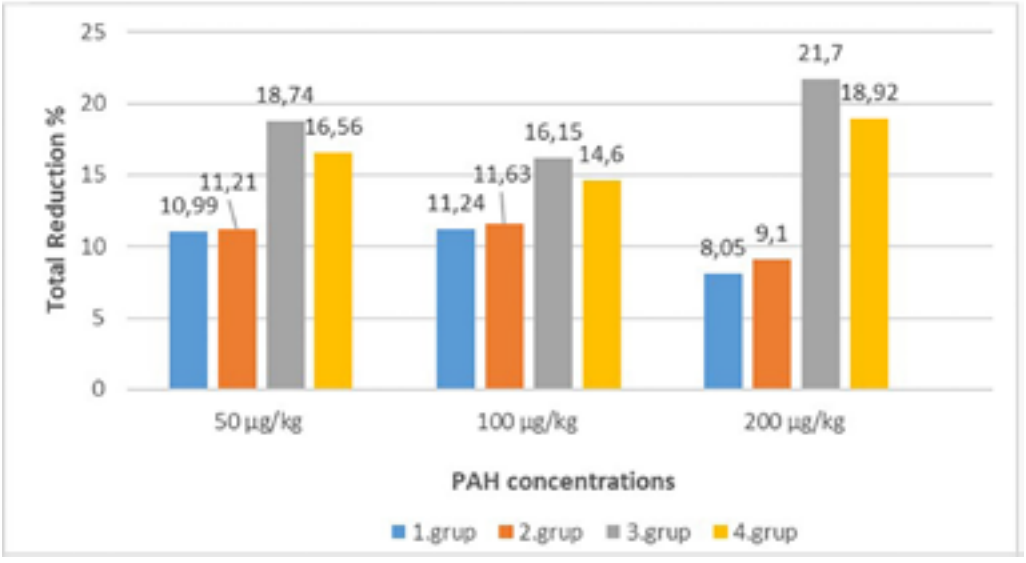

Figure 2. PAH degradation rates in yogurts made with different cultures after adding 50, 100 and 200 µg kg^{-1} PAH to milk.

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).

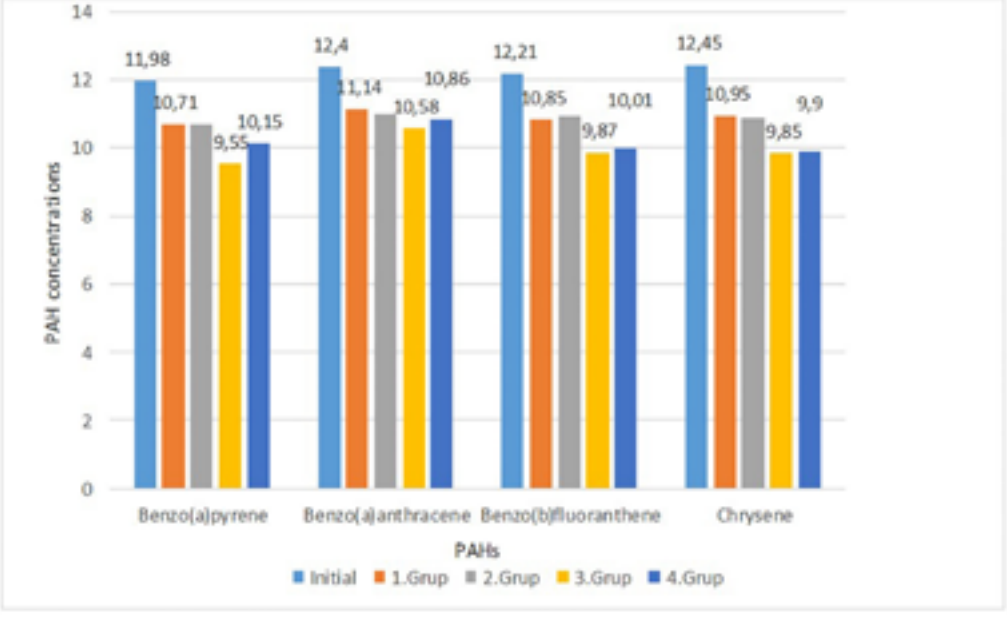

Figure 3. PAH concentrations measured in yogurts made after adding 12.5 µg kg^{-1} PAH to milk.

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*

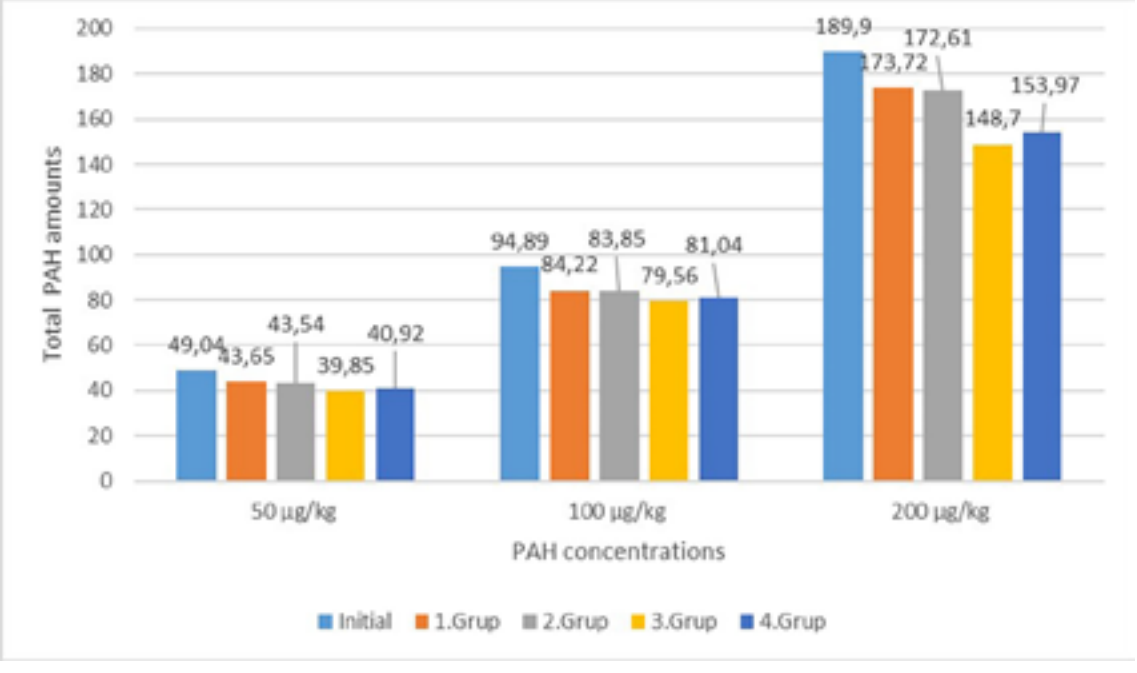

Figure 4. Concentrations of PAH compounds added to milk at concentrations of 50, 100 and 200 µg kg^{-1} measured in yogurts made with different yogurt cultures

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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Original Article
Özgün Araştırma

In vivo anthelmintic effect of *Artemisia annua* L. on oxyurid nematodes of laboratory mice

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Abstract: Oxyurids, common intestinal parasites, pose a recurrent health risk, particularly among children. Seeking natural and repeatable cures, this study explored the antinematodal effects of *Artemisia annua* L. n-hexane extract on oxyurids in vivo. The extract was orally administered to mice naturally infected with *Syphacia obvelata* and *Aspiculuris tetraptera* at doses of 300, 600, and 1200 mg/kg for seven days. Changes in oxyurid egg numbers were assessed through fecal flotation. On the 8th day, nematodes were compared across groups in necropsy for number, gender, and species.

As a positive control, Albendazole (ABZ) was administered at 5 mg/kg for three days, with corn oil serving as the solvent control. Fecal flotation revealed a 43.51% decrease in oxyurid egg counts in the ABZ group and a 21.12% increase in the *A. annua* 1200 mg/kg group on the last day of application. In necropsy, compared to the corn oil group, ABZ 5 mg/kg, *A. annua* 300 mg/kg, and *A. annua* 600 mg/kg groups exhibited reductions of 35.76%, 68.50%, and 63.64%, respectively, in terms of nematode numbers. The likely contributors to these results are the non-polar compounds of *A. annua* L., the very low concentration of ABZ, and the dilution solvent corn oil.

Key words: Anthelmintic; *Artemisia annua* L.; laboratory mice; oxyurid; worm counting

Laboratuvar farelerinin oksiyür nematodları üzerine *Artemisia annua* L.'nin *in vivo* antihelmintik etkisi

Özet: Oksiyürler, yaygın olarak görülen bağırsak parazitleridir ve özellikle çocuklarda tekrarlayan sağlık riski taşırlar. Doğal ve tekrarlanabilir bir tedavi arayan bu çalışma, *Artemisia annua* L. n-hekzan ekstraktının oksiyürler üzerindeki antinematodal etkisini *in vivo* olarak araştırdı. *A. annua* L. n-hekzan ekstraktı *Syphacia obvelata* ve *Aspiculuris tetraptera* türleri ile doğal olarak enfekte olmuş farelere 300, 600 ve 1200 mg/kg dozlarında 7 gün süreyle oral olarak uygulandı. Oksiyür yumurta sayısında meydana gelen değişim fekal flotasyon yöntemi ile değerlendirildi. Nekropsideki nematodlar gruplardaki sayı, cinsiyet ve tür açısından 8. günde karşılaştırıldı. Çözücü kontrol olarak hizmet eden mısır yağı ile birlikte pozitif ilaç kontrolü olarak Albendazol (ABZ) 5 mg/kg dozunda üç gün süreyle uygulandı. Uygulamanın son gününde fekal flotasyonda oksiyür yumurtalarının sayısı ilaç kontrol ABZ grubunda %43,51 azalma ve *A. annua* 1200 mg/kg grubunda %21,12 artış gösterdi. Nekropside, Mısır yağı grubu ile karşılaştırıldığında ABZ 5 mg/kg, *A. annua* 300 mg/kg ve *A. annua* 600 mg/kg gruplarında nematod sayısı açısından sırasıyla %35,76, %68.50 ve %63.64 azalma saptandı. Muhtemelen bu sonuçlara katkı sağlayanlar polar olmayan *A. annua* L. bileşenleri, ABZ'nin çok düşük dozda olması ve çözücü solventi olarak mısır yağı kullanılmasıdır.

Anahtar kelimeler: Antihelmintik; *Artemisia annua* L.; laboratuvar fareleri; oksiyür; solucan sayımı

Introduction

The drug resistance problem is the essential cause of the discovery of new medicines. On the other hand, infections from soil-transmitted helminths are endemic specifically in some regions of Asia, and Africa, and they are repeated frequently. Therefore, it is necessary to refer to herbal medicines because constant or combined usage of anthelmintic drugs affects kidney and liver functions negatively (Arise and Malomo, 2009).

Syphacia obvelata (Rudolphi, 1802) and *Aspiculuris tetraptera* (Nitzch, 1821) are nematodes from Oxyurid groups commonly found in mice and some rodents (Soulsby, 1982). Both nematodes inhabit in the gastrointestinal tract of the host animals and can be transmitted to humans through the ingestion of

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eggs. *Aspiculuris tetraptera* is frequently used in anthelmintic research to determine the efficacy of various chemotherapeutic agents (Theodorides, 1976).

A. tetraptera has a direct life cyclus and infection occurs after ingesting the eggs by the host, with a prepatent period of 21-35 days (Pritchett-Corning and Clifford, 2012). In the first stage nematode larvae stay for a week in the submucosa of the colon and develop into third-stage larvae, then pass to the colonic lumen to develop into adult larvae (Pritchett-Corning and Clifford, 2012). The mature females settle down large intestine to lay, who spend 45 to 50 days before laying their eggs. The eggs that released at night can stay alive for weeks outside the host and become contagious in 6-7 days at 24°C (Stepek et al., 2006). Table 1 shows the some characteristic properties of *A. tetraptera* and *S. obvelata*.

Table 1. Some characteristic properties of *S. obvelata* and *A. tetraptera*

	S. obvelata	A. tetraptera
Characteristics		
Female length	3.5-6 mm	3-4 mm
Male length	1-1.5 mm	2-3 mm
Female tail	Long and pointed	Conical
Cervical alae	Subtle	Prominent
Egg	~ 134 × 36 µm, banana shape	~ 86 × 37 µm, ovoid and symmetrical
Location in host	Caecum and colon	Colon
Location of egg	Perianal skin	Feces
Prepatent period	11-15 days	21-25 days
Infection time of egg	5-20 hours	5-8 days

Table was obtained and changed from Pritchett-Corning and Clifford (2012)

Nematodes of the genus *Syphacia* Seurat, 1916 (family Oxyuridae) are parasitic pinworms possesing host-specificity in several rodents (Perec et al., 2006). Because there are not enough specific characters to differentiate between pinworms, molecular advanced tools are used to identify closely related oxyurid species (Omer et al., 2020). The nuclear small subunit of ribosomal DNA (18S) and the mitochondrial cytochrome c oxidase subunit 1 (cox1) (Stewart *et al*., 2018) are evaluated for molecular discrimination of *Syphacia* species. Both sequences of 18S rDNA and the internal transcribed spacer (ITS1, 5.8S, and ITS2) regions are used the identification of *S. obvelata* showing morphological characteristics (Mohammed et al., 2024).

Several techniques can be used for identification of oxyurid nematodes beside using molecular tools. Because the eggs of oxyurids have some characterictics, they can be seen and detected under optic microscope after application of flotation or perianal tape methods. Fecal flotation or McMaster techniques are used for *Aspicularis tetraptera*, while perianal tape method is prefered for detection of *Syphacia obvelata* eggs classically (Hedrich, 2012).

Although infection with these nematodes does not cause significant symptoms; they drive changes that will affect the results of the experiment (Theodorides, 1976; Fox et al., 1984; Pinto et al. 1994; Gonzalez 1996; Sueta et al. 2002). Therefore, it is crucial to ensure that laboratory animals are free from parasites. *Artemisia annua* L. known locally in Türkiye "Peygamber süpürgesi" is an annual plant. It has 50-150 cm length. It is used as an infusion (2-3%) internally against dysentery and tuberculosis (Baytop, 1999). *A. annua* L. (Chinese: "qing hao", namely "green herb") is native to China. Utilized in Traditional Chinese Medicine for over 2000 years to reduce fever, the plant gained global recognition after the discovery that the artemisinin substance isolated from *A. annua* L. was effective in treating malaria in 1970, leading to its widespread adoption (Willcox et al. 2004). *A. annua* L. has been used not only for malaria but also for headache, fever, infection and inflammation (Memorial Sloan Kettering Cancer Center, 2010). According to a 2010 investigation, *Artemisia annua* L. was recommended in the oldest Chinese prescriptions found in the Mawagndui tomb, dating back to BC 168, depicting its usage for hemorrhoids in females (McGovern et al., 2010). The plant, *Artemisia annua*, is from the Asteraceae family, it has fern-like leaves, yellow flowers and a particularly pleasant smell. Trichomes contain various active compounds, including flavonoids, chromens, and various terpenoids (sesquiterpenoids and triterpenoids) (Brown, 2010). One of the most significant components is artemisinin, used for treating malaria, which has demonstrated anticancer properties, effective against breast cancer and leukemia (Singh and Lai, 2001; Singh and Lai, 2005). Additionally, the anthelmintic effect of artemisinin was shown experimentally (Cala et al., 2014). Artemisinin-derived drugs have also proven effective against various parasites, including *Fasciola hepatica* and gastrointestinal nematodes in ruminants, as well as *Plasmodium* spp., *Coccidia* spp., *Babesia* spp., *Leishmania* spp., *Neospora caninum*, and *Schistosoma* spp. (Tariq et al., 2009).

When the anthelmintic activity of *Laurus nobilis* extract in mice naturally infected with *Aspiculuris tetraptera* was investigated, it was seen that the concentration of 400 ml/kg alcoholic extracts of *L. nobilis* showed the 96% and 100% lethal effects for worms on three and six days after giving the treatment (Mares et al., 2023).

It is a need to learn a new and accurate pharmaceutical procedure that contains new application styles, dilution agents and the effective lowest concentrations of a known marked drug used in control groups in order to discover novel herbal treatment methods against parasites of animals.

The objective of the present study was to demonstrate the anthelmintic activity of the extract, with different application way (by using corn oil for dilution) and with a different drug dose (being lower than normal according to literature), by assessing the reduction in both oxyurid egg count and worm numbers. It was aimed to demonstrate the anthelmintic activity of the extract by evaluating the reduction in oxyurid egg counts and worm numbers.

Materials and Methods

Plant Material and Extract Preparation: *Artemisia annua* L. leaves were collected in 2010 from the Acemler district of Bursa (40º12´ 106 47.12´´N, 29º 0´53.01´´ E),Türkiye. Leaves were dried under shade, pulverized with a fine grinder and extracted with n-hexane in the Soxhlet apparatus. The solvent was evaporated with a rotary evaporator. The yield of extract was 4.5% (w/w). The crude extract was kept at -20°C.

Pharmacological Procedures

Animals: Balb-c mice of both sexes (28-35 g) were obtained from the animal breeding center of Bursa Uludag University, Bursa, Türkiye. The animals were kept in standard polypropylene cages, at 20-24ºC, with 55% relative humidity, they were fed standard pellets and water *ad libitum*. Naturally infected experimental animals for *S. obvelata* and/or *A. tetraptera* were selected from 150 mice by using perianal cellophane tape and fulleborn fecal flotation methods, respectively. Each group was made up of six animals, and each mouse was taken separate cage. All experiments were approved by the Experimental Animals Local Ethics Committee of Bursa Uludag University (no: 2017-10/07).

Infected mice were divided into six groups, four treated and two control groups by selection in the condition that approximately the same number of eggs would be in every group according to the results of fulleborn fecal flotation and perianal tape methods performed on two different days. Each group consisted of six animals three males and three females. Group I was 0.2 ml corn oil solvent control, Group II, III and IV of mice were treated with 300, 600 and 1200 mg/kg b.w. (body weight) doses of n-hexane extract of *A. annua* L. for seven days, respectively. Group V of mice was treated with a 5 mg/kg b.w. dose of reference drug Albendazole for three consecutive days. Group VI was the group drinking water. For oral application, except for Group VI, all groups were fed via steel esophageal gavage in the same manner and volume (by completing a total of 0.2 mL with corn oil). Corn oil was used as solvent for dilution of *A. annua* n-hexane extract because, according to literature (Harvey, 1996), corn oil could be used in the animal experiments in order to observe anthelmintic drug effectiveness.

Macroscopic Examination: Fecal materials were collected from cages of all mice by using pliers. Examples in the labeled plastic bags were weighed on electronic analytical balance. Fecal materials were transferred to petri dishes and physiological saline solution was added on it. It was waited for 10-15 minutes in order to be softened. Fecal materials were examined under stereo microscope by using plastic spatula to slightly spread the cumulative material. Founded worms were taken with a pin and transferred to optic microscope by microscopic slide preparation in order to determine the species and gender.

Fecal Egg Counting: After the acclimatization of mice, perianal tape and fecal flotation methods were used to be detected to be sure whether all mice had oxyurid infection.

The efficacy of plant extract on egg numbers was calculated by egg counting of fecal material collected from each mouse before the 7th and 1st days before the treatment and 1., 3., 5., and 7. days of treatment. Fecal materials were collected by searching through cages' shavings in which each mouse was held separate cage. Firstly, it was investigated whether adult forms of helminths and proglottids were in collected fecal samples. They were then passed to the flotation method (Kasım, 1994). To apply fecal flotation method, weighed feces was homogenized with approximately 10 mL of water, they were filtrated from a metal filter by spraying water on them, two lamels were put on this mixture and all the eggs on microscope slight were counted and the amount in grams of feces was found.

In the fecal flotation, decreasing % of oxyurid eggs was counted as follows:

Decreasing% = 100 × (C-T)/C

C is the arithmetic mean of oxyurid eggs of untreated control mice, and T is the arithmetic mean of oxyurid eggs in the treatment group obtained from fecal flotation.

Worm Counting: On the last day of the application, the mice were euthanized under anesthesia with sevoflurane. After the internal organs were taken into the petri dish, all organs were separated and taken into labeled vials containing 70% alcohol. All organs in the body cavity were investigated by stereomicroscope for the presence of helminths. They were opened seperately in petri dishes by pouring 70% alcohol on them. Organs were cut very carefully with a small metal scissors by avoiding from any damage on helminths. Intestines firstly were seperated according to regions, then all regions (jejunum, duedonum, cecum, and large intestine) opened in different petri dishes. Parts of the intestine were put on one side by pliers and were cut with small scissors by above side through all part. Contents of intestine were investigated by spraying 70% alcohol or physiological saline solution on them in order to diluate. A pin was used to disperse the content and to take the helminths. The collected helminths were identified under the light microscope, were separated, and counted according to their species, genders.

The percentage reduction, indicative of efficacy against oxyurid nematodes, was calculated as follows:

(%)E = 100 × (C-T)/C

In this formula, E is Efficacy, C is the geometric mean of worm numbers obtained from the necropsy of the corn oil solvent control group, and T is the geometric mean of worm numbers obtained from the necropsy of the treatment group. (%)Efficacy is calculated with this formula according to Kozan et al. (2011) and Turel et al. (2013).

The pictures were taken with Olympus CX23 microscope. The samples were sealed with Entellan® and preserved as permanent preparations.

Hematocrit Counting: The hematocrit counting was made in three groups; *A. annua* 600 mg/kg b.w., corn oil 2 ml and ABZ 5 mg/kg b.w. The blood taken from the hearts of mice were collected in two Eppendorf tubes one of them was added EDTA. Hematocrit counting was made using Wintrobe tubes.

Statistical Analysis

The post hoc test was chosen by making the normality analysis. Shapiro Wilk test was used to detect normality. The results of Levene's statistics were evaluated to detect the homogeneity of variance. For comparing the means of parasite egg numbers Kruskal Wallis non-parametric one-way ANOVA analysis method, comparing the means of helminths in the treatment groups, the Tamhane-T2 test and also Kruskal Wallis tests were used. $P<0.05$ was admitted as significant.

Results

Macroscopically, the first finding was that the adults of *S. obvelata* in the fecal examinations were found in the mice group of *A. annua* L. extract at 600 mg/kg b.w. dose (Group III), whereas they were not in groups of ABZ (Group V) and corn oil (Group I). *S. obvelata* adults began to be seen three days after the starting of application and no more than 2-3 worms. In the extract groups (Group II, III and IV), while the decreases in the number of oxyurid eggs were observed in some of the mice, there was no significant difference between the first and last days of the application in terms of the group of mice.

Considering the average number of helminths per group of mice according to the necropsy results, the anthelmintic activity in the *A. annua* 600 and the *A. annua* 300 group was found to be at the similar level as shown in Table 2. The anthelmintic activity, which was detected as approximately 35.76% efficacy, shows the application of ABZ at a dose of 5 mg/kg b.w.for three days have a mild effect. *A. annua* 600 mg/kg b.w. dose also shows a very similar effect (63.64%) in terms of worm count reduction comparing a 300 mg/kg b.w.dose of *A. annua* L. n-hexane extract for seven days (Table 2).

Table 2. Worm count at necropsy

	Worm count at necropsy			
Treatment	**Total number**	**Min-Max**	**Geometric Mean**	**% Efficacy**
Corn oil 2 ml	233	4-107	20.16	-
ABZ 5 mg/kg b.w.	152	2-78	12.95	35.76
A. annua 300 mg/kg b.w.	128	2-115	6.35	68.50
A. annua 600 mg/kg b.w.	196	0-125	7.33	63.64
A. annua 1200 mg/kg b.w.	98	3-38	17.43	13.54

ABZ: Albendazole, *A. annua*: *Artemisia annua*

Although *A. annua* 1200 mg/kg b.w. dose was expected to be at these values, only a slight decrease was observed (13.54%). Based on the fecal flotation results, there was a 68.56% increase observed in the corn oil group on the 7th day of the application compared to the initial count (the arithmetic mean of the counts on the -7th and -1st days). As indicated in Table 3 and Figure 1, the ABZ group exhibited the most significant decrease, although this decrease of 43.51% suggests a moderate effect.

Table 3. The arithmetic mean of EPG (Egg per gram) counted at fecal flotation

	-7th day	-1st day	1st day	3rd day	5th day	7th day
Drinking water	416.3	434.5	415.7	445.3	245.1	466.4
Corn oil	230.4	166.5	327.5	575.8	469.5	631.2
ABZ 5 mg/kg b.w.	70.4	78.5	59.0	77.7	20.7	42.0
A. annua L. 300 mg/kg b.w.	165.1	59.8	76.2	270.8	64.5	112.6
A. annua L. 600 mg/kg b.w.	325.1	163.0	83.5	133.6	165.1	233.8
A. annua L. 1200 mg/kg b.w.	52.3	76.0	76.2	133.0	213.9	81.3

ABZ: Albendazole

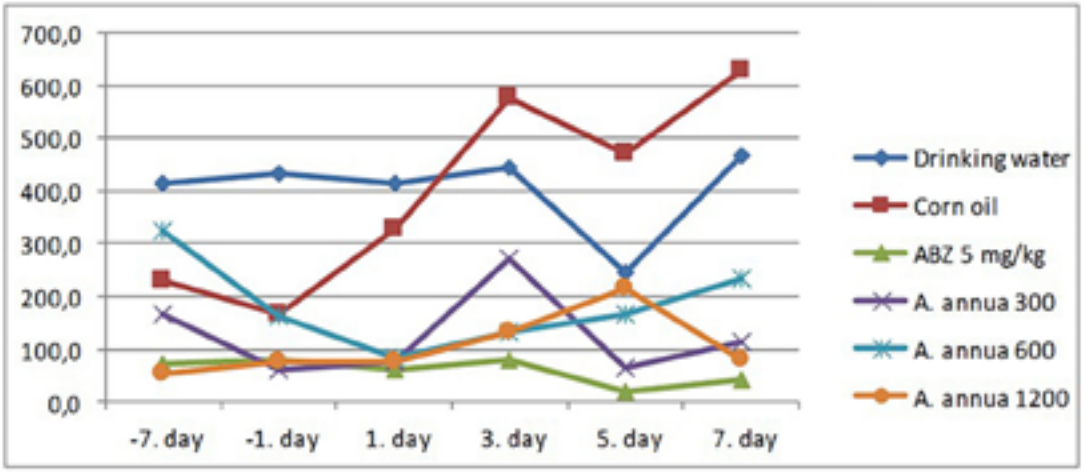

Figure 1. Arithmetic mean of *A. tetraptera* EPG for each mice group at fecal flotation

As shown in Figure 2, a slight decrease (4.22%) in the *A. annua* 600 group and an increase of 21.12% in the *A. annua* 1200 group indicated that the antinematodal effect of the extract could not be detected by fecal flotation or it would take a little longer to see for the result.

The oxyurid eggs seen in the fecal flotation have belonged to *Aspicularis tetraptera*, *Syphacia obvelata* and *Syphacia muris*. Their photographs are given in Figure 3.

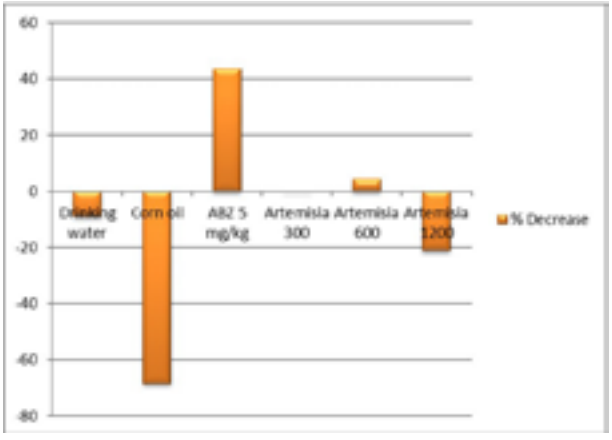

Figure 2. Decrease rate of EPG counted at fecal flotation on the last day comparing the arithmetic mean of -7th and -1st days. *Artemisia*: *Artemisia annua* L. n-hexane extract

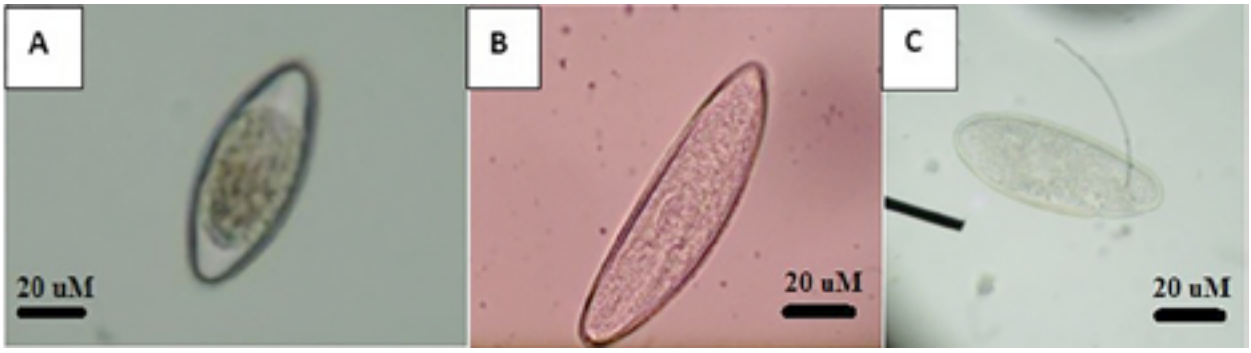

Figure 3. Oxyurid eggs in fecal flotation A) *A. tetraptera* B) *S. obvelata* C) *S. muris*

When comparing the numbers of helminths collected from the internal organs of each mouse during necropsy, the homogeneity of variance is found to be unacceptable according to Levene's statistics. While ANOVA is robust against violations of the assumption of normality and homogeneity of group variance when the group sizes are equal, in this study, some treated mice died before the eighth day, resulting in unequal group sizes in the final assessment.

Based on the results of the Tamhane 2 test, no significant difference was observed between the groups in terms of the number of helminths collected during necropsy. However, according to Figure 4, designed for the comparison of independent samples, there is a considerable disparity between group means and standard deviations, with the *A. annua* 600 group being notably prominent. This disparity in group means and standard deviations can be attributed to the substantial variation in the distribution of infection within the groups from the beginning. Contrary to expectations, the necropsy results did not reveal the anticipated effects from the extracts.

Because the normality could not be provided, non-parametric tests were used. According to the Kruskal Wallis test, there was a statistically significant difference (P=0.019) only between ABZ and corn oil in terms of eggs counted in fecal flotation. This result showed that *A. annua* n-hexane extracts applied at three different doses did not have a significant effect on *A. tetraptera* egg numbers. The statistical correlation between the treatment groups is shown in Figure 5.

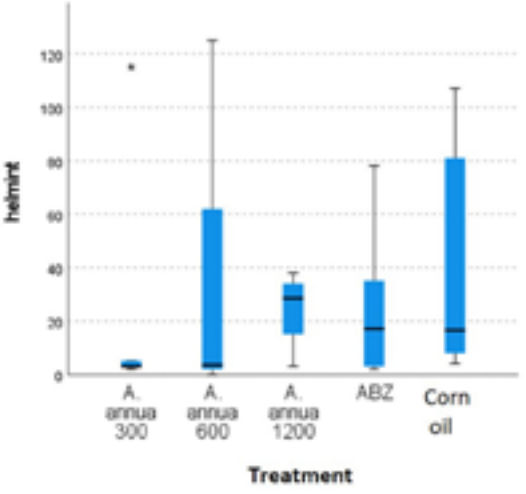

Figure 4. The comparison of the helminths found at necropsy via Kruskal Wallis

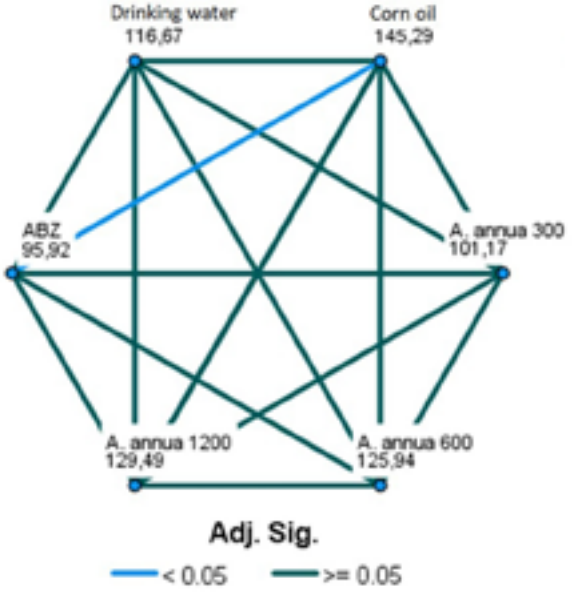

Figure 5. Relationships for adjusted significance (Adj. Sig.) in administration groups in fecal flotation

The results of the blood values of the mice showed that the mice in the ABZ-treated group had higher hematocrit levels than the other mice. This result is in line with the expected. A comparison of the arithmetic mean of hemoglobin and hematocrit count in groups of mice is shown in Figure 6.

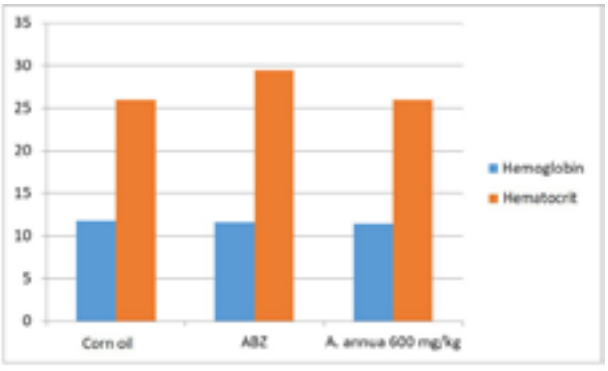

Figure 6. The arithmetic mean of the blood values of the mice in each group on the 8th day of the administration

Both the hematocrit and hemoglobin values of the mice in the other two groups (the mice in the group treated with corn oil as solvent control and the group treated with *A. annua* extract at a dose of 600 mg/kg b.w.) were found to have more intense infections, which were determined by the results of both fecal flotation and necropsy, were quite close to each other. This showed that this dose of the extract did not affect the hemoglobin and hematocrit counts of the mice.

Discussion and Conclusion

Various model organisms are employed in laboratory experimental animals to study soil-transmitted helminths, with *Trichuris muris* being a perfect model for trichuriosis in mice (Keeling, 1961), and *Strongyloides ratti* utilized for strongyloidiasis in rats (Wertheim and Lengy, 1965). Infected rodents with *Ascaris suum* serve as models for the onset of *Ascaris lumbricoides* infection (Tritten et al., 2011).

In our study, *Aspicularis tetraptera* and *Syphacia obvelata*, both belonging to Oxyurid nematodes, were selected as relevant organisms to demonstrate the antinematodal effect of plant extract in the gastrointestinal system. The significance of these two nematodes lies not only in their prevalent existence in laboratory mice and rats but also in their zoonotic characteristics. Given that most commonly used antinematodal drugs are generally broad-spectrum, the present study provides evidence that *A. annua* L. affects nematodes in the gastrointestinal system, suggesting its potential efficacy against soil-transmitted helminths.

Syphacia spp. and *A. tetraptera* were the common parasites of mice. In a parasitology research in Istanbul, Türkiye, the laboratory and pet mice (n=75) had been detected by Çetinkaya et al. (2017) with flotation technique in terms of the species of nematodes. According to distribution of them, *Syphacia* spp. and *Aspicularis tetraptera* were found as %20 and %40 in mice, respectively. If it was used the perianal tape method to detect the *S. obvelata* eggs, it could be found higher infection rate for *S. obvelata*.

According to a study by Turel et al. (2008), giving *Urtica dioica* L. leaves and seeds methanolic extracts orally for seven days to mice naturally infected with *A. tetraptera*, *Urtica dioica* L. seed methanolic extract had not a significant effect but leaves methanolic extract showed a potent anthelmintic effect. A study in sheep infected with *Haemonchus contortus* showed that oral administration of *A. annua* L. water extract and artemisinin had a low-level anthelmintic effect (Cala et al., 2014). In sheep infected with *Fasciola hepatica*, a single dose of 40 mg/kg b.w. (i.v.) of artesunate from *A. annua* L. reduced the egg count by 69% and the worm count by 77%. The same application of artemether obtained from the same plant reduced the number of eggs by 97.6% and the number of worms by 91.9% (Keiser et al., 2010). Extracts of *A. annua* L. were found to be 81.6–83.2% suppressive against *Cryptosporidium parvum* in mice (Youn and Noh, 2001).

In a recent study (Taljaard et al., 2022) that infusions of *Artemisia afra* and *Artemisia annua* were submitted to liquid-liquid partitioning with n-hexane and dichloromethane to provide samples for *in vitro* bioassays using newly transformed schistosomulas (NTS) and adult *Schistosoma mansoni* worms obtained from infected mice. *A. afra* and *A. annua* infusions and extracts were found that they possessed potent *in vitro* antischistosomal activities against NTS, at 100 µg/ml.

According to the results of the present study, since the *A. annua* L. n-hexane extract contains the non-polar active components of the plant, these components may have a mild anthelmintic effect. In the macroscopic examination, only a few helminths were found in the feces of mice in the *A. annua* 600 groups. These worms may have fallen due to the extract or may have been discarded randomly due to the intensity of the infection. As indicated in Table 3, corn oil increased the number of oxyurid

eggs. Despite the expected reduction in the number of eggs and helminths due to the laxative effect of corn oil, the observed increase on the last day of the application in fecal flotation could be related to the stress induced by gavage application, leading to a weakened immune system. The *A. annua* n-hexane extract being diluted in corn oil might have made it challenging to observe the anthelmintic effect, contributing to the 21.12% increase in the average number of eggs at a dose of 1200 mg/kg b.w. This effect may be attributed to the use of corn oil. According to some studies (Harling et al. 1988; Harvey, 1996; National Toxicology Program, 1998) corn oil was preferred as solvent controls. Harvey (1996) used to corn oil as solvent in a patent study to show anthelmintic properties of some macrocyclic lactones. However, it is recommended to add an alcohol type with at least four carbon atoms to the mixture as a carrier when delivering macrocyclic anthelmintic drugs (Harvey, 1996). In our study, alcohol was not added to the mixture to avoid potential interference with the anthelmintic effect. The absence of alcohol might have affected the solubility of the extract, potentially causing it to remain relatively undissolved, despite observations indicating homogenization in corn oil. Therefore, in investigating the anthelmintic activity of *A. annua* L., it may be worthwhile to explore extracts prepared with polar solvents via *in vitro* experiments or consider adding the extract to the foods of experimental animals instead of employing gavage for *in vivo* experiments.

It was stated by Brisibe et al. (2009) that *Artemisia annua* L. has nutritious ingredients and they can be a good food supplement. In our study, the observed increase in the number of oxyurid eggs with the *A. annua* extract at a dose of 1200 mg/kg b.w. may be attributed to this nutritive feature. While this plant is known for its anthelmintic properties, it is also recognized for its nutritional richness. Consequently, one could expect a simultaneous reduction in the number of worms and an increase in ovulation, as indicated by the results of our study. In addition, as a reaction to the extract, the number of eggs in helminths may be increased. Many comments can be made on the fact that *A. annua* L. n-hexane extract increases ovulation.

In this study, both perianal tape and fecal flotation methods were used to select the infected mice. It is advised that the perianal tape method be used for counting *Syphacia obvelata* eggs and fecal flotation for *Aspiculuris tetraptera* eggs (Hedrich, 2012). According to the literature, the combined use of these two methods is more effective in determining *S. obvelata* and *A. tetraptera* eggs. While *A. tetraptera* eggs are rarely found with the perianal tape test, the fecal flotation method is considered less successful for detecting *S. obvelata* eggs (Hedrich, 2012). However, in our study, the fecal flotation method was found to be as effective as the perianal tape method in detecting the presence of *S. obvelata* eggs, even in some cases where there were no eggs on the cellophane band, there were many *S. obvelata* eggs seen in flotation. The recommended usage of Albendazole in oxyurid treatment is 10 mg/kg b.w. a single dose. This study was administered at a dose of 5 mg/kg b.w. for three days with a different application, and its effect in this situation was investigated. In the literature, an *in vivo* anthelmintic effect of ABZ at a dose of 5 mg/kg b.w. was found to be %32.6 against lungworm larvae *Metastrongylus apri* (Ferguson, 1981). In our study, ABZ was suspended in corn oil and given by gavage. Although ABZ was given with gavage, and there was its dissolution problem, it was observed that the average number of eggs per gram in fecal flotation decreased by 43.51% on the seventh day compared to the pre-application. This relatively low effect of ABZ may be attributed to its administration after suspension in corn oil. Another contributing factor could be the administration of ABZ for three days, whereas extracts and corn oil were given for seven days. In necropsy, the number of worms in the ABZ group (Group V) decreased by 35.76% compared to the corn oil group (Group I).

A. annua L. n-hexane extract showed a mild anthelmintic effect when it was given to infected mice by dilution in corn oil. It is an interesting result that the 1200 mg/kg b.w.dose of the extract on the seventh day caused an increase of 21.12% in the number of eggs, compared to the average of the first days. ABZ caused a 43.51% reduction of eggs at the dose of 5 mg/kg. This study has shown the slight anthelmintic activity of the n-hexane extract of *A. annua* L. against oxyurid nematodes of laboratory mice. However, further investigations are required to isolate the active compounds responsible for the anthelmintic activity and to predict the mechanisms of action via *in silico* modeling.

Ethics committee for the use of experimental animals and other ethical committee decisions and permissions: All experiments were approved by the Experimental Animals Local Ethics Committee of Bursa Uludag University (no: 2017-10/07).

Conflict of interest: All authors declare that there is no conflict of interest.

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Contribution of authors: D K made in vivo experiments and wrote the article. A O G read and corrected the article. O G collected the plants and prepared the n-hexane extract. H M contributed to the collecting of *A. annua* from field.

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Original Article
Özgün Araştırma

Effects of propylene glycol in different doses on metabolic parameters in dairy cows

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Abstract: The study evaluated the effects of propylene glycol (PG) drenched in different doses in dairy cows with a positive energy balance on serum metabolic parameters. Twenty-four Simmental dairy cows in 60-190 days of lactation period with positive energy balance were included in this study. PG was drenched three hours after morning feeding, at a dose of 300 mL (8 dairy cows, Group I) and 500 mL (8 dairy cows, Group II) once a day for 3 days. Eight dairy cows were included in the control group (Group III). Blood samples were collected once before PG drenching, daily after drenching of PG, and finally on day 4. Serum biochemical parameters were determined.

With regard to energy metabolism, glucose concentrations from serum biochemical parameters significantly increased on day 3 in the 500 mL PG group compared to the control group, and there were no significant changes in BHBA and NEFA concentrations. Decreased urea and increased chloride concentrations were determined within reference limits. In dairy cows with positive energy balance, 500 mL PG oral drenching had a positive effect on energy balance as determined by serum glucose measurements, might not have adverse effects on hepatic and renal function, and may cause serum electrolyte changes within reference limits.

Keywords: Dairy cows, propylene glycol, serum metabolic parameters

Süt ineklerinde farklı dozlarda propilen glikolün metabolik parametreler üzerine etkileri

Özet: Çalışma pozitif enerji dengesine sahip süt ineklerinde farklı dozlarda propilen glikol (PG) uygulamasının serum metabolik parametreleri üzerine etkilerini değerlendirdi. Çalışmaya 60-190 günlük laktasyon döneminde pozitif enerji dengesine sahip 24 adet Simental ırkı süt ineği dahil edildi. PG, sabah yemlemesinden 3 saat sonra, günde bir kez 300 mL (8 süt sığırı, Grup I) ve 500 mL (8 süt sığırı, Grup II) dozlarında 3 gün boyunca uygulandı. Kontrol grubu (Grup III) için sekiz süt sığırı dahil edildi. Kan örnekleri PG uygulamasından önce bir kez, PG uygulamasından sonra günlük olarak ve son olarak 4. günde toplandı. Serum biyokimyasal parametreleri belirlendi. Enerji metabolizması ile ilgili olarak, serum biyokimyasal parametrelerinden glikoz konsantrasyonları kontrol grubuna kıyasla 500 mL PG grubunda 3. günde önemli ölçüde arttı, BHBA ve NEFA konsantrasyonlarında ise önemli bir değişiklik olmadı. Üre konsantrasyonlarında azalma, klorür konsantrasyonlarında artma ise referans sınırlar içerisinde tespit edildi. Pozitif enerji dengesine sahip süt ineklerinde, 500 mL PG'nin oral uygulanması serum glikoz ölçümleri ile belirlendiği gibi enerji dengesi üzerinde olumlu etkiye sahipti, hepatik ve renal fonksiyonlar üzerinde olumsuz etkileri olmayabilir ve referans sınırlar içinde serum elektrolit değişikliklerine neden olabilir.

Anahtar kelimeler: Propilen glikol, serum metabolik parametreleri, süt ineği

Introduction

Propylene glycol (PG) is a gluconeogenic substrate that has beneficial effects on carbohydrate and lipid metabolism in the early lactation period in dairy cattle (Gordon et al. 2013; Piantoni and Allen 2015; Gordon et al. 2017; Jeong et al. 2018). In a recent study, a long time PG drenching to dairy cattle in the transition period has been reported to decrease subclinical ketosis incidence and increase milk production (El-Kasrawy et al. 2020). Also, PG can reduce the incidence of fatty liver (Kristensen and Raun 2007). Some farmers and veterinarians have expressed that clinical signs such as ataxia, salivation, depression, and shallow breathing develop in some cattle (Nielsen and Ingvartsen, 2004). High-dose PG treatment for ketosis treatment (800-1800 g/day) has been determined to cause clinical signs such as salivation and ataxia, but 200-500 g PG treatment once a day in cows with ketosis has been reported not to cause toxicity signs (Johnson 1954). A study

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has reported that PG toxicity develops in one of three cattle-drenched PGs (Bertram et al. 2009). PG drenching dose in the treatment of ketosis related to dairy cattle is reported as 300 mL/day for three days (Mann et al. 2017) and 500 mL (Maurer et al. 2017). In dairy cattle, the maximum dose of PG is limited to 500 mL (Nielsen and Ingvartsen 2004; Zhang et al. 2020). During PG treatment, taking care of the development of toxicity signs is required (Zhang et al. 2020). A high dose of PG treatment increases the toxicity risk in the case of renal and liver function disorders (Zar et al. 2007). According to the authors' knowledge, there is no study in which the effects of PG at 300 and 500 mL doses are evaluated on serum biochemical parameters in dairy cattle with positive energy balance. Thus, this study evaluated the effects of PG at 300 and 500 mL doses once a day for three days orally on serum biochemical parameters of dairy cattle in positive energy balance.

Material and Method

Animal material

This study was approved by Atatürk University Ethics Committee (13.01.2020/5) and was funded by Atatürk University Scientific Research Unit (TDK-2021-9002).

Feeds of dairy cows comprised dry roughage (29.14%), corn silage (37.47%), and forage (33.38%) were prepared by a veterinary specialist (Sunar Feed Company, Osmaniye, Turkey, Kardelen 21 Dairy Cattle Feed). 24 Simmental breed dairy cattle in positive energy balance, 3-4 years old, with normal physical examination findings, 60-190 days of lactation, were used. The study animals comprised 300 mL PG (Group I), 500 mL PG (Group II) and control (Group III) groups. PG was drenched at a dose of 300 mL (Mann et al. 2017) and 500 mL (Maurer et al. 2017) once a day for three days after morning feeding.

Blood sampling

The venous blood samples were collected into vacutainer tubes 3 hours after PG drenching, centrifuged after 30 minutes at 4 C° (Heraeous sepatec, Labofuge 200, Germany). Sera samples were stored until analyses at -20 °C.

Serum biochemical analyses

Serum triglyceride, total cholesterol, total protein, total bilirubin, urea, creatinine, aspartate aminotransferase (AST), sodium, potassium and chloride concentrations were measured using the device for serum biochemical analyses (Siemens Atellica Solution, Germany).

Blood beta-hydroxybutyric acid (BHBA) and glucose concentrations were measured using the blood ketone (CentriVet™ ACON Laboratories, ABD) and blood glucose (On-call plus, USA) measurement devices.

Serum nonesterified fatty acid (NEFA) (Cat. No: E0021Bo Bioassay Technology Laboratory, China) and glutamate dehydrogenase (GLDH) (Cat. No: E2262Bo Bioassay Technology Laboratory, China) concentrations were measured using ELISA kits as specified by producing company.

Statistical analysis

All data were statistically analyzed using SPSS 20.00 (Windows, IBM, USD). Parametric and nonparametric data were presented as mean±standard error of means (SE) and median (minimum-maximum), respectively. Parametric and nonparametric data were analyzed using repeated measurements ANOVA and repeated measurements using the Kruskal-Wallis test, respectively. Triglyceride was analyzed with chi square test. Statistical significance for all data was considered as $P<0.05$.

Results

Serum metabolic parameters of dairy cows with positive energy balance were presented in Table 1.

BHBA concentrations were not significantly different at all the time points among groups and within groups ($P>0.05$). Glucose concentrations were significantly increased at the third time point in the 500 mL PG group compared to the control group ($P<0.05$), but there was no significant difference within the group. NEFA concentrations were not significantly different at all the time points among groups and within groups ($P>0.05$).

Urea concentrations were significantly decreased at the third time point in the 500 mL PG group compared to the control group and were significantly decreased at the first and third time points than the zeroth time point within the 500 mL PG group ($P<0.05$). Creatinine concentrations were not significantly different at the first, second and third time points among groups, and there was no significant difference at the fourth time point in 300 mL PG and 500 mL PG than the control group ($P>0.05$). Within the 500 mL PG group, creatinine concentrations were significantly increased at the first and third time points than the zeroth time point ($P<0.05$). AST concentrations were not significantly

different at all the time points in 300 and 500 mL PG groups than in the control group and within the groups (P>0.05). GLDH concentrations were not significantly different among the three groups and within groups (P>0.05).

Total protein concentrations were not significantly different at the first, second, third and fourth time points among groups (P>0.05). Total bilirubin concentrations were not significantly different at the first, second, third and fourth time points among groups and within groups (P>0.05). Total cholesterol concentrations were not significantly different at all the time points among groups and within groups (P>0.05). Triglyceride concentrations were not significantly different among groups (P>0.05). Sodium concentrations were not significantly different at all the time points among groups and within groups (P>0.05).

Potassium concentrations were not significantly different at all the time points among groups (P>0.05). In addition, the increase in potassium concentrations was more significant at the first and second time points than the zeroth time point within the 300 mL PG group (P<0.05). Within the 500 mL PG group, a tendency for non-significant increase and decrease was observed in potassium concentrations.

Chloride concentrations were significantly increased at the first, second, and fourth time points than the zeroth time point within the 300 mL PG group, and at the second time point in the 300 mL PG group than the control group (P<0.05), and at the first time point than the zeroth time point within 500 mL PG group (P<0.05).

Table 1. Serum metabolic parameters in dairy cows with positive energy balance in the PG groups and the control group.

Parameters	Time (Day)	300 mL PG Group	500 mL PG Group	Control Group
BHBA (mmol/L)	0	0,60 (0,50-0,90)	0,65 (0,50-0,80)	0,55 (0,40-0,90)
	1	0,50 (0,40-0,70)	0,65 (0,50-0,90)	0,60 (0,50-0,80)
	2	0,70 (0,50-0,80)	0,70 (0,40-0,70)	0,65 (0,00-0,80)
	3	0,60 (0,50-0,70)	0,60 (0,50-0,80)	0,60 (0,50-0,80)
	4	0,70 (0,50-0,90)	0,70 (0,60-0,90)	0,65 (0,60-0,90)
Glucose (mg/dl)	0	41 (32-56)	43,50 (35-78)	42 (33-56)
	1	48 (35-62)	54,50 (40-75)	44,50 (32-63)
	2	47 (40-54)	51,50 (44-62)	45 (36-63)
	3	47 (37-56)^{ab}	52 (48-58)^{a}	43 (36-52)^{b}
	4	39,50 (31-55)	41,50 (36-68)	41,50 (33-56)
NEFA (µmol/L)	0	77,82 (60,48-199,85)	147,98 (53,54-226,95)	94,81 (72,30-160,66)
	1	97,57 (40,00-219,43)	83,07 (46,49-297,40)	71,22 (52,90-203,09)
	2	129,84 (62,31-933,13)	124,12 (47,49-724,61)	88,54 (57,44-337,02)
	3	76,78 (53,86-134,90)	87,59 (54,84-775,00)	103,06 (76,51-125,58)
	4	94,32 (62,29-196,06)	105,50 (71,00-194,00)	92,50 (75,00-139,00)
Urea (mg/dl)	0	18,62±0,80 18,00 (16-23)^{AB}	18,25±0,55^{B} 18,00 (16-20)	18,87±0,98^{AB} 18,50 (15-24)
	1	17,00±0,56 17,00 (15-19)^{B}	16,25±0,49^{C} 16,00 (14-18)	17,50±0,90^{B} 17,00 (14-22)
	2	18,00±0,77 17,50 (15-21)^{AB}	16,50±0,42^{BC} 16,50 (15-18)	18,87±0,63^{AB} 19,00 (16-21)
	3	16,62±1,11 17,00 (10-21)^{Bab}	16,25±0,49^{C} 16,00 (14-18)^{a}	19,12±0,63^{AB} 19,50 (16-22)^{b}
	4	20,00±0,70 19,50 (18-24)^{A}	19,25±0,45^{A} 19,00 (18-21)	21,00±0,73^{A} 21,00 (18-24)

Parameters	Time (Day)	300 mL PG Group	500 mL PG Group	Control Group
Creatinine (mg/dl)	0	0,98±0,03 1,01 (0,82-1,08)	0,92±0,03^{BC} 0,93 (0,76-1,02)	0,96±0,04 0,94 (0,82-1,18)
	1	1,03±0,04 1,07 (0,81-1,14)	1,07±0,01 ^{A} 1,07 (1,01-1,12)	0,98±0,03 0,94 (0,88-1,15
	2	1,05±0,02 1,08 (0,91-1,14)	1,01±0,02^{ABC} 1,01 (0,92-1,11)	0,98±0,04 0,94 (0,86-1,21)
	3	1,05±0,03 1,08 (0,91-1,16)	1,07±0,02^{A} 1,08 (0,99-1,15)	1,04±0,04 0,99 (0,91-1,25)
	4	1,14±0,11 1,05 (0,92-1,91)^{a}	0,93±0,02^{C} 0,94 (0,82-1,03)^{b}	0,98±0,04 0,92 (0,90-1,20)^{ab}
AST (U/L)	0	109,50 (89-122)^{a}	83,50 (72-102)^{b}	101 (72-125)^{ab}
	1	109 (87-120)^{a}	87 (80-102)^{b}	101,50 (79-130)^{ab}
	2	109,50 (84-121)^{a}	86,50 (77-107)^{b}	99 (75-123)^{ab}
	3	110 (86-119)^{a}	90 (78-104)^{b}	102 (78-114)^{ab}
	4	109 (76-118)^{a}	88 (76-98)^{b}	100,50 (70-120)^{ab}
GLDH (ng/mL)	0	13,21 (9,30-19,52)	12,87 (8,77-22,00)	13,71 (10,36-17,95)
	1	13,09 (8,61-18,41)	11,12 (8,53-21)	14,49 (9,77-20,00)
	2	15,75 (11,32-19)	12,88 (8,17-17,46)	12,09 (8,78-24)
	3	11,79 (9,28-15,64)	10,77 (7,93-18,94)	12,59 (10,51-21)
	4	12,80 (11,48-15,79)	12,17 (9,67-16,28)	12,91 (11,01-16,42)
Total Protein (g/L)	0	70,00 (59-76)^{a}	62,50 (55-66)^{Bb}	69,00 (60-71)^{a}
	1	71,50 (60-88)	67,00 (62-80)^{ABCD}	73,00 (61-88)
	2	76 (58-89)	65,50 (60-78)^{BCD}	70,00 (63-87)
	3	73,50 (63-88)	70,00 (60-82)^{AC}	70,00 (63-85)
	4	69,50 (55-91)	77,50 (72-85)^{A}	83,50 (61-86)
Total Bilirubine (mg/dl)	0	0,04 (0,02-0,09)^{a}	0,03 (0,01-0,05)^{ab}	0,01 (0,01-0,05)^{b}
	1	0,04 (0,01-0,09)	0,01 (0,01-0,04)	0,03 (0,00-0,06)
	2	0,05 (0,01-0,07)	0,02 (0,01-0,04)	0,03 (0,01-0,08)
	3	0,01 (0,01-0,04)	0,02 (0,01-0,15)	0,01 (0,01-0,06)
	4	0,04 (0,01-0,06)	0,02 (0,01-0,06)	0,02 (0,01-0,06)
Total Cholesterol (mg/dl)	0	135 (94-166)	136,50 (126-149)	132,50 (79-188)
	1	133,50 (93-163)	143 (125-159)	136 (82-200)
	2	131,50 (91-166)	140,50 (120-156)	130,50 (79-192)
	3	128 (96-161)	139 (122-163)	137,50 (80-188)
	4	136 (90-161)	143 (133-160)	130 (87-188)

Parameters	Time (Day)	300 mL PG Group	500 mL PG Group	Control Group
Sodium (mmol/L)	0	139 (137-140)	138 (134-142)	138 (137-139)
	1	138 (136-144)	140 (137-144)	138 (136-142)
	2	139 (139-144)	139 (136-142)	138 (136-144)
	3	139 (137-141)	140 (137-143)	139 (137-142)
	4	141 (138-146)	140,50 (137-147)	140 (135-144)
Potassium (mmol/L)	0	3,89±0,09 3,84 (3,54-4,36)^{B}	3,94±0,05 4 (3,69-4,09)	3,94±0,07^{AB} 4 (3,67-4,19)
	1	4,28±0,07 4,22 (4,01-4,65)^{A}	4,12±0,09 4,15 (3,78-4,56)	4,25±0,10^{A} 4,21 (3,92-4,65)
	2	4,23±0,07 4,19 (3,99-4,49)^{A}	4,07±0,05 4,05 (3,88-4,41)	4,05±0,09^{AB} 3,99 (3,69-4,53)
	3	4,06±0,06 4,04 (3,79-4,40)^{ABC}	3,91±0,11 3,88 (3,29-4,28)	3,99±0,09^{AB} 3,94 (3,72-4,55)
	4	3,80±0,08 3,86 (3,35-4,02)^{BC}	3,93±0,11 3,93 (3,37-4,44)	3,85±0,10^{B} 3,86 (3,42-4,45)
Chloride (mmol/L)	0	102,02±0,63 102 (99,20-105)^{B}	101,42±0,72^{B} 102 (98,20-104)	100,45±0,80 101 (97,70-103)
	1	104,25±0,45 104 (103-106)^{A}	104,12±0,58^{A} 104 (102-107)	102,60±0,66 102,50 (99,80-106)
	2	104,87±0,39 105 (103-106)^{Aa}	103,50±0,56^{AB} 103,50 (101-106)^{ab}	101,55±0,84 101,50 (98,20-106)^{b}
	3	103,50±0,59 103 (101-106)^{AB}	102,31±0,66^{AB} 102,50 (99,50-105)	101,77±0,70 101,50 (98,20-105)
	4	104,12±0,47 104,50 (102-106)^{Aa}	102,87±0,51^{AB} 102,50 (101-105)^{ab}	101,51±0,76 101,50 (98,50-104)^{b}

^{a,b}: Lower letters in the same row indicate statistical significance (P<0.05).

^{A,B,C}: Capital letters in the same column indicate statistical significance (P<0.05).

Discussion and Conclusion

Propylene glycol (PG) is a gluconeogenic substrate that has beneficial effects on carbohydrate and lipid metabolism in the early lactation period in dairy cattle (Gordon et al. 2013; Piantoni and Allen 2015; Gordon et al. 2017; Jeong et al. 2018). Mikula et al. (2008) have reported that 4 hours after top dressing PG in the morning, blood glucose and NEFA concentrations are not significantly affected in dairy cows after parturition. Cozzi et al. (1996) have reported that 200 and 400 mL PG mixed with TMR have an increased effect on the blood glucose increase during 0-6 hours in mid-lactating dairy cattle. Mikula et al. (2020) have revealed a non-significant effect on the blood glucose in the PG delivery methods, a significant decrease of the blood BHBA concentration 1.5 and 2.5 h after oral PG drenching, a decreasing trend of the blood NEFA concentration 1.5 h after oral PG drenching in dairy cattle with positive energy balance. Maurer et al. (2017) have stated that oral PG drenching at 100, 300 and 500 mL doses has dose-dependent effects on serum glucose and insulin, and 500 mL PG administration provides a long-lasting decrease of blood NEFA concentrations in lactating dairy cows. In this study, 500 mL PG had a significant effect on the blood glucose concentration at the third time point compared to the control group, but the blood glucose concentration had no significant increase at different time points within the 500 mL PG group. Thus, a 500 mL PG dose rather than a 300 mL PG dose could contribute to an increase in blood glucose concentrations. In addition, in the present study, the blood BHBA and serum NEFA concentrations were not significantly affected at different doses of PG administration. Similarly, the blood BHBA concentrations have been reported not to be affected by PG administration in dry and close-up periods of dairy cows (Maurer et al. 2017). Thus, in the present study, the ineffective PG on the blood BHBA concentrations could be related to positive energy balance in dairy cows during the lactation period.

Different results have been obtained from various studies on the gluconeogenic effects of PG in dairy cows. This could be attributed to the PG delivery method, blood sampling times after PG ad-

ministration, and the lactation period of dairy cows (Maurer et al. 2017).

In ruminants, GLDH is an important marker of hepatic disorders (Smith, 2014). Skeletal and liver tissues have large amounts of AST enzyme. In hepatic disorders, increased serum AST activity is evaluated with specific liver enzymes (Constable et al. 2017). Hoedemaker et al. (2004) have reported that in dairy cows before parturition, PD administration has no effects on the increase of AST and GLDH activities, and AST and GLDH activities should be assessed as metabolic profile tests in monitoring liver health. Maurer et al. (2017) have found that oral PG administration has no effect on cholesterol concentration and AST and GLDH activities, but a decrease of bilirubin concentrations is carried out with 500 mL PG in early lactating periods of dairy cows. In the present study, oral PG administration did not have a significant effect on GLDH activities and total cholesterol, total bilirubin and triglyceride concentrations among groups and within groups, as well as on AST activities and total protein concentrations between PG groups and control group. Thus, the present study revealed that 300 mL and 500 mL PG doses could not have adverse effects on liver function as indicated by serum GLDH and AST activities and total protein, total cholesterol, total bilirubin, and triglyceride concentrations.

An increase in serum urea nitrogen concentration can be a marker for kidney failure. However, if serum creatinine is not increased, kidney disorder may not be present. Serum creatinine and urea increase appear in kidney disorders and dehydration (Constable et al., 2017). In addition, Xu et al. (2020) have revealed that in dairy cows with negative energy balance, energy balance is related to the mobilization of muscle proteins.

In the present study, serum urea concentrations significantly decreased at the third time point in the 500 mL PG group compared to the control group. They were significantly decreased at the first and third time points than the zeroth time point within the 500 mL PG group. Serum creatinine concentrations were not significantly different among groups. Within the 500 mL PG group, serum creatinine concentrations were significantly increased at the first and third time points than at the zeroth time point. However, Mikula et al. (2020) found increased serum urea concentration 30 min after oral PG drenching at 400 mL dose and then decreased after 2.5 hours and reached the concentrations of the control group. Miyoshi et al. (2001) have reported a tendency to increase blood urea nitrogen concentration after oesophagal PG administration. Conversely, Chibisa et al. (2008) have not found the effect of PG on BUN concentration. Conversely, in the present study, 500 mL PG dose had an effect on serum urea decreases and creatinine increases. Thus, because serum creatinine concentrations were not significantly different in the PG groups compared to the control group, serum urea increases were not found, and urea and creatinine concentrations were not significantly different at the fourth time point in the PG groups compared to the control group, it was concluded that kidney functions could not have been negatively affected.

Mann et al. (2017) have found that in dairy cows during the early lactation period, 300 mL PG administration once a day for three days did not significantly affect serum sodium, potassium, and chloride concentrations. In the present study, serum sodium and potassium concentrations did not differ significantly among groups. Potassium concentrations in the PG groups and the control group showed a tendency to increase and decrease according to the time points. Chloride concentrations significantly increased at the second point in the 300 mL PG group compared to the control group. In addition, increases in serum chloride concentrations were within acceptable limits (95-110 mEq/L) (Constable et al. 2017).

In dairy cows with positive energy balance, 500 mL PG oral drenching has a positive effect on energy balance as determined by serum glucose measurements, might not have adverse effects on hepatic and renal function, and might cause serum electrolyte changes within reference limits.

Ethics Committee Approval: This study was approved by Atatürk University Animal Experiments Local Ethics Committee (13.01.2020/5).

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Original Article
Özgün Araştırma

Tumor cells and microenvironmental interaction in natural course of canine transmissible venereal tumour

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Abstract: Canine transmissible venereal tumors (CTVT) are naturally occurring tumors that are mostly transmitted between dogs through coitus. This study aims to investigate the effect of CTVT on molecular expression and disease progression by studying the tumor microenvironment. For this purpose, biopsy samples taken from ten female dogs were evaluated histopathologically and CTVT was diagnosed. The expression of markers such as CD163, CD68, CD44, TGF-beta and bFGF was evaluated by immunoperoxidase tests. Histopathologically, CTVT cells exhibited pleomorphism, ranging from round to polygonal. Some cells exhibited prominent vacuoles and hypochromatic nuclei, while others exhibited hyperchromatic nuclei containing mitotic figures within the thin fibrovascular wall. Immunohistochemically, TGF-beta and CD44 expression was higher in CTVT cells compared to CD68 and bFGF, while bFGF expression was higher in fibrocytes and spindle cells compared to other markers. The results indicate that CD44 and TGF-beta may play a pivotal role in fibrovascular processes, CD163 and CD68 may facilitate interactions between stromal components and mesenchymal cells, and bFGF, TGF-beta and CD68 may contribute to the arrest of tumoral progression and the initiation of the regression phase. These findings underscore the necessity for further studies to elucidate the role of markers at different stages of CTVT progression.

Keywords: CTVT, dog, histopathology, immunoexpression, microenvironment

Köpek aktarılabilir venereal tümörlerinin doğal seyri sırasında tümör ile mikroçevre etkileşimi

Özet: Köpeklerde Aktarılabilen Aktarılabilir Venereal Tümörler (CTVT), doğal olarak oluşan ve çoğunlukla cinsel temas yoluyla köpekler arasında aktarılan tümörlerdir. Bu çalışma, CTVT'nin tümör mikroçevresini inceleyerek moleküler ekspresyon ve hastalık ilerlemesi üzerindeki etkisini araştırmayı amaçlamaktadır. Bunun için; on dişi köpekten alınan biyopsi örnekleri histopatolojik olarak değerlendirildi ve CTVT tanısı konuldu. CD163, CD68, CD44, TGF-beta ve bFGF gibi belirteçlerin immünoperoksidaz testlerle ekspresyonları değerlendirildi. Histopatolojik olarak; CTVT hücrelerinin yuvarlaktan poligonal şekle kadar değişen pleomorfizm gösterdiği, bazı hücrelerin belirgin vakuoller ve hipokromatik çekirdeklere sahip olduğu bazılarının ise ince fibrovasküler duvar içinde mitotik figürler içeren hiperkromatik çekirdeklerinin olduğu görüldü. İmmünohistokimyasal olarak, CTVT hücrelerinde TGF-beta ve CD44 ekspresyonu CD68 ve bFGF'ye kıyasla daha yüksek olduğu; bFGF ekspresyonunun ise fibrositlerde ve iğsi hücrelerde diğer belirteçlere göre daha yüksek olduğu gözlendi. Sonuçlar, CD44 ve TGF-beta'nın fibrovasküler süreçlerde önemli roller oynayabileceğini, CD163 ve CD68'in ise stromal bileşenler ve mezenkimal hücreler arasındaki etkileşimleri kolaylaştırabileceğini ve bFGF, TGF-beta ve CD68'in tümöral ilerlemenin durdurulmasına ve gerileme evresinin başlatılmasına katkı sağlayabileceğini göstermektedir. Bu bulgular, CTVT ilerlemesinin farklı aşamalarında belirteçlerin rolünü anlamak için daha fazla çalışma gerektiğini vurgulamaktadır.

Anahtar kelimeler: CTVT, histopatoloji, immünoekspresyon, köpek, mikroçevre

Introduction

Canine Transmissible Tenereal Tumor (CTVT), or Sticker's sarcoma, is a contagious and transplantable tumor by coitus between male and female dogs and other canidae. The mass grow rapidly in cauliflower-like shape and later regressed spontaneously. Its consistency may show friable to firmness. Ulceration and bleeding may occur. It is more encountered in mongrel dogs and kennels, where stay dogs are kept together. The tumor cells are almost round to polygonal and sometimes contain mesenchymal and histiocytic cells. CTVT cells contain sometimes distinct, clear, and cytoplasmic vacuoles (Cangul, 2001; Thangathurai et al., 2008; Ganguly et al., 2016; Alkan et al., 2017). The histoarchitecture of CTVT is distinct. - In the progressive

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(P) phase of CTVT, proliferating round to polygonal shape cells are closely packed in rows or cords by thin mesenchymal (fibrovascular) septum. During the stable (S) phase, the developing tumor parenchyma and stroma stop growing, and the inflammatory response is initiated. At the regressive (R) phase, fibroblasts and spindle cell transformation are rapidly developed at intercepts of mesenchymal bundles and the periphery of CTVT cells. (Hiblu et al., 2019; Setthawongsin et al., 2019).

The tumor is known to have some immunogenic properties, and the immunity provides the inhibition of tumoral progression for several decades (Cohen, 1973). But, there is limited knowledge regarding the interaction between CTVT cells and the tumor microenvironment (TME). Even though CTVT has been well studied (Hiblu et al., 2019; Ke et al., 2022). At that course, tumor cell behaviour (tissue invasion capability) by stromal interaction and molecular profiling during transmission phases of CTVT is unfortunately not known. In fact, there is a close interaction between host immune system and tumor cells. So, the tumor cells together with their microenvironment and molecular expression in CTVT are thought to be highly complex. Stem-like cancer cells are developed in a niche together by infiltrating inflammatory cells (TILs), immune cells, fibroblasts, and angioblast. In the cellular communication process, extracellular matrix proteins (such as MMP), cytokines (IFNγ, TNF-alpha, and interleukin) and chemokines, growth factors, singaling systems (Wnt/ß-catenin pathway), as well as genetic elements (miRNA, mRNA, MHC) and genetic mutations (LINEs) play roles as well. (Hsiao et al., 2004; Siddle and Kaufman, 2015; Kanca et al., 2018; Frampton et al., 2018; Zayas et al., 2019; Skytthe et al., 2020). Thus, the more understandable/comprehensible, highly complex changes may give more facilitate the insight on potential therapeutic strategies for treating contagious CTVTs.

In this context, macrophages (such as M2) involving drug-targeting of the surface marker CD163 and CD68 expresses. Macrophages are phagocytic cells showing versatile functions. They also have several roles in cellular architecture, tissue remodeling, and fibrosis, i.e., TME and tumorigenesis, as well as regulation of inflammation (induction of anti-inflammatory activity) as tumor-infiltrating cell against tumor cell proliferation (Skytthe et al., 2020; Zhang et al., 2022). CD44 is known as a complex transmembrane glycoprotein. Its expression has formerly showed formerly shown to have a close relationship with the morphogenesis of cancer stem cells (or cytoskeleton) and epithelial-mesenchymal plasticity as well as roles in tumor cell proliferation, metastasis, and resistance against chemotherapeutics. Thus, CD44 has a versatile molecule providing interaction between extracellular matrix (ECM) and matrix metalloproteinases (MMPs), several growth factors. It has also played an important function in the stimulation of receptor tyrosine kinases (RTKs) while tumor cells proliferating (Ponta et al., 2003; Xu et al., 2015; Primeaux et al., 2022). Basic Fibroblast Growth Factor (bFGF) over fibroblast proliferation has a role in fibroblast proliferation due to being mitogenic agent. There are also several essential roles in regulating cell growth, angiogenesis, and organizing the tumor microenvironment. bFGF binds to and activates its tyrosine kinase receptors during cancer progression. Additionally, there have been several similar roles as aforementioned in CDs (Turner and Grose, 2010; Ardizzone et al., 2022). Transforming Growth Factor-beta (TGF-β) stimulates tumor cell progression and recruits fibroblasts, other mesenchymal stem cells, and angioblasts while undergoing cells to apoptosis and inhibiting T cell activation, proliferation, differentiation, and migration. As such, it regulates tumor microenvironment and immunocyte infiltration (Hao et al., 2019; Chan et al., 2023).

This study aims to evaluate the microenvironment of CTVT tumor cells to understand how it affects molecular expression and disease progression.

Material and Method

Pathological Evaluation of Collected Samples

In total, ten collected samples were the biopsies fixed in buffered formalin solution, and 10% were sent to the Department of Pathology, Faculty of Veterinary Medicine at Kirikkale University, TURKIYE The samples were selected from eight vaginal and two clitoral sites, in accordance with the site of lesions in female dogs. Tumor suspected-materials were evaluated suitably to general macroscopic criteria including their size, color, shape, and appearance of the upper and sectional faces of the mass. Tissue samples were trimmed, passed through ethanol and xylol series and liquid paraffin series on a tissue tracking device (Leica TP1020, Germany), and blocked in paraffin (Thermo Shandon, EG1150, Germany). Sections were taken from blocks with a thickness of 5 microns (Shandon, AS325, Germany). Tissue sections were stained using the Hematoxylin-Eosin (H&E) staining method (Luna, 1968).

Immunohistochemical Analysis

The Avidin-Biotin Complex Peroxidase (ABC-P) method was utilized to observe changes in the progression and regression of CTVT and mesenchymal tumor components. The secondary antibody employed was the Avidin Biotin Complex Peroxidase (ABC-P, HRP/DAB, ab64264, Abcam, France). CD163, CD68, CD44, TGF-beta, and bFGF expression was evaluated (Table 1). The ABC-P staining method was used for the rest of the procedure, following the instructions provided in the kit. For this purpose, serial sections were taken on 5-micron-thick adhesive slides. Glass slides were deparaffinized and dehydrated in Phosphate Buffered Saline (PBS-pastile, Sigma) by passing through xylol and alcohol series. Then, they were gently placed in a citrate buffer (pH=6.0, 10× concentration, Bioptica-Italy) solution in the microwave oven to reveal the antigen in the tissue. They were kept at 800 watts for 25 minutes in a microwave oven. To remove the endogenous peroxidase activity, they were kept in a 3% hydrogen peroxide-methanol mixture at room temperature for 5 minutes; and then taken into a humid chamber. The sections were kept in a 37°C oven for 25 minutes by dripping a drop of normal blocking serum on. Primary sera containing antibodies (aforementioned immunomarkers) were dripped onto the sections and incubated for 60 minutes in an oven at 37°C. Then, appropriate secondary antibodies labeled with biotinized and Horse Radish Peroxidase (HRP) were used and incubated in the incubator (Nüve, EN055, Turkiye) at the specified temperature and time. At the end of each step up to herein, tissue sections were washed twice by PBS at 5 minutes except for post-primary antibody dripping. For the reaction to become visible, 3-3'Diaminobenzidine (DAB, Abcam) chromogen and buffer were gently mixed in good proportion. The chromogen mixture was dropped on the sections and waited for 5 minutes. The sections were washed with distilled water. Finally, Gill's hematoxylin (Bioptica, Italy) was used for background staining. The slides were passed through ethanol and xylene series. The slides were covered with a coverslip and nonaqueous mounting medium (Entellan®, Merck, Germany). Findings were evaluated under an optic Brightfield microscope (Olympus BX51, Japan) and photographed (Olympus DP25 camera, Japan) as in other histopathological examinations, and the results were scored.

Table 1. Antibody panel and detailed information using in immunohistochemical analysis

Markers	Trademark	Catalog number	Dilution	Properties	Antigen retrieval (pH 6.0, Citrate buffer)
CD163	Abcam	ab182422	1:200	**Polyclonal** rabbit IgG	Yes
CD68	Abcam	ab125212	1:200	**Monoclonal** rabbit IgG	Yes
CD44	Abcam	ab189524	1:200	**Monoclonal** rabbit IgG	Yes
TGF-beta	Santa Cruz	sc-130348	1:200	**Monoclonal** mouse IgG1	Yes
bFGF	Antibodiesonline	AA 143 250	1:200	**Polyclonal** rabbit IgG	Yes

Semiquantitative Scoring and Statistical Analysis of Pathological Data

Serial tissue histosections were examined at ×200 magnification to see neoplastic changes after diagnosis of CTVT. These criteria included CTVT neoplastic cell and clear cell, pleomorphism, mitotic index, polychromasia, inflammatory cell infiltrations (neutrophil, macrophage, lymphocyte, and plasma cell), and spindle cells to be like in fibrocyte and fibroblasts in neoplastic tissue in three different areas of high power fields (HPFs). Histologically, the cells in histostained sections were counted semiquantitatively. The results were divided into 0 (no finding), 1,2,3,4,5,6 (for cases with lesions, according to the degree of finding) index slices according to Cingi et al. (2020). After converting them into equally divided percentiles and scoring, these data were analyzed by unpaired-t-test and Wilcoxon test. For immunostaining sections, positive cells were counted in three different areas (or HPFs). Mean and standard error were calculated for all samples and analyzed with the one-way ANOVA method and posthoc Tukey test. Between the criteria of mean and standard error-values, the statistical difference was evaluated significantly for $p<0.001$ (GraphPad Prism 8.0, USA).

Results

Histopathological findings

CTVT cells had polymorphism varying from round to polygonal shape. These cells contained hypochromatic nuclei and distinct nucleoli or hyperchromatic nuclei. Mitotic figures were widespread. Some of the cells had clear vacuoles and hypochromatic

nuclei. But, these clear cells were not so evaded in proliferating tumor cells. Thin fibrous or fibrovascular stroma was interrupted by CTVT cells in chords or packages (**Figure-1a and b**). In some areas, the periphery of mass, in particular, fibroblasts and fibrocytes or spindle-shaped cells covered the microscopic fields. In such areas, of many capillaries and arterioles were dispersed in connective tissue. Inflammation gets accorded in CTVT proliferation and involved neutrophil leucocyte, lymphocyte, macrophage, and plasma cells, consecutively (**Figure1-c**).

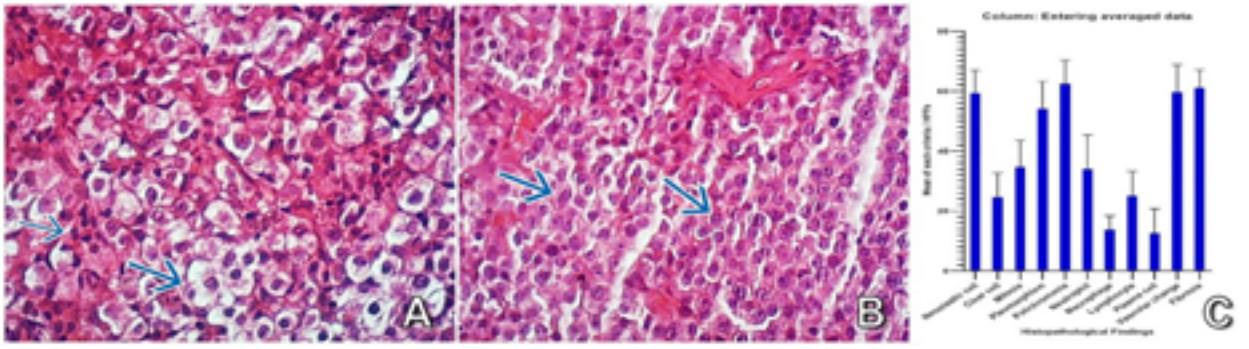

Figure 1. (A) In CTVT tumor, clear cells (thick arrow) and inflammatory cells (thin arrow). ×200 magnification H&E staining, **(B)** CTVT cells showing polygonal to round shape (arrows). ×200 magnification, H&E staining. **(C)** The bar graph represents histopathological findings scores of Canine Transmissible Venereal Tumor

Immunoexpression Results

CD163 and CD68 expressions were intensive in the cytoplasm of CTVT cells and clear cells (undergoing apoptosis or cellular alteration with clear vacuoles). Spindle-shaped cells were also reacted in cytoplasms with CD163 and CD68 immunomarker. Some of the reactions belonged to macrophage and macrophage-like cells. However, the reaction was not encountered in each microscope field by CD163 and in some microscope fields by CD68. However, there is no meaningful statistical difference. Thus, the reaction was not as much as in neoplastic cells. Polymorphonuclear cells, lymphocytes, and plasma cells did not react with both immunomarkers. The reactions in tumor cells and spindle cells were more dense in CD163 when compared to those in CD68 (**Figure-2a and b**). CD44 expressions were dense in both cytoplasms of CTVT cells and clear cells. The total reactions were more than those obtained from other CDs, TGF-beta, and bFGF markers. The reactions in these tumor cells were encountered in almost all microscope fields. However, the reactions in spindle-shaped cells were less when compared to reactions obtained from other markers. The last reaction was obtained by CD44 in spindle cells among all markers (**Figure-2c**). The immunohistochemical results by the TGF-beta marker were almost similar to the CD163 marker in CTVT cells, clear cells, and spindle-shaped cells. In other words, there is no meaningful statistical difference when the total cellular components were considered. However, the reaction in spindle cells is found in fibrocyte and fibroblast-like appearance due to being together with wide collagen bundles. The reaction by the bFGF marker was similar to the figure of the TGF-beta marker. However, fibrocyte-fibroblasts and spindle-shaped cells were more reacted with the bFGF marker (**Figure-2d and e**). Tumor cells were also reacted by bFGF marker as much reaction by CDs and TGF-beta markers as in tumor cells. Thus, there is no statistical difference at total positive cell counts. However, bFGF immunoexpression in clear cells undergoing apoptosis and / or cellular degeneration was not reacted as potential as in other immunomarkers in CTVT cells and clear cells. (see in **Figure-2f**).

Table 2. The mean, standard deviation, and p-values of the immunoperoxidase tests

	CD163			CD68			CD44			TGF-beta			bFGF			p
	n	m	SD	n	m	SD	n	m	SD	n	m	SD	n	m	SD	
Neoplastic Cell	10	7.44	4.55	10	4.44	1.49	10	11.88	5.16	10	7.77	4.21	10	9	4.23	
Clear Cell	10	10	5.04	10	10.55	4.55	10	12.55	4.51	10	12.55	5.49	10	3.11	1.85	0.0593
Inflammation	10	0	0	10	0	0	10	0	0	10	0	0	10	0.44	0.44	
Spindle Cell	10	5.22	3.15	10	3.55	2.33	10	1.44	0.62	10	4.66	2.39	10	6.44	1.57	

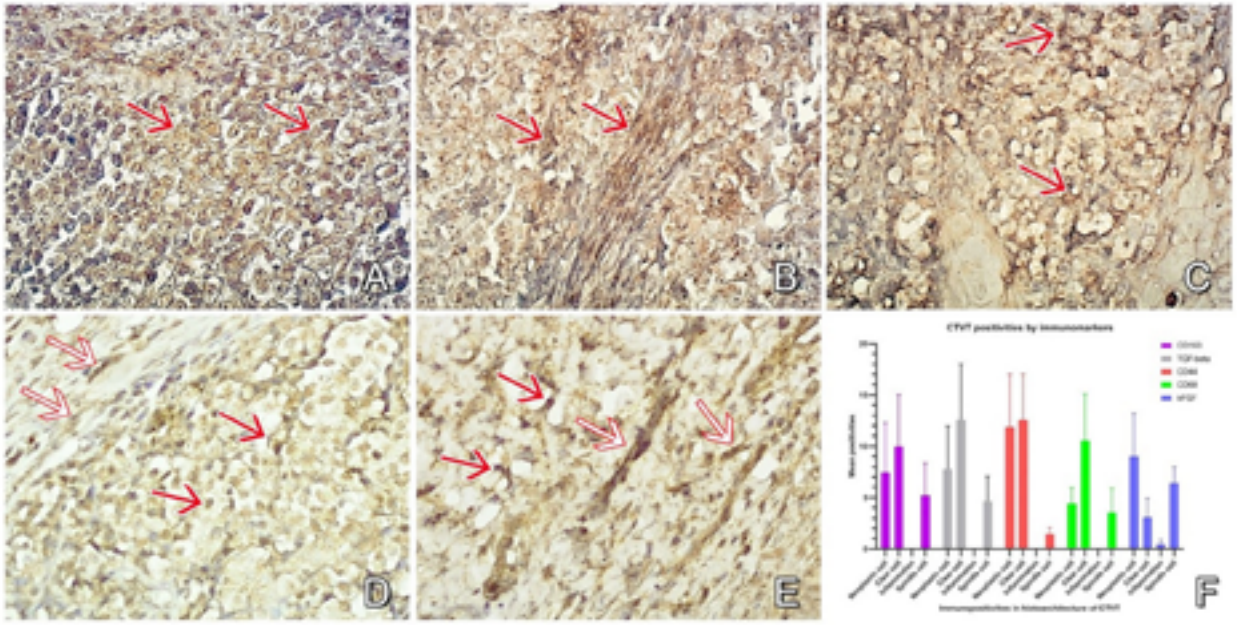

Figure 2. (A) CD163 positivity in polygonal to round shape tumor cells, immunoperoxidase staining, ×200 magnification, DAB chromogen and counterstaining with Mayer's hematoxylin. **(B)** CD68 positivity in round to spindle-like cells, immunoperoxidase staining, ×200 magnification, DAB chromogen and counterstaining with Mayer's hematoxylin. **(C)** CD44 positivity in polygonal to round shape cells, immunoperoxidase staining, ×200 magnification, DAB chromogen and counterstaining with Mayer's hematoxylin. **(D)** bFGF positivity in polygonal to round shape cells carrying clear vacuoles (red arrows) and fibrocytes and fibroblastic cells (white arrows), immunoperoxidase staining, ×200 magnification, DAB chromogen and counterstaining with Mayer's hematoxylin. **(E)** TGF-beta positivity in round shape cells having clear vacuoles (red arrows) and spindle-like cells (white arrows), immunoperoxidase staining, ×200 magnification, DAB chromogen and counterstaining with Mayer's hematoxylin. **(F)** The graph depicts the immunoexpression of CD163, CD68, CD44, TGF-beta, and bFGF according to cell type.

Discussion

Canine Transmissible Venereal Tumor is a transmissible neoplastic change during coitus between adult animals. It has been known for over one hundred years (Alkan et al., 2017; Birhan and Chanie, 2015). Although many studies are focusing on revealing several cell characteristics (such as cytogenetic, immunophenotypic, apoptotic, histopathologic, ultrastructural, and biochemical etc.) of the tumor, it has not been fully elucidated (Mukaratirwa et al., 2003; Sidha et al., 2015; Birhan and Chanie, 2015; Flórez et al., 2017; Frampton et al., 2018); So, there have been still some mysterious and dilemma to wait for its exploration. CTVT cells have ubiquitous morphology: spindle cells (fibroblast and histiocyte like-cells) and round to polygonal cells (Cangul, 2001; Thangathurai et al., 2008; Ganguly et al., 2016; Ujvari et al., 2016; Alkan et al., 2017). It is stated that its morphology has been related to plasmacytoid, lymphocytoid, and mixed-shaped cells (Flórez et al., 2016; Duzanski et al., 2017). We also observed this different morphology and cellular types in tumor parenchyma and microenvironment. The histoarchitecture of CTVT can be interchangeable from the progression to the regression phase. At initial, microscope fields are covered by polygonal-shaped cells. These cells are intercepted with fibrovascular septum. Later, the figure of the microscope field can transform into fibroblast and spindle cells. In our study, we also observed several round to polygonal-shaped cells and spindle cells or spindle-like cells in microscope fields of different cases. In particular, spindle-shaped cells were covered in the microscope field in a few cases. As expected, the other histomorphological components were accorded with CTVT cell riched-cases in that cases. Thus, we thought for the general view of cases constituted the subject of study that they were in either P or S phases rather than R-phases. We also thought that some CTVT cell poor-cases might be transformed into R-phases. Meanwhile, inflammatory cells (in particular lymphocytes and

macrophages) can infiltrate tumor parenchyma during the regressional process (Hiblu et al., 2019; Setthawongsin et al., 2019). On the other side, CTVT immunophenotype can be different from cellular morphology. Some cells represent a dilemma in immunoexpressions. It is shown that CTVT tissue can give immunoexpression in T-lymphocyte by CD8, tumor-infiltrating T-lymphocytes (TILs) by CD3, T-regulatory cells (Treg) by CD4, B-lymphocyte by CD79 as well as dendritic cells and bone-marrow-derived stem cells dendritic cells from bone marrow by CD1a, CD11c, CD40, CD80, CD83 and CD86 (Shankaran et al., 2001; Pai et al., 2011; Silveira et al., 2009; do Prado Duzanski et al., 2022).

Indeed, CTVT cells and tumor microenvironment (TME) are always found in complete interaction. Recent documents have been signed to powerfull evidence regarding cellular components and stromal companents including fibrocyte and vessels in tumor niche (Hiblu et al., 2019; Ke et al., 2022). In this study, we tried to show the role of induced inflammatory cells as well as CTVT cells and undergoing cellular degeneration in tumor niche in accordance with the knowledge (Skytthe et al., 2020; Zhang et al., 2022). Differently from previous documents, we picked out specific markers to show potent effects in both neoplastic and inflammatory cells. In the results, fibrovascular stroma, including spindle-like cells, also gave an expression. In this context, those macrophages expressed CD163 and CD68 in CTVT cells and stromal infiltrating cells. We obtained an expression with CD44 in those cells, showing formerly their roles in cancer stem cells (or cytoskeleton) and epithelial-mesenchymal plasticity. Basic Fibroblast Growth Factor (bFGF), mitogenic agent for fibroangioblast, and Transforming Growth Factor-beta (TGF-β) recruiting agent for both CTVT cells and fibroangioblasts gave an expression as mentioned in CDs (Shankaran et al., 2001; Silveira et al., 2009; Pai et al., 2011; Turner and Grose, 2010; Ardizzone et al., 2022; do Prado Duzanski et al., 2022). In all phases (P, S, and R) of current cases, we encountered high expressional levels in both CTVT cells and clear cells. In the P and S phases, clear cells undergoing apoptosis or degeneration gave relatively more expression by CD163, CD44, and TGF-beta according to CD68 and bFGF. Inflammatory cells including lymphocytes (TILs, Treg) and neutrophils, did not give an exact result, but histiocyte-like cells and spindle cells gave less expression by bFGF. Spindle cells comprising fibrocytes and fibroblasts and spindle-like cells gave relatively less expression by CD163, CD68, CD44, and TGF-beta despite much more in inflammatory cells when compared to in bFGF. This situation showed us that TGF-beta and CD44 might co-play a role in the fibrovascular process by triggering inflammatory reactions by means of CD68 expression. Induced- macrophages can facilitate the interaction between fibrous stromal components and possibly mesenchymal stem cells by CD44, CD68b, and FGF expressions. As such, the development of fibrous tissue of the tumor niche might realize a transition into R-phase from P and S phases.

In conclusion, we arrive come to the conclusions: First of all, the CTVT tumor niche is highly complicated. Round to polygonalshaped cells and spindle cells, spindle-like cells can interact. This situation selects destiny; either the tumor regresses or keeps stable according to undergoing degeneration and mesenchymal tissue development. Secondly, excepting out bFGF role, TGF-beta and CD68 can provide together for stopping tumoral progression and passing into regression phases. However, we thought that CD163 did not have a great role in inducible macrophages as much as CD68. Lastly, for CTVT cells, CD44 is thought to be more effective in recruiting of round to polygonal shaped-tumor cells. CD44 molecule can also be a determination factor for cells undergoing degeneration in clear cells. We strongly believe that this study's results show that there might be useful showing an important interaction between tumor cells and mesenchymal cells (such as fibroblasts, stem cells, and bone marrow-resourced inflammatory cells) in tumor stroma. We recommended researchers focus on such transmissible tumors so that further studies can be cross-checked together with these studied markers and several mesenchymal cell markers and stem cell markers in each of the progression, stable, and regression phases. The marker results should definitely be compared between those phases.

Ethics approval: The study samples consisted of tumor tissues sent to Kırıkkale University, Faculty of Veterinary Medicine, Department of Pathology. Ethical approval was not required.

Conflict of interest: No person or organization provided funding for this study, and the authors have no conflicts of interest.

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Özgün Araştırma
Original Article

Deney hayvanlarında probiyotikli yem kullanımının bağırsak mikrobiyotasına etkisi

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Özet: Bilimsel çalışmalarda laboratuvar hayvanı kullanımı büyük önem taşımakta ve önemli bir yer tutmaktadır. In-vivo çalışmalarda araştırılanın kesin olarak etkinliğini ortaya konulabilmesi için deneyde kullanılan hayvanların türü, soyu ve yaşının yanı sıra çevre koşulları gibi pek çok koşul kontrol ve test gruplarında bir örnek hale getirilmektedir. Çalışmalarda gastrointestinal sistemin işlevi ve bütünlüğünde, bağışıklık homeostazının korunmasında ve konak enerji metabolizmasında önemli rol oynayan bağırsak mikrobiyotasının kullanılan deney hayvanlarında farklılıklar gösterebileceği dikkate alınmalıdır. Ancak, bu farklılıkların çalışma sonuçlarını olumsuz etkileyebileceği çoğu zaman gözden kaçmaktadır. Bu çalışmada, bilimsel araştırmaların öncesinde adaptasyon periyodunda kullanılmak üzere laboratuvar hayvanlarının (rat ve fare) bağırsak mikrobiyotalarının senkronizasyonunda kullanım potansiyeli olan probiyotik içerikli yemlerin üretimi ve bağırsak mikrobiyotası üzerine etkinliğinin belirlenmesi amaçlanmıştır. Analizler neticesinde üretilen sıvı yemlerin muhafaza süresi sonunda toplam aerob genel canlı sayısı ve laktik asit bakteri sayısının (*L. acidophilus* ve *L. plantarum*) 10^9 kob/ml ulaştığı buna karşın koliform bakteri, maya-küf ile diğer patojen mikroorganizmalara rastlanmamıştır. Mikrobiyota analizleri neticesinde ise hem fare hem de ratlarda 10 günlük probiyotikli yem uygulamasının kontrol grupları ile 0. gündeki test gruplarına göre bağırsak mikrobiyotası kompozisyonu üzerine etkili olduğu ortaya konmuştur. Günümüze kadar yapılan literatür incelemelerinde Türkiye'de bu kapsamda bir çalışma olmadığı belirlenmiştir. Bu bağlamda in-vivo çalışmalar öncesinde deney hayvanlarının bu çalışma kapsamında üretilen probiyotik içerikli yemler ile beslenmesinin hayvanların bağırsak mikrobiyotasının geliştirilmesine katkı sunacağı sonucuna varılmıştır.

Anahtar kelimeler: Laboratuvar hayvanları; *L. acidophilus; L. plantarum*; mikrobiyota; senkronizasyon

The effect of probiotic feed use on intestinal microbiota in experimental animals

Abstract: Laboratory animals are frequently used in scientific studies and are of great importance. To clearly demonstrate the effectiveness of the researched in *in-vivo* studies, many conditions such as the species, lineage and age of the animals used in the experiment, as well as environmental conditions, should be uniform in the control and test groups. It should be considered that the intestinal microbiota, which plays an important role in the function and integrity of the gastrointestinal tract, maintenance of immune homeostasis and host energy metabolism, may differ in the experimental animals used in studies. However, it is often overlooked that these differences may negatively affect study results. In this study, it was aimed to produce probiotic-containing feeds which have the potential to synchronize intestinal microbiota of laboratory animals (rat and mouse) to be used in the adaptation period before scientific studies and to investigate the effectiveness of feeds on the intestinal microbiota. According to the analysis, at the end of the storage period of the produced liquid feeds, the total number of general aerobic organisms and the number of lactic acid bacteria (*L. acidophilus* and *L. plantarum*) reached to 10^9 cfu/ml, however, coliform bacteria, yeast-mold and other pathogenic microorganisms were not detected. As a result of microbiota analysis, it was revealed that 10-day probiotic feed application was effective on intestinal microbiota composition in both mice and rats compared to control groups and 0. day test groups. In the literature reviews carried out to date, it has been determined that there is no study in this scope in Turkey. In this context, it was concluded that feeding experimental animals with probiotic-containing feeds that produced within the scope of this study before in-vivo studies will contribute to the improvement of the intestinal microbiota of the laboratory animals.

Key words: Laboratory animals; *L. acidophilus; L. plantarum*; microbiota; synchronization

Giriş

Bilimsel çalışmalarda laboratuvar hayvanı kullanımı büyük önem taşımakta ve önemli bir yer tutmaktadır. Avrupa Birliği istatistiklerine göre 2017 yılı itibari ile yaklaşık 10 milyon laboratuvar hayvanının bilimsel çalışmalarda tercih edildiği, fare ve ratların

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ise toplam sayının %73'ünü oluşturduğu rapor edilmiştir. Laboratuvar hayvanlarının refahı da dikkate alındığında hayvanların sağlıklı olmaları yapılan çalışmaların güvenilirliği açısından büyük önem taşımaktadır (Anonim 2010; Anonim 2019). Bu kapsamda laboratuvar hayvanlarının tüm fizyolojik sistemlerinin sağlıklı olması gerekmektedir. Sindirim sistemi de bu durumun en önemli parçalarından birini oluşturmaktadır.

Sindirim sistemi mikrobiyotası bu sistem içerisinde toplam mikroorganizma sayı ve çeşidini ifade eden, hayvanın sağlıklı olup olmadığını gösteren, aynı zamanda hem insanlar hem de hayvanlar için hastalık oluşturan mikroorganizmaları taşıyıp taşımadığını anlamamızı sağlayan en önemli göstergedir. Bu mikrobiyota canlının tüm metabolizmasını, fizyolojisini, immünolojik yapısını, hatta davranışlarını dahi direk olarak etkilemektedir (Barko ve ark. 2018).

Sindirim sistemi mikrobiyotası; en çok yemlerden, çevresel faktörlerden (ısı, ışık vb.), bakım, besleme ve yetiştiricilik uygulamalarından (havalandırma, altlık su vb.) etkilenmektedir. Bunların sonucu olarak farklı laboratuvar hayvanı yetiştirme ve araştırma birimlerinde hatta aynı birimlerdeki her bir bireyde büyük farklılıklar gözlenebilmektedir. Bu hayvanların kullanım alanı dikkate alındığında sindirim sisteminin sağlıklı ve bir örnek (veya benzer) olmaması yapılan bilimsel çalışma verilerini olumsuz bir şekilde etkiler, bilimsel çalışmaların tekrarlanabilir olmasına imkân tanımaz. En basit bir çalışmada dahi hayvanların mikrobiyota farklılıkları kontrol ve deney grupları arasındaki verilerin doğruluğunu etkileyebilir. (McCoy ve ark. 2017).

Laboratuvar hayvanlarının sağlıklı olmaları deneysel kullanımları için en önemli ön koşuldur. Subklinik enfeksiyonlar aynı zamanda zoonoz hastalıklar açısından endişe yaratmaktadır. Bu durum deney modellerinin fenotipik varyasyonunu arttırmakta olup istatistiksel hatalara yol açmakta ve bu nedenle daha fazla sayıda hayvanın gereksiz yere kullanımı ile; yerine koyma, arıtma ve indirgeme (3R kuralı) temel kurallarına aykırıdır (Nicklas 2008).

Bu bilgiler ışığında, bir bilimsel çalışma öncesi kullanılacak hayvanların tüm dış faktörlerin bir örnek olmasına dikkat edilmesi zorunludur. Bu kapsamda çalışmalarda aynı tür, soy, cinsiyet ve yaşta hayvanlar seçilir, hayvanlar aynı yemle bir ortamda bakılır. Böylelikle pek çok dış çevresel değişken faktör elimine edilmiş olur. Ancak hayvanların iç faktörleri olan bağırsak mikrobiyotaları senkronize edilmediği sürece mevcut şartlarda deneylere, başlama kriterlerinin sağlanması yeterli olmayabilir. Genel olarak mikrobiyolojik standardizasyon; hayvanlarda bulunan mikroorganizma türlerine ve buna bağlı olarak yetiştirilme ortamlarına göre sınıflandırılması mikrobiyolojik durumlarının belgelendirilmesi esaslarına dayanır. Bu sınıflama, bariyerli spesifik patojen free; (SPF) ve bariyersiz konvansiyonel (CV) hayvanlar şeklindedir. Mikrobiyolojik standardizasyonun izlenmesi iki şekilde yapılmakta olup bunlar bariyer sistemlerinin kontrolü (süreç kontrolü) ve deney hayvanlarının kontrolüdür. Bariyer Sistemlerinin Kontrolü (Süreç Kontrolü); hayvanın mevcut mikrobiyolojik kalitesinin korunmasının yanı sıra çalışanın korunması ve laboratuvar kaynaklı enfeksiyonların önlenmesi için önemlidir. Bununla beraber bu çalışmanın konusu olan mikrobiyota senkronizasyonu ile ilgili kapsamlı ve tanımlı protokollerin eksikliği göze çarpmaktadır (Nicklas 2008).

Avrupa ve Amerika'da laboratuvar hayvanları üretimi büyük çiftliklerde ve üretim merkezlerinde yapılmaktadır. Oldukça gelişmiş teknik alt yapı ve laboratuvar olanaklarına sahip bu kuruluşlar kendi standartlarını ve kalitelerini denetlemek amacıyla FELASA'nın (Avrupa Laboratuvar Hayvanları Bilimi Dernekleri Federasyonu-Federation of European Laboratory Animal Science Associations) önerileri doğrultusunda çeşitli mikrobiyolojik kalite kontrol programlarını izlemektedirler. Ülkemizde de laboratuvar hayvanı üretim ve yetiştiriciliği alanında FELASA'nın hazırlamış olduğu rehberler kabul edilmektedir. Ancak Türkiye'de konu ile ilgili sıkıntılar olduğu düşünülmektedir (Nicklas 2008; Anonim 2020a; 2020b). Tarım ve Orman Bakanlığı'nın şimdiye kadar verdiği çalışma izinleri konvensiyonel ünite iznidir. Bu nedenle laboratuvar hayvanlarının mikrobiyolojik standardizasyonu sağlanamamaktadır.

Son yıllarda ülkemizde kamu ve özel sektörde laboratuvar hayvanı üretim ve yetiştiriciliği yapan kuruluşların sayısı artmıştır. Türkiye'de 2020 yılı itibariyle bilimsel çalışmalarda toplam 209.212 hayvan kullanılmış olup bunların %17,2 sini rat ve %11,9'unu fare oluşturmuştur (Anonim 2021). Ancak burada önemli olan rakamsal olarak artışlar değil refah düzeyi yüksek, mikrobiyolojik standartların uygulandığı ve sağlık kontrollerin yapıldığı ünitelerin bulunmasıdır. Tarım ve Orman Bakanlığı'nın "Deneysel ve Diğer Bilimsel Amaçlar için Kullanılan Hayvanların Refah ve Korunmasına Dair Yönetmelik" çerçevesinde çalışma izni verilen deney hayvanı kuruluşları sayısı 192 olup bunların büyük bölümünün üretim, tedarik ve deney için rat ve fare kullandığı ve konvensiyonel özellikte olduğu görülmektedir (Anonim 2023). Hayvanların patojen mikroorganizmalara karşı korunmalarında

bağırsak mikrobiyotası önemli bir rol oynar. Normal bağırsak floralarını oluşturan bakteri türlerinin sayısı kolon içeriklerinde yaklaşık olarak $1x10^{11}$-10^{12} kob/gram bakteri bulunmaktadır (Lee ve ark. 2013). Konvensiyonel hayvanlar genellikle hastalık belirtisi göstermezken enfeksiyon ve paraziter etkenleri taşımaktadırlar.

Probiyotikler canlı mikroorganizmalar olup yeterli miktarda ve sürede uygulandıkları takdirde verildikleri canlıda bağırsaklarda kolonize olarak olumlu sağlık etkileri oluşturdukları ifade edilmektedir (Anonim 2006; Hill ve ark. 2014). Probiyotiklerin, enfeksiyonları önleyerek, mide-bağırsak koşullarını iyileştirerek ve bağışıklık bozukluklarını gidererek sağlığı geliştirme özelliklerinin olduğu pek çok çalışmada gösterilmiştir (Wilkins ve Sequoia 2017; Wang ve ark. 2019). Probiyotiklerin bağırsak bütünlüğünün korunması (Khailova ve ark. 2010) ve bağırsak mikrobiyal taksonomik yapısının modülasyonunu sağladığı belirtilmiştir (Ferrario ve ark. 2014; Gargari ve ark. 2016). Probiyotiklerin ana hedefi olan bağırsak mikrobiyotasının bağırsak kanalı boyunca değişiklik gösterebildiği ve bağırsaklardaki karmaşık etkileşimler nedeniyle probiyotiklerin etki mekanizmasının mutlaka in-vivo çalışmalarla desteklenmesi gerektiği ifade edilmektedir (Farzi ve ark. 2018; Taverniti ve ark. 2021).

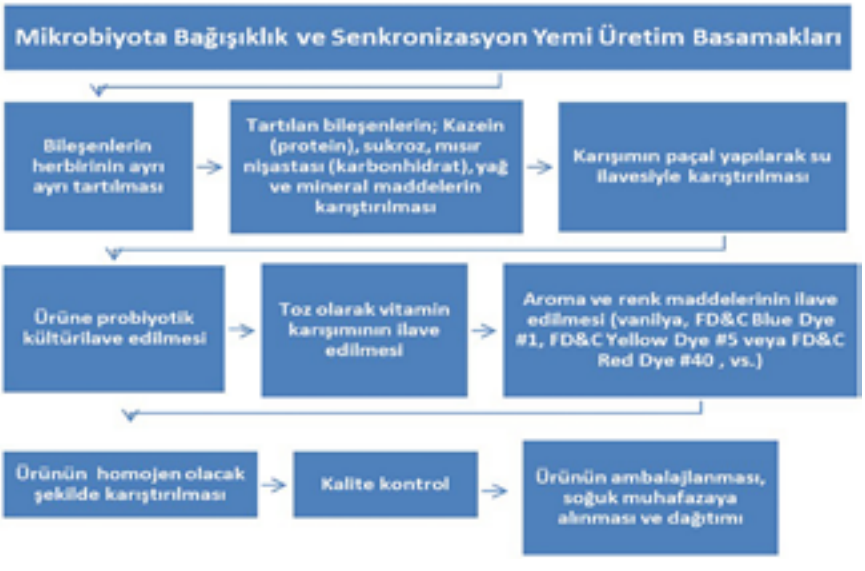
Tüm bu bilgiler ışığında, bu çalışmada dünyada genel ve kabul edilmiş bir standardizasyonu olmayan laboratuvar hayvanlarının (rat ve fare) bağırsak mikrobiyotalarının senkronizasyonunun sağlanması için bilimsel çalışmalar öncesi adaptasyon periyodunda kullanılmak üzere probiyotik içerikli yemlerin üretimi ve etkinliği *in-vivo* olarak araştırılmıştır.

Gereç ve Yöntem

Probiyotik içerikli yemlerin üretimi

Mikrobiyota senkronizasyon yemi olarak probiyotik yem üretimi gerçekleştirilmiştir. Üretilen yemler, kullanım kolaylığı, tüketici talepleri, verimliliği ve etkinliği göz önünde bulundurularak sıvı formda üretilmiştir. Çalışmada yemlerin formülasyonunun oluşturulması, üretim yönteminin geliştirilmesi ve optimizasyon çalışmaları gerçekleştirilmiştir.

Yemlere probiyotik kültür olarak *Lactiplantibacillus plantarum* ATCC 14917 ve *Lactobacillus acidophilus* (ATCC 4356) katılarak test grubu (T) yemleri hazırlanmıştır. Yemlere karıştırılmak üzere -18°C'de tutulan suşlar buz içerisinde çözdürüldükten sonra Tryptone Soy Broth'a (TSB, LAB004 Acumedia) geçilerek 24 saat 37° C'de inkübe edilmiştir. Daha sonra suşun kontrol ve deneyde kullanılacak konsantrasyonunun belirlenmesi amacıyla De Man, Rogosa & Sharpe agar (MRS, Merck 64271) kullanılmıştır. Bu kapsamda bir gece TSB'de inkübe edilen suşlar MRS Agar'a çizme plak metodu ile ekilerek 24-48 saat 37° C'de anaerob ve aerob ortamda inkübe edilmiştir. Anaerob koşullarda, inci beyazı koloniler değerlendirilmiştir. Yemlere aktif katılmak üzere ilgili kolonilerden tekrar TSB'lere ekimler yapılarak 37° C'de 24 saat inkübe edilmiştir. Miktarın belirlenmesi amacıyla da deney günü tekrar MRS Agar'lara ekimleri gerçekleştirilmiştir. Miktarı belirlendikten sonra yemlere aynı miktarda probiyotik verilmesi amacıyla *L. plantarum* ve *L. acidophilus* bakterilerinden 10^8 kob/ml olacak şekilde steril tüplerde hazırlıklar tamamlanmıştır. Probiyotik kültür ilavesi yapılmamış yemler ise kontrol grubunu (K) oluşturmuştur. Sıvı yem üretim basamakları sırasıyla Şekil 1'de belirtilmiştir.



Şekil 1. Sıvı yem üretim basamakları.

Yemlerin mikrobiyolojik analizleri

Paketlenmiş yemlerin muhafaza süresi başında ve sonunda mikrobiyolojik analizleri gerçekleştirilmiştir. Hazırlanmış yemler probiyotik mikroorganizma içerdiğinden raf ömrü +4°C'de 1 hafta olarak belirlenmiştir. Yemlerde gerçekleştirilen mikrobiyolojik analizler şu şekildedir; Aerob genel canlı tayini (ISO 4833-2), koliform bakteri sayısı (ISO 4832), *Salmonella* spp. (ISO 6579), *Listeria monocytogenes* (ISO 11290-1), *Staphylococcus aureus* (ISO 6888-1), küf-maya sayımı (ISO 21527-2) ve probiyotik mikroorganizma sayımı (ISO 15214).

Mikrobiyota senkronizasyon yemlerinin deney hayvanlarına uygulanması

Mikrobiyota senkronizasyon yemlerinin etkinlikleri ve optimal uygulama sürelerinin belirlenmesi amacıyla deney hayvanı çalışmaları ve laboratuvar analizleri gerçekleştirilmiştir. Çalışmada deney hayvanları çalışmaları Kırıkkale Üniversitesi Hüseyin Aytemiz Deney Hayvanları Biriminde etik kurul izni alınarak gerçekleştirilmiştir (Etik kurul karar no: 2021-02-08). Çalışmada Kontrol (K grup) ve Test (T grupları, üretilen her bir yem için) olarak gruplar oluşturulduktan sonra, erkek ve dişi olarak ayrı ayrı her bir grup için 9'ar adet 200-250 g ağırlığında, 8-10 haftalık uluslararası standartlara uygun sayıda Wistar albino rat ve 20-25 g ağırlığında 8-10 haftalık BALB/c fare rastgele seçilmiştir. Grupların gerekli adaptasyon işlemleri ve süresi (7 gün) tamamlandıktan sonra başlangıç ağırlıkları kaydedilmiş, mikrobiyota analizleri için dışkı örnekleri alınmış ve DNA ekstraksiyonları gerçekleştirilmiştir.

Sonrasında hayvanlar belirlenen gruplara göre (Kontrol ve Test) ad-libitum olarak 10 günlük periyodlarda 30 gün süreyle standart kontrol yemi veya mikrobiyota senkronizasyon yemi ile beslenmiştir. Deney periyodu sonunda dışkı örnekleri alınarak mikrobiyota analizleri tekrarlanmıştır. Çalışma süresi sonunda başlangıç-bitiş mikrobiyota sonuçları kontrol grubu verileriyle karşılaştırılmıştır.

Deney hayvanlarının mikrobiyota analizleri

Bu kapsamda steril pens ve kaşık yardımıyla taze dışkı örnekleri kontrol ve test gruplarına ait fare ve ratlardan alınarak steril kaplara konulmuş ve analiz için soğuk zincir altında laboratuvara taşınmıştır.

Örneklerden DNA izolasyonu

Dışkı örneklerinden genomik DNA izolasyonu Quick-DNA TM Fungal/Bacterial Miniprep Kit (Zymo Research, Cat. No: D6005) kullanılarak yapılmıştır. İzole edilmiş DNA'nın miktar ve saflığı florometrik olarak QUBIT3™ ile tayin edilmiştir (Thermo Fisher Scientific, Wilmington, DE, USA).

16s rRNA V3-V4 bölgesinin amplifikasyonu

Tür tayininde kullanılacak olan 16s rRNA genine ait V3-V4 bölgeleri üniversal 341F-805R primer dizileri ile SimpliAmp™ Thermal Cycler kullanılarak amplifiye edilmiştir. Kullanılan primer dizileri ve PCR koşulları aşağıda verilmiştir (Thermo Fisher Scientific, Wilmington, DE, USA).

Primer dizileri

341F: CCTACGGGNGGCWGCAG

805R: GACTACHVGGGTATCTAATCC

PCR koşulları

95°C 10 dakika – ilk denaturasyon (HS enzim kullanılmıştır), 35 döngü:

- 95°C for 30 saniye - denaturasyon

- 53-48°C for 30 saniye – annealing (touchdown PCR)

- 72°C for 15 saniye – extension, sıcaklık 4°C'ye düşürülüp PCR tamamlanmıştır.

Kütüphane hazırlama ve dizileme işlemi

16s rRNA V3-V4 amplikon ürünleri için kütüphane hazırlama Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, Cat. No.: FC-131-1096) ile index işlemi ise TG Nextera XT Index Kit v2 Set A (96 Indices, 384 Samples), (Illumina, San Diego, CA, Cat. No.: TG-131-2001) ile gerçekleştirilmiştir. PCR pürifikasyonu-AMPure XP beads ile yapılmıştır (Beckman Coulter, High Wycombe, UK). Dizileme işlemi Illumina'nın Miseq platformu ile paired-end (PE) 2x150 bazlık okumalar olarak yapılmıştır. Örnek başına minimum ≥ 50.000 okuma yapılmıştır.

Ham verinin biyoinformatik analizi

Ham veri okumaları (FASTQ) QC kontrol yapılmış, trim edilmiş ve Kraken Metagenomik sistemi ile OTU sınıflarına ayrılmıştır. Kraken uygulaması, yüksek hassasiyet ve hızda kısa DNA sekanslarına taksonomik etiketler atarak gerçekleştirilmiştir (Wood ve Salzberg 2014).

Bulgular

Yemlerin muhafaza süresince raf ömrünün belirlenmesi ve mikrobiyolojik kalitesinin takibi amacıyla mikrobiyolojik analizler gerçekleştirilmiştir. Buna göre yemlerin yedinci gün sonunda toplam aerob genel canlı sayısı ve laktik asit bakteri sayısı (*L. aci-*

dophilus ve *L. plantarum*) 10^9 kob/ml olarak belirlenmiştir. Analizlerde koliform bakteri, maya-küf ile diğer patojen mikroorganizmalara (*Salmonella* spp., *L. monocytogenes* ve *S. aureus*) rastlanmamıştır.

Mikrobiyota analiz sonuçları

Mikrobiyota analizlerinde örnekler deney gruplarına göre analiz sonuçları aşağıda belirtilmiştir. Deney grupları numaralandırılmış olup buna göre; KF10- Kontrol fare grubu 10. gün analiz sonuçlarını; TF10- Test fare grubu 10. gün analiz sonuçlarını; KF0- Kontrol fare grubu 0. gün analiz sonuçlarını; TF0- Test fare grubu 0. Gün analiz sonuçlarını; KR10- Kontrol rat grubu 10. gün analiz sonuçlarını; TR10- Test rat grubu 10. gün analiz sonuçlarını; KR0- Kontrol rat grubu 0. gün analiz sonuçlarını; TR0- Test rat grubu 0. gün analiz sonuçlarını temsil etmektedir.

Simpsons indeksi 0-1 arasında bir değer alır. 1 çeşitliliği, 0 ise çeşitlilik yok anlamına gelmektedir ve shannon indeksi genellikle 1,5-3,5 arasında bir değer alır ve bu indeks arttıkça çeşitlilik de artmaktadır.

Tablo 1. Deney gruplarının dizileme istatistiği

Grup no	Okuma sayısı	Ortalama okuma uzunluğu	Sınıflanmış okuma
KF10	34088	149.7	33931 / 99.54%
TF10	21935	149.7	21839 / 99.56%
KF0	26444	149.7	26319 / 99.53%
TF0	42432	145.7	40345 / 95.08%
KR10	25087	133.9	20737 / 82.66%
TR10	15458	149.6	15356 / 99.34%
KR0	24530	149.9	24465 / 99.74%
TR0	23282	149.9	23233 / 99.79%

Tablo 2. Deney gruplarının taksonomi istatistiği

Tür Seviyesinde Çeşitlilik		
Grup no	**Shannon Index (H) / (H / LN (N))**	**Simpson Indeks (1-D)**
KF10	4.154 / 0.717	0.9721
TF10	3.68 / 0.6129	0.9425
KF0	4.296 / 0.7226	0.9756
TF0	3.779 / 0.631	0.9386
KR10	4.145 / 0.6959	0.9616
TR10	3.741 / 0.6578	0.9441
KR0	4.316 / 0.7156	0.9756
TR0	4.206 / 0.7245	0.9745

Tablo 3. Fare ve Rat test gruplarına göre taksonomik dağılımlar

Krallık	Test gruplarına göre oran (%)							
	KF0	**TF0**	**KF10**	**TF10**	**KR0**	**TR0**	**KR10**	**TR10**
Bacteria	100.0	99.98	100.0	100.0	99.97	99.99	99.9	99.91
Archaea	0.0	0.01	0.0	0.0	0.02	0.01	0.06	0.08
Eukaryota	0.0	0.0	0.0	0.0	0.01	0.0	0.04	0.01

Tablo 4. Fare test gruplarına göre filum seviyesinde taksonomik dağılımlar*

KF0		TF0	
Filum	**%**	**Filum**	**%**
Firmicutes	52.04	*Firmicutes*	50.5
Bacteroidetes	31.33	*Bacteroidetes*	41.52
Proteobacteria	12.61	*Proteobacteria*	5.58
Tenericutes	1.78	*Actinobacteria*	1.02
Actinobacteria	1.67	*Tenericutes*	1.02
Chloroflexi	0.25	*Chloroflexi*	0.12
Cyanobacteria	0.08	*Cyanobacteria*	0.05
Candidatus saccharibacteria	0.05	*Candidatus saccharibacteria*	0.04
Thermotogae	0.05	*Spirochaetes*	0.03
Deferribacteres	0.04	*Gemmatimonadetes*	0.02

KF10		TF10	
Filum	**%**	**Filum**	**%**
Firmicutes	64.36	*Bacteroidetes*	64.01
Bacteroidetes	28.14	*Firmicutes*	30.63
Proteobacteria	6.02	*Proteobacteria*	3.65
Actinobacteria	0.87	*Actinobacteria*	0.73
Spirochaetes	0.17	*Tenericutes*	0.45
Tenericutes	0.15	*Chloroflexi*	0.22
Chloroflexi	0.06	*Cyanobacteria*	0.12
Coprothermobacterota	0.04	*Spirochaetes*	0.07
Gemmatimonadetes	0.04	*Planctomycetes*	0.05
Candidatus saccharibacteria	0.03	*Verrucomicrobia*	0.01

* Örneklerdeki en yüksek okuma dizisi bulunan ilk 10'a ait tablo verilmiştir.

Tablo 5. Rat test gruplarına göre filum seviyesinde taksonomik dağılımlar*

KR0		TR0		KR10		TR10	
Filum	**%**	**Filum**	**%**	**Filum**	**%**	**Filum**	**%**
Firmicutes	69.03	*Firmicutes*	67.88	*Firmicutes*	59.54	*Bacteroidetes*	44.09
Bacteroidetes	14.66	*Bacteroidetes*	24.42	*Bacteroidetes*	31.83	*Firmicutes*	44.06
Proteobacteria	12.97	*Proteobacteria*	4.26	*Proteobacteria*	5.79	*Proteobacteria*	6.74
Actinobacteria	1.96	*Tenericutes*	1.65	*Actinobacteria*	1.03	*Verrucomicrobia*	2.28
Tenericutes	0.83	*Actinobacteria*	1.14	*Spirochaetes*	0.96	*Actinobacteria*	2.21
Spirochaetes	0.19	*Chloroflexi*	0.21	*Tenericutes*	0.33	*Tenericutes*	0.41
Chloroflexi	0.12	*Spirochaetes*	0.15	*Chloroflexi*	0.09	*Basidiomycota*	0.07
Candidatus saccharibacteria	0.08	*Planctomycetes*	0.13	*Elusimicrobia*	0.07	*Cyanobacteria*	0.04
Gemmatimonadetes	0.03	*Cyanobacteria*	0.04	*Verrucomicrobia*	0.05	*Armatimonadetes*	0.03
Verrucomicrobia	0.02	*Armatimonadetes*	0.03	*Candidatus thermoplasmatota*	0.04	*Deferribacteres*	0.02

* Örneklerdeki en yüksek okuma dizisi bulunan ilk 10'a ait tablo verilmiştir.

Tablo 6. Fare test gruplarına göre sınıf seviyesinde taksonomik dağılımlar*

KF0		TF0		KF10		TF10	
Sınıf	**%**	**Sınıf**	**%**	**Sınıf**	**%**	**Sınıf**	**%**
Clostridia	43.86	*Clostridia*	41.96	*Clostridia*	55.41	*Bacteroidia*	63.61
Bacteroidia	31.11	*Bacteroidia*	40.48	*Bacteroidia*	27.65	*Clostridia*	22.26
Epsilonproteobacteria	7.22	*Bacilli*	7.6	*Bacilli*	7.55	*Erysipelotrichia*	4.21
Bacilli	5.17	*Epsilonproteobacteria*	2.97	*Gammaproteobacteria*	3.08	*Bacilli*	3.35
Erysipelotrichia	2.89	*Gammaproteobacteria*	1.6	*Epsilonproteobacteria*	1.88	*Gammaproteobacteria*	1.61
Mollicutes	1.66	*Flavobacteriia*	1.18	*Erysipelotrichia*	1.06	*Flavobacteriia*	1.28
Gammaproteobacteria	1.4	*Mollicutes*	0.76	*Actinomycetia*	0.62	*Deltaproteobacteria*	0.5
Coriobacteriia	1.27	*Alphaproteobacteria*	0.7	*Flavobacteriia*	0.47	*Betaproteobacteria*	0.43
Alphaproteobacteria	1.27	*Coriobacteriia*	0.57	*Deltaproteobacteria*	0.4	*Mollicutes*	0.43
Flavobacteriia	1.25	*Erysipelotrichia*	0.51	*Betaproteobacteria*	0.38	*Coriobacteriia*	0.35

* Örneklerdeki en yüksek okuma dizisi bulunan ilk 10'a ait tablo verilmiştir.

Tablo 7. Rat test gruplarına göre sınıf seviyesinde taksonomik dağılımlar*

KR0		TR0		KR10		TR10	
Sınıf	**%**	**Sınıf**	**%**	**Sınıf**	**%**	**Sınıf**	**%**
Clostridia	57.4	*Clostridia*	53.12	*Clostridia*	49.88	*Bacteroidia*	41.56
Bacteroidia	14.02	*Bacteroidia*	24.68	*Bacteroidia*	30.11	*Clostridia*	36.01
Gammaproteobacteria	7.72	*Bacilli*	10.39	*Bacilli*	8.01	*Bacilli*	5.33
Erysipelotrichia	5.69	*Gammaproteobacteria*	3.0	*Gammaproteobacteria*	2.09	*Erysipelotrichia*	4.31
Bacilli	5.53	*Erysipelotrichia*	2.91	*Flavobacteriia*	2.06	*Gammaproteobacteria*	3.66
Deltaproteobacteria	2.59	*Negativicutes*	1.58	*Alphaproteobacteria*	1.84	*Verrucomicrobiae*	2.45
Negativicutes	1.81	*Mollicutes*	1.46	*Deltaproteobacteria*	1.24	*Coriobacteriia*	2.16
Coriobacteriia	1.46	*Coriobacteriia*	0.45	*Spirochaetia*	0.99	*Negativicutes*	1.12
Alphaproteobacteria	0.98	*Deltaproteobacteria*	0.32	*Negativicutes*	0.94	*Betaproteobacteria*	0.69
Flavobacteriia	0.83	*Alphaproteobacteria*	0.27	*Erysipelotrichia*	0.85	*Alphaproteobacteria*	0.57

* Örneklerdeki en yüksek okuma dizisi bulunan ilk 10'a ait tablo verilmiştir.

Tablo 8. Fare test gruplarına göre sıra seviyesinde taksonomik dağılımlar*

KF0		TF0		KF10		TF10	
Sıra	**%**	**Sıra**	**%**	**Sıra**	**%**	**Sıra**	**%**
Eubacteriales	43.82	*Eubacteriales*	42.02	*Eubacteriales*	55.52	*Bacteroidales*	61.51
Bacteroidales	30.65	*Bacteroidales*	40.01	*Bacteroidales*	27.18	*Eubacteriales*	22.29
Campylobacterales	7.25	*Lactobacillales*	7.31	*Lactobacillales*	6.51	*Erysipelotrichales*	4.23
Lactobacillales	4.51	*Campylobacterales*	2.97	*Campylobacterales*	1.89	*Lactobacillales*	3.04
Erysipelotrichales	2.9	*Flavobacteriales*	1.19	*Pseudomonadales*	1.61	*Marinilabiliales*	2.4
Acholeplasmatales	1.48	*Cellvibrionales*	0.69	*Erysipelotrichales*	1.07	*Flavobacteriales*	1.29
Eggerthellales	1.26	*Eggerthellales*	0.56	*Bacillales*	1.04	*Enterobacterales*	0.6
Flavobacteriales	1.26	*Marinilabiliales*	0.56	*Enterobacterales*	0.77	*Cellvibrionales*	0.49
Hyphomicrobiales	0.92	*Rhodospirillales*	0.54	*Marinilabiliales*	0.52	*Burkholderiales*	0.4
Desulfovibrionales	0.72	*Acholeplasmatales*	0.52	*Flavobacteriales*	0.47	*Eggerthellales*	0.33

* Örneklerdeki en yüksek okuma dizisi bulunan ilk 10'a ait tablo verilmiştir.

Tablo 9. Rat test gruplarına göre sıra seviyesinde taksonomik dağılımlar*

KR0		TR0		KR10		TR10	
Sıra	**%**	**Sıra**	**%**	**Sıra**	**%**	**Sıra**	**%**
Eubacteriales	59.78	*Eubacteriales*	53.01	*Eubacteriales*	49.68	*Bacteroidales*	40.29
Bacteroidales	14.7	*Bacteroidales*	24.67	*Bacteroidales*	30.16	*Eubacteriales*	34.53
Erysipelotrichales	5.97	*Lactobacillales*	8.01	*Lactobacillales*	5.94	*Erysipelotrichales*	4.18
Lactobacillales	4.16	*Erysipelotrichales*	2.92	*Flavobacteriales*	2.07	*Lactobacillales*	4.11
Myxococcales	2.06	*Bacillales*	2.38	*Bacillales*	2.06	*Bacteroidetes Order II. Incertae sedis*	3.52
Enterobacterales	1.83	*Cellvibrionales*	2.02	*Rhodospirillales*	1.71	*Verrucomicrobiales*	2.38
Bacillales	1.63	*Acidaminococcales*	1.34	*Desulfovibrionales*	1.13	*Pseudomonadales*	2.09
Flavobacteriales	0.87	*Acholeplasmatales*	1.12	*Spirochaetales*	0.99	*Coriobacteriales*	1.98
Selenomonadales	0.85	*Eggerthellales*	0.36	*Erysipelotrichales*	0.85	*Bacillales*	1.01
Pasteurellales	0.83	*Pasteurellales*	0.35	*Selenomonadales*	0.65	*Acidaminococcales*	0.87

* Örneklerdeki en yüksek okuma dizisi bulunan ilk 10'a ait tablo verilmiştir.

Tablo 10. Fare test gruplarına göre aile seviyesinde taksonomik dağılımlar*

KF0		TF0		KF10		TF10	
Aile	**%**	**Aile**	**%**	**Aile**	**%**	**Aile**	**%**
Lachnospiraceae	28.52	*Lachnospiraceae*	29.6	*Lachnospiraceae*	39.57	*Prevotellaceae*	26.82
Muribaculaceae	10.83	*Prevotellaceae*	20.14	*Muribaculaceae*	11.76	*Lachnospiraceae*	15.83
Oscillospiraceae	10.57	*Muribaculaceae*	10.89	*Oscillospiraceae*	8.94	*Bacteroidaceae*	13.13
Helicobacteraceae	7.95	*Oscillospiraceae*	6.59	*Prevotellaceae*	6.95	*Muribaculaceae*	12.37
Prevotellaceae	7.7	*Lactobacillaceae*	6.56	*Lactobacillaceae*	5.57	*Oscillospiraceae*	5.41
Bacteroidaceae	6.11	*Bacteroidaceae*	4.14	*Bacteroidaceae*	5.35	*Erysipelotrichaceae*	4.86
Lactobacillaceae	3.56	*Helicobacteraceae*	3.13	*Eubacteriaceae*	2.82	*Marinilabiliaceae*	2.77
Erysipelotrichaceae	3.19	*Eubacteriaceae*	2.12	*Helicobacteraceae*	2.05	*Rikenellaceae*	2.66
Clostridiaceae	2.13	*Rikenellaceae*	2.08	*Rikenellaceae*	1.46	*Lactobacillaceae*	2.63
Rikenellaceae	1.82	*Clostridiaceae*	1.82	*Clostridiaceae*	1.41	*Tannerellaceae*	1.84

* Örneklerdeki en yüksek okuma dizisi bulunan ilk 10'a ait tablo verilmiştir.

Tablo 11. Rat test gruplarına göre aile seviyesinde taksonomik dağılımlar*

KR0		TR0		KR10		TR10	
Aile	**%**	**Aile**	**%**	**Aile**	**%**	**Aile**	**%**
Lachnospiraceae	31.64	*Lachnospiraceae*	26.62	*Lachnospiraceae*	24.05	*Lachnospiraceae*	21.31
Oscillospiraceae	18.73	*Oscillospiraceae*	16.33	*Prevotellaceae*	20.71	*Prevotellaceae*	19.53
Prevotellaceae	11.55	*Prevotellaceae*	15.32	*Oscillospiraceae*	19.58	*Muribaculaceae*	16.82
Erysipelotrichaceae	6.58	*Muribaculaceae*	7.76	*Muribaculaceae*	7.71	*Oscillospiraceae*	9.38
Clostridiaceae	5.0	*Lactobacillaceae*	6.42	*Lactobacillaceae*	5.08	*Erysipelotrichaceae*	4.37
Muribaculaceae	2.53	*Clostridiaceae*	3.38	*Clostridiaceae*	2.54	*Rhodothermaceae*	3.68
Lactobacillaceae	2.46	*Erysipelotrichaceae*	2.93	*Acetobacteraceae*	1.84	*Akkermansiaceae*	2.48
Enterobacteriaceae	1.86	*Bacillaceae*	2.29	*Bacillaceae*	1.71	*Lactobacillaceae*	2.41
Bacillaceae	1.67	*Spongiibacteraceae*	2.2	*Bacteroidaceae*	1.67	*Coriobacteriaceae*	1.98
Peptostreptococcaceae	1.6	*Eubacteriaceae*	1.75	*Desulfovibrionaceae*	1.23	*Bacteroidaceae*	1.82

* Örneklerdeki en yüksek okuma dizisi bulunan ilk 10'a ait tablo verilmiştir.

Tablo 12. Fare test gruplarına göre cins seviyesinde taksonomik dağılımlar*

KF0		TF0		KF10		TF10	
Cins	**%**	**Cins**	**%**	**Cins**	**%**	**Cins**	**%**
Helicobacter	8.88	*Prevotella*	20.63	*Duncaniella*	8.71	*Prevotella*	24.85
Prevotella	7.43	*Lachnoclostridium*	8.09	*Lachnoclostridium*	8.69	*Bacteroides*	13.24
Bacteroides	6.83	*Duncaniella*	7.89	*Anaerocolumna*	7.45	*Phocaeicola*	11.47
Lachnoclostridium	6.23	*Bacteroides*	4.36	*Prevotella*	7.24	*Duncaniella*	7.93
Duncaniella	5.95	*Anaerotignum*	4.26	*Bacteroides*	5.8	*Lachnoclostridium*	3.3
Muribaculum	3.82	*Lactobacillus*	3.92	*Butyrivibrio*	4.0	*Alkalitalea*	2.79
Phocaeicola	3.78	*Helicobacter*	3.3	*Fastidiosipila*	3.7	*Alistipes*	2.68
Ruminococcus	3.78	*Coprococcus*	2.65	*Eubacterium*	2.93	*Muribaculum*	2.26
Fastidiosipila	3.27	*Mediterraneibacter*	2.52	*Lactobacillus*	2.83	*Ruminococcus*	2.16
Anaerotignum	2.98	*Fastidiosipila*	2.27	*Muribaculum*	2.62	*Mediterraneibacter*	2.12

* Örneklerdeki en yüksek okuma dizisi bulunan ilk 10'a ait tablo verilmiştir.

Tablo 13. Rat test gruplarına göre cins seviyesinde taksonomik dağılımlar*

KR0		TR0		KR10		TR10	
Cins	**%**	**Cins**	**%**	**Cins**	**%**	**Cins**	**%**
Prevotella	11.14	*Prevotella*	13.28	*Prevotella*	20.39	*Prevotella*	14.79
Lachnoclostridium	9.96	*Lachnoclostridium*	6.12	*Ruthenibacterium*	5.28	*Duncaniella*	13.21
Ruminococcus	6.04	*Ruminococcus*	6.01	*Ligilactobacillus*	4.57	*Anaerostipes*	9.3
Lacrimispora	4.96	*Duncaniella*	5.38	*Muribaculum*	4.48	*Rhodothermus*	4.22
Clostridium	4.71	*Blautia*	5.09	*Oscillibacter*	3.59	*Ruthenibacterium*	3.71
Fastidiosipila	3.85	*Fastidiosipila*	3.87	*Lachnoclostridium*	3.01	*Anaerocolumna*	3.55
Faecalitalea	3.56	*Ligilactobacillus*	3.61	*Duncaniella*	2.7	*Akkermansia*	2.84
Mediterraneibacter	2.77	*Ruthenibacterium*	2.94	*Ruminococcus*	2.64	*Faecalitalea*	2.73
Intestinimonas	2.44	*Oceanicoccus*	2.42	*Intestinimonas*	2.61	*Muribaculum*	2.65
Anaerostipes	2.28	*Clostridium*	2.36	*Flavonifractor*	2.59	*Lacrimispora*	2.57

* Örneklerdeki en yüksek okuma dizisi bulunan ilk 10'a ait tablo verilmiştir.

Tablo 14. Fare test gruplarına göre tür seviyesinde taksonomik dağılımlar*

KF0		TF0	
Tür	**%**	**Tür**	**%**
Helicobacter typhlonius	7.53	*Prevotella dentalis*	20.5
Duncaniella dubosii	4.26	*Clostridium scindens*	7.34
Fastidiosipila sanguinis	4.22	*Anaerotignum propionicum*	5.35
Prevotella sp. WR041	3.89	*Duncaniella dubosii*	5.19
Prevotella dentalis	3.88	*Coprococcus comes*	3.15
Anaerotignum propionicum	3.85	*Fastidiosipila sanguinis*	2.86
Ruminococcus champanellensis	3.69	*Eubacterium cellulosolvens*	2.66
Clostridium scindens	3.45	*Helicobacter apodemus*	2.62
Bacteroides uniformis	2.99	*Prevotella sp. WR041*	2.56
Clostridium hylemonae	2.76	*Lachnoanaerobaculum umeaense*	2.46

KF10		TF10	
Tür	**%**	**Tür**	**%**
Prevotella dentalis	7.05	*Prevotella dentalis*	14.81
Duncaniella dubosii	6.5	*Prevotella sp. WR041*	12.14
Lachnoclostridium phocaeense	5.57	*Phocaeicola dorei*	8.55
Fastidiosipila sanguinis	5.21	*Duncaniella dubosii*	5.34
Clostridium hylemonae	4.17	*Phocaeicola coprophilus*	5.32
Eubacterium cellulosolvens	4.11	*Bacteroides uniformis*	4.43
Anaerocolumna sp. CTTW	3.34	*Alkalitalea saponilacus*	3.64
Helicobacter apodemus	3.08	*Ruminococcus champanellensis*	2.4
Bacteroides uniformis	2.71	*[Ruminococcus] torques*	2.35
Anaerocolumna sedimenticola	2.7	*Bacteroides caccae*	2.18

* Örneklerdeki en yüksek okuma dizisi bulunan ilk 10'a ait tablo verilmiştir.

Tablo 15. Rat test gruplarına göre tür seviyesinde taksonomik dağılımlar*

KR0		TR0	
Tür	**%**	**Tür**	**%**
Ruminococcus champanellensis	5.49	*Prevotella copri*	7.09
Prevotella copri	5.42	*Ruminococcus champanellensis*	5.36
Lacrimispora saccharolytica	5.16	*Fastidiosipila sanguinis*	5.22
Fastidiosipila sanguinis	4.64	*Prevotella dentalis*	4.42
Faecalitalea cylindroides	4.28	*Ruthenibacterium lactatiformans*	3.96
Lachnoclostridium phocaeense	4.28	*Clostridium hylemonae*	3.56
Clostridium scindens	4.24	*Oceanicoccus sagamiensis*	3.26
Clostridium hylemonae	3.37	*Intestinimonas butyriciproducens*	3.15
Intestinimonas butyriciproducens	2.95	*Anaerobutyricum hallii*	2.59
Clostridium sp. SY8519	2.62	*Paraprevotella xylaniphila*	2.48

KR10		TR10	
Tür	**%**	**Tür**	**%**
Prevotella copri	13.86	*Prevotella copri*	15.8
Ruthenibacterium lactatiformans	7.13	*Anaerostipes hadrus*	12.12
Prevotella dentalis	6.23	*Rhodothermus marinus*	5.57
Intestinimonas butyriciproducens	3.52	*Ruthenibacterium lactatiformans*	4.9
Flavonifractor plautii	3.5	*Anaerocolumna cellulosilytica*	4.47
Fastidiosipila sanguinis	3.16	*Akkermansia muciniphila*	3.76
Komagataeibacter rhaeticus	2.84	*Faecalitalea cylindroides*	3.6
Flintibacter sp. KGMB00164	2.61	*Lacrimispora saccharolytica*	3.19
Ruminococcus champanellensis	2.49	*Collinsella aerofaciens*	2.99
Clostridium hylemonae	2.19	*Fastidiosipila sanguinis*	2.73

* Örneklerdeki en yüksek okuma dizisi bulunan ilk 10'a ait tablo verilmiştir.

Tartışma ve Sonuç

In-vivo çalışmalarda araştırılanın kesin olarak etkinliğini ortaya koyabilmek için deneyde kullanılan hayvanların türü, soyu ve yaşının yanı sıra çevre koşulları gibi pek çok koşul kontrol ve test gruplarında bir örnek hale getirilmektedir. Böylelikle deney sonucunda test ve kontrol gruplarından elde edilen sonuçlar arasındaki farkın tek değişken olduğu düşünülen uygulamadan kaynaklandığı varsayılmaktadır. Ancak bu deneylerde gastrointestinal sistemin işlevi ve bütünlüğünde, bağışıklık homeostazının korunmasında ve konak enerji metabolizmasında önemli bir rol oynayan bağırsak mikrobiyotasının (Pflughoeft ve Versalovic 2012) kullanılan deney hayvanlarında farklılıklar gösterebileceği bu durumun ise çalışma sonuçlarını olumsuz etkileyebileceği çoğu zaman gözden kaçmaktadır. Yapılan çalışmalar, bir dizi faktörün mikrobiyotada bazı değişikliklere neden olabileceğini göstermiştir. Bunlarla sınırlı olmamak üzere bağırsak

mikrobiyotasını modüle edebilen faktörler arasında, üretici/satıcı (tek bir üretici içindeki farklı tesisler dahil), beslenme, altlık tipi, barınak muhafazası, nakliye, terapötik müdahale, su, dekontaminasyon yöntemleri ve çoğaltma yer almaktadır (Ericsson ve ark. 2015; Rasmussen ve ark. 2019). Dolayısıyla özellikle farklı üreticilerden temin edilen deney hayvanlarıyla yapılan çalışmalar öncesinde kullanılan hayvanların mikrobiyotalarının senkronize edilmesinin çalışmaların güvenilirliği açısından önemli olduğu düşünülmektedir. Yapılan deneysel gözlemler probiyotiklerin bağırsak mikrobiyomunun yapısını ve genel metabolik işlevini etkileyebileceğini öne sürmektedir (Lavasani ve ark. 2010; McNulty ve ark. 2011). Günümüzde yeni nesil dizileme, kemirgen kolonilerinde var olan karmaşık mikrobiyal florayı daha iyi karakterize edilebilinmesine ve bağırsak mikrobiyotasındaki farklılıkları dışarıdan müdahaleler ile ilişkilendirilmesine imkân tanımaktadır (Franklin ve Ericsson 2020). Bu bağlamda probiyotik uygulaması ile deney hayvanlarının mikrobiyotasının senkronize edilebileceği hipotezi üzerine bu çalışma kurgulanmıştır.

Yapılan analizler neticesinde çalışma öncesinde farelerde kontrol (KF0: %52) ve test (TF0: %50) gruplarında mikrobiyotanın dominant florasının filum düzeyinde *Firmicutes*'ten oluştuğu gözlenmiş olup çalışmanın 10. gününde kontrol grubunda (KF10) bu dengenin yine %64 ile yine *Firmicutes* yönünde olduğu buna karşın test grubunda ise (TF10) *Bacteroidetes*'in %64 oranına yükselirken *Firmicutes*'in %30,6'ya gerilediği gözlenmiştir. Bu değişim rat deney gruplarında da benzer şekilde tespit edilmiştir. Analiz sonuçlarında görüleceği gibi sınıf, sıra, aile ve cins düzeyinde yapılan değerlendirmelerde hem fare hem de ratlarda probiyotik uygulamasının mikrobiyotanın kompozisyonu üzerine etkili olduğu ortaya konmuştur. Bununla beraber sonuçlar incelendiğinde oluşan farkların fare ve ratlar arasında farklılık gösterebildiği gibi aynı hayvanlarda sınıf, aile, cins ve tür düzeyinde de farklılıklar olabildiği görülmektedir. Bu açıdan daha kapsamlı araştırmaların faydalı olacağı düşünülmektedir.

Tür düzeyinde yapılan değerlendirmede, farede en yüksek oranda tespit edilen *Prevotella dentalis* ve ratta *Prevotella copri* dışında diğer türlerin sayılarında önemli değişikliklerin olduğu gözlenmiştir. Sayısında azalma görülen bakterilerin arasında *Clostridium* ve *Helicobacter* olması dikkat çekicidir. Kemirgenlerde helikobakterlerin, özellikle de *Helicobacter hepaticus* ve *Helicobacter bilis*'in, enflamatuar bağırsak hastalığı modellerinde yangıda provokatör olarak görev yaptıkları çeşitli araştırmalarda gösterilmiştir (Fox 2007; Jergens ve ark. 2007). Bu sonuçlara istinaden bu durumun özellikle bazı deneysel çalışmalarda problem yaratabileceği düşünülmektedir.

Antibiyotik tedavisi ile eşzamanlı olarak veya farelerin antibiyotik tedavisini takiben iyileşme aşamasında probiyotiklerin etkisinin incelediği bir çalışmada, probiyotiklerin bağırsağı kolonize etmediği veya bağırsak mikrobiyotasının genel çeşitliliğini değiştirmediği tespit edilmiştir. Bununla birlikte, probiyotik takviyesinin, mevcut olan bakteri türlerini önemli ölçüde değiştirdiği belirtilmektedir. Özellikle iyileşme fazı sırasında probiyotiklerin, *Enterobacteriaceae*'nin (*Shigella* ve *Escherichia*) baskılanmasına neden olurken, özellikle *Anaerotruncus* cinsinden *Firmicutes*'in artışını teşvik ettiği bildirilmiştir (Grazul ve ark. 2016). Aynı çalışmada, kontrol gruplarında özellikle *Firmicutes* ve *Bacteroidetes* baskın profilleri olarak belirlenmiştir. Ayrıca probiyotik ilavesi sonrasında bu sonuçlar bizim çalışma sonuçlarımız ile büyük oranda benzerlik göstermektedir. Bununla beraber çalışmamızda *Firmicutes* baskınlığının *Bacteroitedes'*e oranla çok daha yüksek olduğu belirlenmiştir.

Çalışmada aynı hayvan türlerine ait gruplar kendi içinde bir uyum gösterse de fare ve rat mikrobiyotalarının tür düzeyinde farklılık gösterdiği tespit edilmiştir. Bu durum diğer araştırmacılar tarafından da tespit edilmiş olup bağırsak mikrobiyotasının genetik ve cinsiyete göre değişiklik gösterebileceği belirtilmiştir (Benson ve ark. 2010). Franklin ve Ericsson (2020) her ne kadar deney amacıyla kullanılan rodentlerin sindirim sistemi mikrobiyotaları hakkında sınırlı bilgi sahibi olsak da hayvanların mikrobiyotalarının karmaşık ve kapsamlı varyasyonlar içerdiğini belirtmişlerdir. Bir başka çalışmada ise deney hayvanlarının sadece kalıtsal olarak aldıkları bağırsak mikrobiyotasına bağlı olarak maruz bırakıldıkları hastalığın ciddiyetinin değiştiği belirlenmiştir. Bu durumun kemirgen kolonilerinde bulunan farklı mikrobiyotaların fenotipik farklılıklara da neden olabileceğini göstermesi açısından önemli bulunmuştur (Hart ve ark. 2017). Bir fenotipik etkinin konakçı genotipinden mi, mikrobiyotadan mı yoksa ikisinin kombinasyonundan mı kaynaklandığını belirlerken deneysel kurulum kritik öneme sahip olduğu belirtilmektedir. Deneysel değişkenliği azaltmak, tekrarlanabilirliği artırmak ve doğru biyolojik yorumlar yapmayı sağlayacak anlamlı bilimsel veriler elde etmek için, yalnızca mikrobiyota-bağışıklık sistemi etkileşimlerini doğrudan araştıranlara değil, tüm deneysel fare modellerine titiz deneysel tasarım ve raporlama uygulanması gerektiği düşünülmektedir. Bu nedenle merkezlerde farklı protokollerin uygulanması istenmektedir (McCoy ve ark. 2017). Bu çalışmalar mikrobiyota senkronizasyonunun ne denli

önemli olduğunun ortaya konması açısından oldukça önemli bulunmuştur.

Mikrobiyotanın geliştirilmesinde çeşitli yöntemler kullanılabilmektedir. Laboratuvar kemirgenlerinin üretildikleri kurumlarla iş birliği yapılabildiği hallerde altlık veya dışkı örnekleri toplanıp hayvanların gönderildiği yeni tesise nakledilebilmektedir. Bu yaklaşım esasen kaprofajiye dayanmaktadır. Önemli bazı bakteri türlerinin kolonizasyonu uygulanmak istenilen deney hayvanlarında şekillenmeyebildiğinden bağırsak mikrobiyotasının transferinin eksik şekillendiği ve dolayısıyla karışık sonuçlarla karşılaşıldığı belirtilmiştir (Franklin ve Ericsson 2020).

Karmaşık bağırsak mikrobiyotasını transfer etmek için en doğrudan yaklaşım fekal transplantasyon olarak kabul edilmektedir. Bu yöntemde alıcı kemirgenlerdeki mevcut mikrobiyotanın önemli ölçüde azaltılması amacıyla hayvanlar geniş spektrumlu antibiyotik kokteyllerine tabi tutulurlar. Sonrasında alıcı kemirgenler donör dışkılarından veya mikrobiyotayı içeren diğer bağırsak numunelerinden hazırlanan karışımla beslenmektedirler. Antibiyotik tedavisi birlikte barınma ve dışkı/kirli yataklara maruz kalma ile ilişkili kolonizasyonun neden olduğu direncin çoğunu ortadan kaldırırken, endojen mikrobiyotanın çoğu üyesi tam olarak ortadan kaldırılamamaktadır. Bu nedenle antibiyotik ortadan kalktıktan sonra bu mikroorganizmalarda yeniden kolonizasyon meydana geldiği belirtilmiştir. Bu, durum donör mikrobiyotasının eksik transferi ile birleştiğinde hibritleşmiş bir mikrobiyota ile sonuçlandığı ifade edilmiştir (Ericsson ve ark. 2017). Ayrıca uygulamanın zorluğu ve yoğun antibiyotik kullanımının neden olduğu olumsuzluklar bu tekniği sınırlandıran diğer faktörlerdir. Dolayısıyla mikrobiyota modifikasyonlarında henüz ideal bir yöntem ortaya konulamamış olup çevre dostu, pratik ve etkin uygulamalara ihtiyaç bulunmaktadır. Son yıllarda bu kapsamda artan oranda çalışmalar yapılmaya başlanmış olup konak-mikrobiyota etkileşimlerinin hem deney hayvanlarının tekrar üretilebilirliğini ve yapılan deneylerin sonuçlarının doğruluğunu etkilediği düşünülmektedir. Sindirim sistemindeki mikrobiyal ortamın doğuştan itibaren süre gelen ve adaptif bağışıklık sistemini etkileyen ve mikrobiyomun araştırılmadığı hastalık modellerinde bile bu tür araştırmaların sonuçlarını belirlediğini göstermektedir (Franklin ve Ericsson 2020).

Sonuç olarak bu çalışmada probiyotik mikroorganizmalar içeren sıvı bir yem hazırlanmış ve deney hayvanlarına 10 gün süreyle verilmiştir. Mikrobiyota analizleri neticesinde probiyotik uygulamasının hem fare hem de ratlarda önemli değişikliklere neden olduğu gözlenmiştir. Günümüze kadar yapılan literatür incelemelerinde Türkiye'de bu kapsamda bir çalışma olmadığı belirlenmiştir. Bu bağlamda deneylere başlamadan önce hayvanların çalışma kapsamında üretilen probiyotik içerikli yemler ile beslenmesi mikrobiyotanın geliştirilmesi açısından umut vaat etmektedir.

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Özgün Araştırma
Original Article

Adıyaman ilinde satışa sunulan çiğ sütlerde Aflatoksin M_1 varlığının araştırılması ve potansiyel risk değerlendirmesi

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Özet: Bu araştırmanın amacı, Adıyaman ilinde Eylül 2021-Nisan 2022 tarihlerinde arasında satışa sunulan çiğ inek süt örneklerinde, Aflatoksin M_1 (AFM_1) varlığıyla miktarlarının belirlenmesidir. ELISA yöntemiyle çiğ inek sütünde (96 adet) AFM_1 miktarları belirlenerek tehlike indeksi (Hazard Index, HI) ve günlük alım miktarı (Estimated daily intake, EDI) hesaplanarak potansiyel risk değerlendirmesi yapılmıştır. Çalışma sonucunda 96 adet çiğ sütün %55,21 negatif (53 örnek), %44,79 pozitif (43 örnek), ortalama 0,028±0,026 µg/kg ve en fazla 0,054 µg/kg AFM_1 bulunmuştur. En çok sonbahar aylarında (n=24) toksin varlığı tespit edilmiştir. Sokak satıcılarından alınan örneklerin tamamı pozitif sonuç vermiştir. Pozitif numunelerden ortalama AFM_1 ile yapılan günlük alım miktarıyla yapılan hesaplamada HI düzeyi 1 olarak belirlenmiştir. Türk Gıda Kodeksinin maksimum yasal sınırlarını aşan 4 örnekten 2 tane örneğin sokakta satışı yapılan süt olduğu ve diğer 2 örneğin ise Besni ilçesinde bulunan entansif bir çiftliğe ait olduğu tespit edilmiştir. Çalışmada 4 tane örneğin TGK yasal limitinin üzerinde olduğu ancak ortalama olarak AFM_1 miktarının HI düzeyi potansiyel risk oluşturacak düzeyde bulunmamıştır. Araştırmamız sonucunda halkın çiğ inek sütü ile Aflatoksin M_1'e maruz kaldığı tespit edilmiştir. Özellikle yetiştiricilerin aflatoksin konusunda bilinçlendirilmesi ile denetimlerin artırılması toplum sağlığının korunmasında önem arz etmektedir.

Anahtar kelimeler: Adıyaman; Aflatoksin M_1; Çiğ süt; ELISA

Investigation of Aflatoxin M_1 presence in raw milk sold in Adıyaman province and potential risk assessment

Abstract: This study aimed to determine the presence and quantities of Aflatoxin M1 (AFM_1) in raw cow milk samples sold in the market between September 2021 and April 2022 in Adıyaman province. Hazard Index (HI) and estimated daily intake (EDI) were calculated by determining the amount of AFM_1 in raw cow milk (96 samples) by ELISA method and potential risk assessment was performed. As a result of the study, 55.21% negative (53 samples), 44.79% positive (43 samples), 0.028±0.026 µg/kg, and a maximum of 0.054 µg/kg AFM_1 was found in 96 raw milk samples. The presence of toxin was detected mostly in the autumn months (n=24). All samples taken from street vendors were positive. The HI level was determined as 1 in the calculation based on the average daily intake of AFM_1 from positive samples. Of the 4 samples that exceeded the maximum legal limits of the Turkish Food Codex, it was determined that 2 samples were milk sold on the street and the other 2 samples belonged to an intensive farm in Besni district. In the study, 4 samples were found to be above the legal limit of the TGK, but on average, the HI level of AFM_1 was not found to be at a level that would pose a potential risk. As a result of our research, it was determined that the public was exposed to Aflatoxin M_1 in raw cow's milk. Especially raising awareness of breeders about aflatoxin and increasing inspections are important for the protection of public health.

Keywords: Adıyaman; Aflatoksin M_1; ELISA; Raw milk

Giriş

Süt ve süt ürünleri, insan yaşamının çeşitli dönemlerinde tüketilerek protein, yağ, karbonhidrat (laktoz), mineral (kalsiyum, fosfor, potasyum, demir, bakır) ve vitamin (A, B1, B2, B6, B12, C ve biotin) içermesi bakımından halk sağlığı açısından önemli bir besindir. Süt ve süt ürünlerinin üretimi sırasında çeşitli kontaminasyonlarla pestisit, ağır metal, ilaç kalıntıları, çevre kirleticileri ve aflatoksinler gibi kanserojen metabolitlerle kirlenebilmektedir (Kubicová ve ark. 2019; Pardakhti ve Maleki 2019). Aflatoksinler insan ve hayvanlara gıdalarla bulaşmaktadır. Bulaşma gıda maddesinde toksin varlığıyla doğrudan; gıda üretiminde kullanılan yardımcı veya hammaddelere toksin bulaşmasıyla dolaylı kontaminasyon ve taşınma kontaminasyonu olmak üzere 3 farklı yolla meydana gelmektedir. Aflatoksin B_1 bulaşmış yemlerin süt ineklerinde Aflatoksin M_1 (AFM_1) dönüşmesiyle sütte bulunan aflatoksin kalıntıları insan ve hayvanlarda fizyolojik değişikliklere, sütün kalite ve kantitesinin bozulmasına neden olmaktadır (Karaoğlan ve ark. 2022). Özellikle inek sütünde AFM_1 plasental maru-

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ziyet yoluyla anne rahminde başlayarak yaşam boyu maruziyeti başta çocuklar olmak üzere insan sağlığı için küresel bir gıda güvenliği sorunu haline gelmektedir (Min ve ark. 2020). Süt ve süt ürünlerinin AFM_1 ile kontaminasyonunun mevsime, ülkeye ve coğrafyaya göre değişiklik gösterdiği ifade edilmiştir (Çelik ve ark. 2005; Karaoğlan ve ark. 2022). AFM_1 kanserojenik, mutajenik, teratojenik, genotoksik ve bağışıklık sistemini baskılayıcı etkileri bulunmaktadır (Min ve ark. 2020). Uluslararası Kanser Araştırma Kuruluşu (IARC) tarafından insan için olası kanserojen B2 grubunda kategorize edilen AFM_1, karaciğer, böbrek ve sindirim sorunlarına sebep olmaktadır (Pardakhti ve Maleki 2019). Aflatoksinler maruziyet miktarına ve süresine bağlı olarak hepatotoksik ve kanserojen etkilere neden olmaktadır (Lewis ve ark. 2005). Akut aflatoksikoz durumunda başlıca karaciğer olmak üzere böbrek, kalp, akciğer ve beyinde yüksek miktarda aflatoksin tespit edilmiştir (Alamu ve ark. 2018). İnsan ve hayvanlarda aflatoksin toksisitesi prognoza, doza, süreye, yaşa, cinsiyete, beslenme ve bağışıklık durumuna göre değişkenlik göstermektedir. Akut maruziyet, bulantı, kusma ve karın ağrısına; yüksek dozda akut zehirlenme çocuklarda ölümcül olabilmektedir. Kronik aflatoksin maruziyeti mutajenite ve kanserojeniteye neden olmaktadır (Sarma ve ark. 2017).

Günümüzde özellikle süt ve süt ürünlerinde tarama amaçlı mikotoksin türlerinin tespitinde AFM_1 tespitinde ELISA, hızlı, güvenilir, ekonomik, tekrarlanma, hazırlama prosedürü ve küçük numune hacimlerinde analize imkân vermesi ile çeşitli analiz yöntemleri (yüksek performans likit kromatografisi (HPLC), floresans tespiti (FL), ince tabaka kromatografisi (TLC) ve kütle spektrometrisi (MS)) arasından hassasiyet ve özgüllüğü nedeniyle yaygın tercih edilen bir yöntemdir (Maggira ve ark. 2021).

Bu çalışma, Adıyaman ili ve ilçelerinden toplanan inek sütü örneklerinde AFM_1 belirlenerek halk sağlığı açısından risk bulunup bulunmadığının belirlenmesi amacıyla yapılmıştır.

Gereç ve Yöntem

Numunelerin Toplanması

Çalışmada AFM_1 analizi için numuneler Eylül 2021-Nisan 2022 tarihleri arasında sonbahar, kış ve ilkbahar mevsimlerinde her mevsim 32 örnek olacak şekilde Adıyaman il ve ilçe merkezlerinden (seyyar satıcılardan ve çiğ süt satışı yapan 9 bölgede) satışa sunulan 96 adet numuneden 30 ml çiğ inek sütü toplanmıştır. Süt numuneleri -20°C'de dondurulmuş ve soğuk zincirle taşınmıştır. Dondurulmuş sütler analiz süresine kadar -20 °C'de saklanmıştır.

Numunelerin Analize Hazırlanması

Süt örneklerinin yağlarının ayırılması için 10°C'de 3500 rpm hızda 10 dakika santrifüj gerçekleştirilmiştir. Üst yağ tabakasının alınmamasına dikkat edilerek, seyreltme yapılmadan ve numuneler doğrudan analize alınmıştır.

Numunelerin Analizi

Aflatoksin seviyelerinin değerlendirilmesi amacı ile spesifik ticari kitleri kullanılmıştır (Veratox for Aflatoxin M1, 8019, Neogen Co., USA). Örnekler firmanın tavsiye ettiği Elisa yöntemi protokolüne uygun olarak belirlenmiştir. Örnekler için 6 adet standart ve 1 adet karıştırma kuyucuğu mikro plaka tutucuya konulmuştur. Her bir örnek 250 µl alınarak karıştırma kuyucuklarına eklenmiştir. Karıştırma kuyucuklarından 100 µl antikor kaplı kuyucuklara aktarılmıştır. 96 kuyucuklu mikroplaka 20 dakika oda sıcaklığında otomatik çalkalayıcıda (600 d/d) bekletilmiştir. Antikor kaplı kuyucuklardan sıvılar boşaltılarak, 5 defa yıkama solüsyonu (PBS with Tween™ 20, Canvax Reagents, S.L.U, BR0005, İspanya) ile yıkanmıştır. Her örnek için kuyucuklara 100 µl konjugat eklenerek 10 dakika oda sıcaklığında otomatik çalkalayıcıda (600 d/d) bekletilmiştir. Antikor kaplı kuyucuklardan sıvılar boşaltılarak, 5 defa yıkama solüsyonu ile yıkanmıştır. Her örnek için kuyucuklara 100 µl substrat eklenmiştir. 15 dakika oda sıcaklığında otomatik çalkalayıcıda (600 d/d) bekletilmiştir. Her örnek için kuyucuklara 100 µl stop solüsyonu eklenerek spektrofotometrik cihazda (Thermo Fisher Scientific, Multiskan Go, 1510-05762-51119200, Japan) 650 nm dalga boyunda okutulmuştur. Analizde 6 adet standart (0, 0,005, 0,015, 0,03, 0,06, 0,1 µg/kg) kullanılmıştır. Standartların absorbans değerleri sırasıyla (1,605, 1,496, 1,208, 0,775, 0,466, 0,294 µg/kg) belirlenmiştir. Sonuçlar Excel 2010 analizleri gerçekleştirilerek yapılmıştır.

Sağlık Risk Değerlendirmesi

Çevre kirleticilerin gıdalar üzerinde sadece kontaminasyon miktarlarının belirlenmesinden ziyade, halk sağlığında oluşturabileceği potansiyel risklerin değerlendirilmesi önem arz etmektedir (Yipel ve Yarsan 2021; Tutun ve ark. 2022). İnsan sağlığında risk değerlendirmesi gıda tüketimi yoluyla maruz kalınan ksenobiyotiklerin belli bir zamanda tüketilen miktarının belirlenmesiyle yapılmaktadır. Tehlike indeksi (Hazard Index, HI) ve günlük alım miktarı (Estimated daily intake, EDI) ile süt tüketimi şeklinde aşa-

ğıdaki verilen denkleme göre insan sağlığı üzerinde potansiyel riski hesaplanmıştır (Tsakiris ve ark. 2013; Milićević ve ark. 2017). EDI ng/kg olarak ifade edilmektedir. AFM_1, tolere edilebilir günlük alım miktarı (TDI) için 0,2 ng/ kg olarak verilmiştir. Yetişkin için günlük süt tüketimi yaklaşık olarak 500 ml/gün ve ortalama vücut ağırlığı 70 kg olarak belirlenmiştir (Kuiper-Goodman 1990; Nejad ve ark. 2019).

Denklemler:

$$\text{Günlük Alım Miktarı (EDI)} = \frac{\text{Ortalama AFM1 X Günlük Tüketim}}{\text{Ortalama vücut ağırlığı}} \text{ X 1000}$$

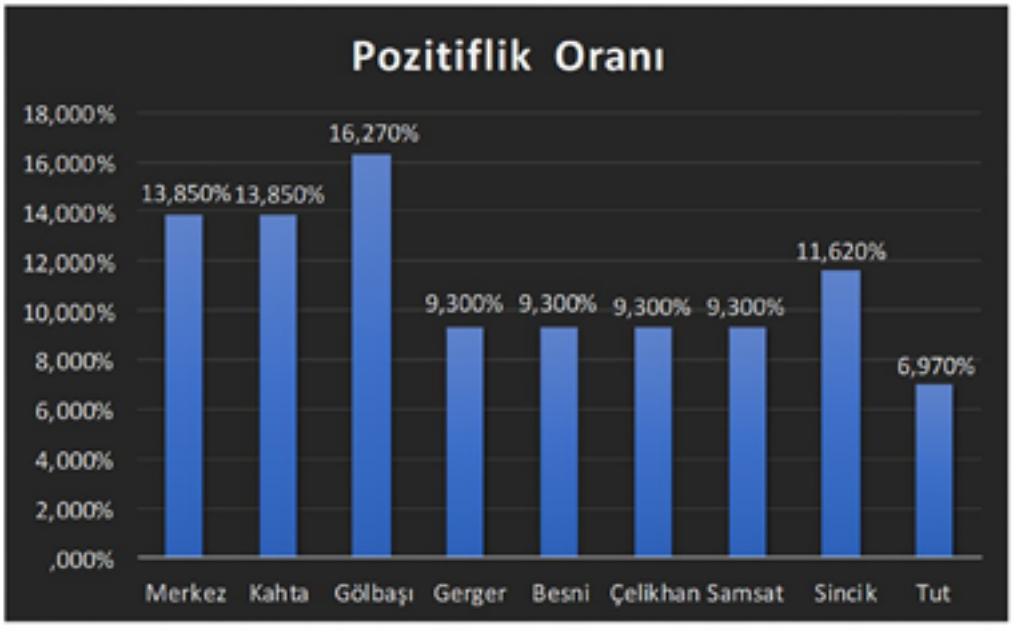
$$\text{Tehlike indeksi (HI)} = \frac{\text{Günlük alım miktarı (EDI)}}{\text{Günlük Tolerans düzeyi}}$$

Bulgular

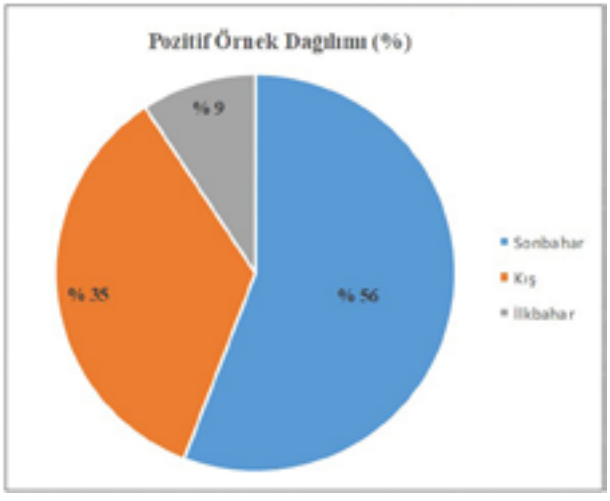
Çalışmada Adıyaman ilinde satışa sunulan çiğ sütlerde AFM_1 miktarları (tespit limiti (LOD) 0.0043 µg/kg olarak) belirtilmiştir. Veriler genel olarak değerlendirildiğinde 96 örnekten 43'ünün (%44,79) değişen miktarlarda AFM_1 içerdiği tespit edilmiştir. Analizi yapılan süt örnekleri içerdiği toksin yönünden dağılımı ise Tablo 1 'te verilmiştir. Türk Gıda Kodeksi (TGK) ve Avrupa Birliği tarafından belirlenen yasal limitin 0,05 µg/kg olduğu göz önünde bulundurulduğunda, çiğ süt örneklerinden 4'ünün (%4,16) yasal limitin üzerinde olduğu tespit edilmiştir (Türk Gıda Kodeksi Yönetmeliği 2011; Maggira ve ark. 2021). Bu 4 örneğin 2'si Kahta bölgesinden alınan sokak sütlerini, 2 örnek ise Besni bölgesindeki işletmelerden alınan süt örnekleri oluşturmaktadır. Gölbaşı bölgesi 7 pozitif örnek ile yasal limiti geçmemesine rağmen en çok toksin varlığının olduğu bölge olarak belirlenmiştir. Bölgelere göre pozitif örneklerin dağılımı verilmiştir (Grafik 1). En çok pozitif örnek %56 sonbahar (24 örnek) ve en az ilkbahar mevsiminde %9 (4 örnek); %35 kışın (15 örnek) pozitif bulunmuştur (Grafik 2). Pozitif numunelerden ortalama AFM_1 ile yapılan günlük alım miktarıyla yapılan hesaplamada HI düzeyi 1 olarak belirlenmiştir.

Tablo 1. Örneklerdeki AFM1'in Miktar ve Yüzde Olarak Dağılımı (µg/kg)

Toplam örnek sayısı	Negatif örnek sayısı	0,001-0,020	0,021-0,040	0,041-0,050	>0,050	AFM_1 en fazla miktar	AFM_1 pozitiflik (%)	Pozitif ortalama AFM_1 miktarı
96	53	15	12	12	4	0,0546	%44,79	0,028



Grafik 1. Bölgelere Göre Pozitif Örneklerin Dağılımı



Grafik 2. Mevsimlere Göre Pozitif Örneklerin Dağılımı

Tartışma

Aflatoksin maruziyetine ilişkin verileri takip etmek ve uzun süreli maruziyetiyle insan sağlığı üzerindeki etkilerini kontrol etmek için çiğ sütte AFM_1 insidansının araştırılması gerekmektedir (Min ve ark. 2020). Adıyaman ilinde 2019 verilerine göre inek sütü üretimi (185.918 kg) diğer süt türlerinin üretimine göre daha fazla olduğundan bu çalışmada inek sütü kullanılmıştır (Ulusal Süt Konseyi 2022).

96 adet çiğ inek sütünde 4 örneğin TGK yasal sınırının üstünde olduğu; pozitif %44,79 (43 örnek) ve negatif %55,21 (53 örnek) tespit edilmiştir. Çalışmada süt örneğinde en fazla AFM_1 miktarı 0,0546 µg/kg şeklinde belirlenmiştir. Benzer araştırmalarda, İşleyici ve ark. (2015), Van'da 100 çiğ inek sütünde 85 pozitif örnekten (%85) 12 örneğin (%12) yasal limit değerlerini aştığını tespit etmişlerdir. Yurt ve Uluçay (2017), Iğdır'da 25 çiğ inek sütü örneğinde 0,46-0,018 µg/kg aralığında pozitif örnekler belirlemişlerdir. Hazer (2011), Ege bölgesinde 81 adet çiğ süt örneğin 20 örneğinin TGK yasal sınırını aştığı ve 61 örneğin değişen miktarlarda AFM_1 içerdiğini ifade etmiştir. Özsunar ve ark. (2007), 135 çiğ süt örneğinde yaptıkları çalışmada 76 pozitif örnek ve 1 örnekte ise TGK yasal sınırını geçtiğini raporlamıştır. Ertaş ve ark. (2011) Kayseri'de ELISA yöntemiyle yaptıkları çalışmada 50 çiğ süt örneğinin %86 oranında (43 pozitif örnek) AFM_1 tespit etmişlerdir ancak yasal sınırı aşmadığı belirtilmiştir. Karadal ve ark. (2018), 30 çiğ inek sütüyle Niğde'de yaptıkları çalışmada %10 (3 pozitif örnek) örneğin yasal limitleri aştığını bildirmişlerdir.

Dünyada yapılan çalışmalarda Sudan'da Elzupir ve Elhussein (2010), yılında yaptığı bir çalışmada 44 inek sütünün 42'sinde (%95,45) AFM_1 bulmuşlardır. El Marnissi ve ark. (2012), yaptıkları çalışmada Fas'ta 48 süt örneğinde 13 numunede AFM_1 tespit ederken 4 örneğin yasal sınırın üzerinde olduğunu tespit etmişlerdir. Lübnan'da El Khoury ve ark. (2011), 64 süt örneğiyle yaptıkları çalışmada %40,62 oranında AFM_1 içerdiğini ifade etmişlerdir. Tomasevic ve ark. (2015), Sırbistan'da 628 çiğ süt örneğiyle yaptıkları çalışmada %56,3 oranında yasal sınırın üzerinde AFM_1 tespit etmişlerdir. Picinin ve ark. (2013), farklı iklim koşullarının aflatoksin üzerine etkisini araştırdığı çalışmada 129 çiğ süt örneğinin tamamının toksin içerdiğini ve yağışlı bölgelerde yaz döneminde aflatoksin varlığının özellikle daha fazla olduğunu ifade etmiştir.

Mevcut çalışmada çiğ sütlerde yapılan AFM_1 analizi sonucunda 0,028±0,026 µg/kg bulunmuştur. Çalışmada sokak satıcılarından alınan sütlerin tamamında (n=18) aflatoksin tespit edilmiştir. Sokak satıcılarından alınan sütler toplam pozitif örnekler (n=43) arasında 18 pozitif örnek ile %41,86'yı oluşturmaktadır. TGK yasal limitini aşan 4 örneğin %50 oranında sokak satıcılarından alınan süt örneği oluşturmaktadır. Özellikle sokakta satılan sütlerin üretimi ve depolanması esnasında hijyen ve mikrobiyolojik yaklaşımların uygun yapılmamasıyla ilişkilendirilebilmektedir. Ayrıca sokak sütlerinin rastgele hanelerden toplanılmasıyla aflatoksin içeren ve içermeyen sütlerin birbirleriyle karışmasından kaynaklanmış olabileceği düşünülmektedir. Sokak sütü olarak bilinen sütler genelde kırsal kesimlerden toplanılan süt örneklerinden oluşmaktadır. Kırsal alanlarda hayvanlar otların bittiği ilkbahar ve yaz mevsimi dışında entansif olarak beslenmesi tercih edilmektedir (Doğan 2012).

Süt ve süt ürünlerinde aflatoksin varlığı rasyona, yem saklama koşullarına, mevsime, besleme yöntemine ve hayvanların sağlık durumu gibi faktörlere bağlı olarak değişkenlik göstermektedir (Karaoğlan ve ark. 2022). TGK'nın yasal limitini aşan diğer iki örneğin ise yüksek oranda küf içeren hayvan yemi rasyonlarından kaynaklanmış olabileceği düşünülmektedir. Adıyaman ili ve ilçelerinde il merkezinde 84.438, Çelikhan'da 105.014, Sincik'te 67.738, Gerger'de 58.658, Besni'de 38.326, Kahta'da 34.942, Samsat'ta 21.450, Tut'ta 12.757 ve Gölbaşı'nda 1.332 hektar mera alanı bulunmaktadır (Adıyaman Gıda, Tarım ve Hayvancılık İl Müdürlüğü 2022). Besni mera bakımından son sıralarda yer almaktadır bu durum entansif beslenmeye uygun olmayan yem muhafaza koşullarından dolayı kontaminasyon riskini artırabilmektedir.

7 pozitif örnek ile Gölbaşı ilçesinin en çok toksin içeren örneklere sahip olması mera alanlarının çok kısıtlı olmasından kaynaklanmış olabileceği düşünülmektedir. Entansif olarak yapılan yetiştiricilikte yem rasyonlarının iyi muhafaza edilmemesi durumunda aflatoksin kontaminasyonu meydana gelebilmektedir. Bölgeler arasındaki mera alanları dışında mevsimsel farklar da küf üremesini etkileyebilmektedir. Küflerin üreme koşullarının sıcaklık ve nem ile doğru orantılı olduğu bilinmektedir. Mevsimsel olarak en çok pozitif örnek (n=24) sonbahar Eylül-Ekim-Kasım aylarında belirlenmiştir (Grafik 2). Bu aylarda meradan ahırda beslemeye geçiş daha yoğun meydana gelmektedir. Taze otların bittiği mevsim koşullarının entansif beslemeye daha uygun olduğu bu dönemlerde yem depolarında küflenme riski de bulunmaktadır. Küflerin üreme koşullarının bu durumun nedeni olduğu düşünülmektedir. Aflatoksijenik küfler minimum 6-8 °C' maksimum 50-60 °C'de üreyebilmelerine rağmen toksin oluşumu için en az 10-13 °C ve en fazla 42 °C sıcaklık uygundur. Bunların uygun gelişme sıcaklıkları 35-38 °C olmasına rağmen maksimum toksin konsantrasyonuna 25-30°C de ulaşılmaktadır (Halkman 2022). Aflatoksin konsantrasyonları kış aylarında yaz aylarına kıyasla daha yüksek görülebilir (Fallah 2010). Kışın nem seviyeleri daha yüksek olmasına rağmen sıcaklık küf oluşumu için yeterli değildir bu bağlamda sonuçları eylül, ekim ve kasım aylarının hem sıcaklık hem de nem açısından aflatoksin üreten küfler için daha uygun bir ortam sağlamasıyla açıklamak mümkündür. Sütteki AFM_1 düzeyi coğrafi koşullara ve mevsime göre değişiklik gösterebilmektedir. Ayrıca Mart-Nisan aylarında mevsime bağlı otların gelişmesiyle hayvanların depolanmış yemler yerine mera-çayır gibi yerlerde otlatıldığı göz önünde bulundurarak durum açıklanabilir. En yüksek aflatoksin konsantrasyonu kış ayında olması bu durum, bu mevsimde daha düşük süt üretiminin daha yüksek aflatoksin konsantrasyonlarına yol açmasına ve depolanan yemin küfle kirlenmesiyle açıklanabilir. Yaz mevsiminde üretilen sütün kış mevsiminde üretilen sütten daha az aflatoksin içerdiği açıklanmıştır (Filazi ve ark. 2010). Hindistan'da 2011 yılında 76 adet inek sütü örneklerinde AFM_1 varlığı üzerine yapılan bir çalışmada kış mevsiminde alınan örneklerin daha fazla aflatoksin içerdiğini bildirmişlerdir (Karaoğlan ve ark. 2022).

Aflatoksin ile küresel kalkınma arasında negatif bir korelasyon olduğu düşünülmektedir. Üretici ve tüketici bilinci, hijyen düzenlemelerine bağlılık ve bunlara uymayan işletmelere yönelik yaptırımlar gibi faktörler AFM_1 varlığını azaltmaktadır (Karaoğlan ve ark. 2022). Sosyoekonomik durum toplumun her alanını etkilediği özellikle bu durum insanların davranışları ve maddi durumları ile açıklanabilir. Tablo 2'te görüldüğü gibi dünyada ve ülkemizde AFM_1 varlığı göz önünde bulundurularak toplumun AFM_1 yönünden risk altında olduğu görülmektedir.

Tablo 2. Ülkemizde ve farklı ülkelerde AFM1 ile ilgili yapılan bazı çalışmalar

Örnek	Örnek sayısı	Pozitif örnek sayısı	Tespit aralığı (µg/kg)	Ülke	Kaynak
Çiğ Süt	20	20	0,005-0,250	Türkiye	(Kireçci ve ark. 2007)
Süt	77	18	0,010-0,030	Arjantin	(Lopez ve ark. 2003)
Pastörize Süt	91	66	0,013-0,250	İran	(Fallah 2010)
Peynir	50	14	0,002-0,020	Türkiye	(Filazi ve ark. 2010)
Bebek Maması	62	5	0,005-0,018	Türkiye	(Kabak 2012)
Süt	38	26	0,021-0,418	Yemen	(Murshed 2020)
Beyaz Peynir	116	68	0,052-0,785	İran	(Fallah ve ark. 2009)
Süt	613	496	0,002-0,020	Kenya	(Kang'ethe ve Lang'a 2009)
Peynir	400	327	0,05-0,25	Türkiye	(Sarımehmetoğlu ve ark. 2004)
Pastörize Süt	85	75	0,001-0,090	Türkiye	(Çelik ve ark. 2005)
Pastörize Süt	16	12	0,01-0,05	Çin	(Guo ve ark. 2019)
Çiğ süt	50	43	0,001-0.03	Türkiye	(Ertas ve ark. 2011)
Çiğ süt	120	107	0,005-0,078	Türkiye	(Eker ve ark. 2019)
UHT Süt	78	24	0,004-0,127	Türkiye	(Köse ve ark. 2019)
Çiğ süt	52	52	0.029-2.159	Etiyopya	(Tadesse ve ark. 2020)

Ülkemizde yapılan araştırmalara göre süt ve süt ürünleri farklı konsantrasyonlarda AFM_1 içermektedir, hatta TGK yasal limitini bile aşmaktadır. Süte toksin bulaşmasıyla o sütten elde edilen süt ürünlerine de toksinler geçmektedir (Sharafi ve ark. 2022). Aflatoksin oranı yüksek olan sütlerin, özellikle de kırsal kesimden toplanan sütlerin insan sağlığına özellikle de çocuklar üzerinde uzun vadede ciddi etkileri olabilmektedir. Türkiye'de süt ve süt ürünlerinde AFM_1 varlığına ilişkin çalışmalar incelendiğinde AFM_1 düzeylerinin bölgeden bağımsız olarak farklılık gösterdiği görülmektedir. Ayrıca aynı ilde yapılan farklı çalışmalarda bile AFM_1 düzeyleri farklılık göstermektedir. Bu farklılık süt ürünlerinin türüne, yemlerdeki küf kontaminasyonuna, mevsime, numune alma noktalarında hijyen ve sanitasyon kurallarına verilen önem düzeyine bağlı olarak değişkenlik göstermektedir (Karaoğlan ve ark. 2022).

Potansiyel risk değerlendirmesinde tehlike indeksinin HI>1 tüketiciler için riskli olduğu ifade edilmiştir (Milićević ve ark. 2017). Pozitif numunelerden ortalama AFM_1 ile yapılan günlük alım miktarıyla yapılan hesaplamada HI düzeyi 1 olarak belirlenmiştir. Tsakiris ve ark. (2013), yaptıkları çalışmada AFM_1 ile risk değerlendirilmesinde en yüksek değerin 1-3 yaş aralığında olduğunu ifade etmişlerdir. Sharafi ve ark. (2022), yaptıkları çalışmada HI indeksini Türkiye için ortalama olarak 0,23 olarak belirlemişlerdir. Çalışmada değerlerin örneklerin konum ve büyüklüğü, süt veya süt ürünleri türü ve ölçüm tekniklerinin farklılığından kaynaklanmış olabileceği düşünülmektedir (Sharafi ve ark. 2022). Mevcut çalışmada da Adıyaman ilinde ortalama olarak AFM1 miktarının HI düzeyi potansiyel risk oluşturacak düzeyde bulunmamıştır.

Sonuç

Çalışmamızda 96 adet çiğ inek süt örneğinin 4 tanesi TGK yasal limitinin üzerinde olduğu belirlenmiştir. Çalışmada 4 tanesi örneğin TGK yasal limitinin üzerinde olduğu ancak ortalama olarak AFM_1 miktarının HI düzeyi potansiyel risk oluşturacak düzeyde bulunmamıştır. Ülkemizde yapılan çalışmalar incelendiğinde AFM_1 varlığının yaygın görüldüğü anlaşılmaktadır. IARC tarafından Grup II kanserojen madde olarak tanımlanan AFM1 Isıl işlem uygulamalarıyla yok olmadığından çiğ süt ve ürünlerindeki miktarının belirlenmesi büyük öneme sahiptir. Gıda ve yemlerdeki mikotoksin kontaminasyonunu azaltmak amacıyla Dünya Sağlık Örgütü (WHO), Avrupa Gıda Güvenliği Otoritesi (EFSA), ABD Gıda ve İlaç Dairesi (FDA) ve Gıda Tarım Örgütü (FAO) gibi birçok ulusal ve uluslararası organizasyon ve örgüt düzenleyici yönergelerle bu küresel sorunu çözmeyi amaçlamaktadırlar. Ülkemizde TGK sütlerde AFM_1 için yasal sınırları belirlemiş olmasına rağmen açıkta veya sokakta satışa sunulan sütlere herhangi bir analiz uygulanmaması halk sağlığının korunması konusunda ciddi tehdit oluşturmaktadır. Çalışmamızda bulunan veriler örneklerin neredeyse yarısının toksinle kontamine olduğunu göstermektedir. Özellikle bağışıklık sistemi zayıf kişiler ve çocuklarda AFM_1 maruziyeti önem arz etmektedir. Aflatoksinlerin önlenmesi için yemlerin kontaminasyonu engellenmesi, halk ve üreticiler aflatoksinlerin zararları hakkında bilgilendirilmesi gerekmektedir. Piyasaya sürülen sütlerle beraber özellikle sokak satıcıları tarafından satılan süt örneklerinin de belirli periyodlarda analize tabi tutulması ve kontamine olmuş sütlerin tüketime sunulmaması halk sağlığı açısından önem arz etmektedir.

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Özgün Araştırma
Original Article

Laktik asit bakterilerinden elde edilen konsantre postbiyotiklerin bazı gıda patojenleri üzerine etkilerinin değerlendirilmesi

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Özet: Çalışmada dokuz farklı laktik asit bakterisi MRS Broth'da 24, 48 ve 96 saat inkübe edildikten sonra postbiyotikleri elde edildi. Elde edilen postbiyotiklerin (*1x*) suyu evaporasyonla uçurularak iki (*2x*) ve dört (*4x*) kat yoğunlaştırıldı. Farklı inkübasyon sürelerinde elde edilen ve farklı yoğunluktaki postbiyotiklerin pH değerleri, titre edilebilir organik asit miktarları ve *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157, metisilin dirençli *Stapylococcus aureus* ve *Brucellla melitensis* üzerine antimikrobiyal etkileri agar difüzyon yöntemi ile inhibisyon zon çapları ölçülerek ortaya konuldu. Çalışmada laktik asit bakterilerinin inkübasyon sürelerindeki artışın postbiyotiklerindeki pH değerleri, titre edilebilir asit miktarları ve patojenler üzerine antimikrobiyal etkide önemli bir değişikliğe neden olmadığı görüldü ($p>0.05$). Postbiyotikler *2x* ve *4x* yoğunlaştırıldıklarında, içerdikleri organik asit miktarları artmasına ve patojenler üzerine daha güçlü bir antimikrobiyal etki göstermelerine rağmen ($p<0.05$), pH değerlerinde önemli bir değişiklik görülmedi ($p>0.05$). Postbiyotiklerin antimikrobiyal etkilerini içerdikleri organik asitler ile meydana getirdikleri, organik asitlerin ise NaOH ile nötralize edildiklerinde antimikrobiyal etkilerinin kaybolduğu tespit edildi. En yüksek titre edilebilir asit miktarları ve patojenlere karşı en güçlü antimikrobiyal etkiler ise *Lactobacillus plantarum*, *L. sakei* ve *L. curvatus*'dan elde edilen postbiyotiklerde görüldü ($p<0.05$).

Anahtar kelimeler: Antimikrobiyal etki, gıda patojenleri, postbiyotik

Evaluation of the effects of concentrated postbiotics from lactic acid bacteria on some food pathogens

Abstract: In the study, nine different lactic acid bacteria were incubated in MRS Broth for 24, 48 and 96 hours and their postbiotics were obtained. The water of the obtained postbiotics (*x*) was evaporated and concentrated two (2x) and four (4x) times. The pH values and titratable organic acid amounts of postbiotics obtained at different incubation times and at different concentrations were determined, and their antimicrobial effects on *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157, methicillin resistant *Stapylococcus aureus* and *Brucellla melitensis* were determined by measuring the inhibition zone diameters with agar diffusion method. In the study, it was detected that the increase in the incubation period of lactic acid bacteria did not cause a significant change in the pH values, titratable acid content and antimicrobial effect on pathogens ($p>0.05$). When postbiotics were concentrated *2x* and *4x*, the amount of organic acids they contained increased ($p<0.05$) and they showed a stronger antimicrobial effect on pathogens ($p<0.05$), but there was no significant change in their pH values ($p>0.05$). It was determined that the postbiotics showed their antimicrobial effects through the organic acids which they produced, and when the organic acids were neutralized with NaOH, their antimicrobial effects disappeared. The highest titratable acid amounts and the strongest antimicrobial effects against pathogens were observed in postbiotics obtained from *L. plantarum*, *L. sakei* and *L. curvatus* ($p<0.05$).

Keywords: Antimicrobial effect, food pathogens, postbiotic

Giriş

İnsanoğlu eski çağlardan beri laktik asit bakterilerini (LAB) gıdaların raf ömrünü uzatmak ve değişik lezzetler elde etmek için fermantasyon amacıyla kullanmasına rağmen, ancak 1900'lu yılların başında bu bakterilerin sağlık üzerine olumlu etkilerinin olduğunun farkına varmıştır. Son yıllarda yapılan çalışmalar bakteriyel metabolizma sonucu üretilen ürünlerin de biyolojik aktiviteye sahip olduklarını ortaya koymuş ve bu ürünlere yeni bir isimlendirmeyle "postbiyotikler" adı verilmiştir. Postbiyotikler [veya diğer isimleriyle metabiyotikler, biyojenikler, probiyotik hücre parçaları, cell free supernatants (CFS)], fermentasyon sırasında LAB'lar tarafından üretilen biyoaktif çözünür ürünler veya metabolik son ürünler olarak tanımlanmaktadır (Barros ve ark. 2020; Aghebati-Maleki ve ark. 2022). Postbiyotik terimi günümüze kadar en sık kullanılan terim olmasına

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rağmen hala evrensel olarak kabul edilmiş tam bir tanımı yoktur (Thorakkattu ve ark. 2022).

Postbiyotik terimi son yıllarda sık kullanılsa da, aslında bazı fermente gıdaların (yoğurt, turşu, kombucha çayı, kefir vs.) doğal olarak postbiyotik içerdikleri ve insanların tarih boyunca bu gıdaları tükettikleri ve fayda sağladıkları unutulmamalıdır. Postbiyotiklerin tespit edilmiş bir toksisitelerinin olmaması, sindirim sistemi enzimlerine dirençli olmaları, uzun raf ömrü, immun sistem düzenleyici, antienflamatuvar, antihipertansif, antioksidan, antimikrobiyal, antikanser ve hipokolesterolemik aktivitelere sahip olmaları gibi avantajları bulunmaktadır (Aguilar-Toalá ve ark. 2018; Wegh ve ark. 2019; Gökırmaklı ve ark. 2021; Rad ve ark. 2021). Ayrıca postbiyotiklerin hazırlanması ve kullanılmasının basit ve uygun olması, geniş pH ve sıcaklık aralıklarında stabil olmaları, kendilerine özgü kimyasal yapılarının ortaya konulabilmesi, mikroorganizma sayısında artışa sebep olmamaları, immun sistemi baskılanmış bireylerde yan etkilere sahip olmaması ve postbiyotik türlerine göre muhafazaları esnasında soğuk zincire gerek duyulmaması da avantajdır (Barros ve ark. 2020; Aghebati-Maleki ve ark. 2022; Sabahi ve ark. 2022).

Son zamanlarda gıdalarda antimikrobiyal olarak kimyasal bileşiklerden ziyade doğal bileşiklerin kullanılmasına yönelik tüketici talepleri öne çıkmaktadır (Balthazar ve ark. 2021; Salantá ve Cropotova 2022). Postbiyotiklerin faydalı etkilerinden biri de bazı mikroorganizmalar üzerine inhibisyon etkilerinin olmasıdır. Kimyasal koruyuculara ve antibiyotiklere göre avantajlara sahip olan postbiyotiklerin gıdalarda bozulma yapan mikroorganizmalara ve gıda kaynaklı patojenlere karşı kullanılması ile ilgili araştırmalar devam etmektedir (Yolmeh ve ark. 2017; Jo ve ark. 2020; İncili ve ark. 2021; 2022a; 2022b; 2023; Mani-López ve ark. 2022). Postbiyotiklerin antimikrobiyal etkileri genel olarak elde edildikleri LAB türlerine, kültür ortamına, gelişme şartlarına, hedef mikroorganizmanın tipine (gram pozitif bakteriler, gram negatiflere göre postbiyotiklere daha dirençlidir) ve postbiyotiğin konsantrasyonuna bağlı olarak değişiklik gösterir (Özçelik ve ark. 2016; Moradi ve ark. 2020; Sabahi ve ark. 2022). Postbiyotiklerin sahip oldukları antimikrobiyal etkinin içerdikleri organik asitler, bakteriyosinler ve bakteriyosin benzeri inhibitör bileşiklerden ileri geldiği, hidrojen peroksit, diasetil, kısa ve uzun zincirli yağ asitleri ve etanolün de antimikrobiyal etkiye katkıda bulunduğu bildirilmektedir (Mani-López ve ark. 2022).

LAB'lardan elde edilen postbiyotiklerin toplam organik asit miktarı ve pH değerlerinin bilinmesi, gıda patojenlerine karşı antimikrobiyal etkilerinin ortaya konulması gıda endüstrisi açısından büyük öneme sahiptir. Gıda güvenliği ve kalitesi açısından uygun LAB'ların seçiminde de bu verilerin önem arz edeceği ileri sürülebilir. Postbiyotiğin içerisindeki bileşiklerin karakterizasyonları ve miktarlarının ortaya konulması için kromatografik yöntemler (HPLC, GC veya TLC) ya da kapiler elektroforez, spektrofotometrik gibi analizlere ihtiyaç duyulmaktadır (Barros ve ark. 2020; Moradi ve ark. 2021; Thorakkattu ve ark. 2022). LAB'lardan elde edilen postbiyotiklerin kimyasal içeriği ve antimikrobiyal etkileri ile ilgili bazı detaylı araştırmalar mevcut olsa da bu konuda yeterli sayıda veri bulunmadığı görülmektedir. İleri teknolojik cihazların kullanılması çoğu laboratuvar için gerçekten pahalı yöntemlerdir ve rutin olarak her elde edilen postbiyotiğe uygulanmaları mümkün değildir. Ayrıca, postbiyotiklerin gıda güvenliği ile ilgili mikroorganizmalara karşı kullanılması için öncelikle kaba kimyasal bileşimlerinin analiz edilmesi ve in vitro antagonistik testlerle gıda patojenlerine karşı etkinliklerinin ölçülmesi uygun olabilir. Bu yüzden, daha basit ve ucuz yöntemlerle postbiyotiklerin antibakteriyel özelliklerini ortaya koymak ve etkili oldukları tespit edilince detaylı analizleri uygulamak yerinde olacaktır. Bu çalışma, starter kültür amacıyla da yaygın olarak kullanılan bazı LAB'ların MRS Broth'da farklı inkübasyon sürelerinde (24, 48 ve 96 saat) ürettikleri postbiyotiklerin normal, iki kat ve dört kat yoğunlaştırılmış formlarının toplam titre edilebilir organik asit (laktik asit cinsinden) miktarını, pH değerini ve in vitro şartlarda bazı gıda patojenleri *(Salmonella* spp.*, Listeria monocytogenes, E. coli* O157, Metisilin dirençli *Staphylococcus aureus ve Brucella melitensis)* üzerine antimikrobiyal etkilerini ortaya koymak amacıyla yürütülmüştür.

Gereç ve Yöntem

Çalışmada kullanılan laktik asit ve patojen bakteri suşları

Çalışmada kullanılan LAB suşları, referans kodları ve fermantasyon tipleri Tablo 1'de verilmiştir. Chr. Hansen firması tarafından üretilen Bactoferm™ B-LC-78 ticari kültürü liyofilize halde *Pediococcus acidilactici* ve *Staphylococcus cornosus* suşları; Bactoferm™ B-FM ticari kültürü ise *Latilactobacillus sakei* ve *Staphylococcus xylosus* suşları içermektedir. Bu ticari kültürler ayrı ayrı De Man Rogosa and Sharpe Broth (MRS Broth) besiyerinde 30°C'de 20 saat inkübe (Memmert, Germany) edilerek aktive edildi. Aktivasyonun ardından MRS Agar'a çizim yapıldı ve gelişen kolonilerden gram boyama yapıldı. Kok ve basil

morfolojiye sahip olanlar *P. acidilactici* ve *L. sakei* olarak izole edilerek çalışmada kullanıldı.

Çalışmada, Besin Hijyeni ve Teknolojisi Laboratuvarı koleksiyonunda bulunan üç adet *Salmonella* spp. (*Salmonella* Enteritidis ATCC 13076, *Salmonella* Typhimurium ATTC 14028 ve NCTC 12416), üç adet *Listeria monocytogenes* (ATCC 7644, 13932 ve 19111), üç adet *Escherichia coli* O157:H7 (ATCC 43890, 43895 ve 35150), bir adet metisilin-dirençli *Staphylococcus aureus* (ATCC 33591) ve saha izolatı olan iki adet *Brucella melitensis* tip III suşu (Pendik Veteriner Kontrol Enstitüsü (İstanbul, Türkiye) ve Balıkesir Üniversitesi Veteriner Fakültesi Mikrobiyoloji anabilim dalından sağlanmıştır) patojen bakteri olarak kullanılmıştır.

Tablo 1: Çalışmada kullanılan laktik asit bakterileri

Laktik asit bakterisi	Kod	Glikoz fermentasyon tipi*
L. plantarum	Bioferm DSMZ 16627	Fakültatif heterofermentatif
L. curvatus	Bactoferm™ B-LC-48	Fakültatif heterofermentatif
L. sakei	Bactoferm™ B-FM	Fakültatif heterofermentatif
L. paracasei	ATCC 11974	Fakültatif heterofermentatif
L. rhamnosus	ATCC 7469	Fakültatif heterofermentatif
L. fermentum	ATCC 9368	Zorunlu heterofermentatif
L. reuteri	DSM 17938, Biogaia®	Zorunlu heterofermentatif
P. acidilactici	Bactoferm™ B-LC-78	Zorunlu homofermentatif
L. delbrueckii subsp. bulgaricus	ATCC 11842	Zorunlu homofermantatif

*: LAB'ın glikoz fermentasyon özellikleri De Angelis ve Gobbetti (2011) kaynağından alınmıştır.

Laktik asit bakteri postbiyotiklerinin eldesi ve evaporasyon ile konsantre edilmesi

Çalışmada kullanılan LAB'ların her biri MRS Broth besiyerinde 30 ˚C'de 24, 48 ve 96 saat geliştirildi. İnkübasyon sonunda besiyerleri 5000 rpm'de 10 dk santrifüj edilerek süpernatantları elde edildi. Elde edilen süpernatantların (postbiyotik ürünler) su kısmı evaporasyon (60 ˚C'de, 0.75 atm basınçta) ile %50 ve %75 oranında uçurularak *2x* ve *4x* yoğunlukta postbiyotik ürünler elde edildi. Yoğunlaştırılmamış (*x*), *2x* ve *4x* yoğunlukta elde edilen postbiyotikler 0.22 mikron gözenekli filtreden geçirilerek steril edildi (İncili ve ark. 2022a).

Postbiyotiklerin pH ve titre edilebilir asitlik değerlerinin ölçülmesi

Postbiyotiklerin pH değerleri dijital pH metre (HI 2211, Hanna Instruments, USA) kullanılarak ölçüldü. Postbiyotiklerin içerdikleri titre edilebilir asit miktarlarının ölçümü için, 5 ml postbiyotik içerisine 0.25 ml fenolftalein indikatörü ilave edildi ve 0.25 N NaOH (Merck, Emplura, Darmstadt, Germany) ile pembe renk oluşumu gözleninceye kadar titrasyon yapıldı. Titrasyon işleminde harcanan 0.25 N NaOH miktarı 20 ile çarpıldı, böylece 100 ml postbiyotik için harcanan NaOH miktarı tespit edildi. Elde edilen sonuç 0.0225 katsayısı ile çarpılarak çıkan sonuç % g laktik asit olarak kaydedildi (Serter ve ark. 2024).

Patojen bakterilerin hazırlanması ve Agar kuyucuk difüzyon testi

Her bir patojen bakteri suşu Tryptic Soy Broth (TSB) besiyerinde 35 ˚C'de 18-20 saat ayrı ayrı çoğaltıldı. İnkübasyon sonunda bakteri kültürü içeren tüpler soğutmalı santrifüjde 5000 rpm'de 5 dakika santrifüj edildi. Süpernatant uzaklaştırıldıktan sonra kalan bakteri peleti 10 ml steril maximum recovery diluent (MRD) içerisinde çözdürüldü ve tekrar santrifüj edilerek metabolit ürünlerden arındırıldı. Her bir bakteri peleti 10 ml steril MRD içerisinde çözdürüldükten sonra patojen süspansiyonları elde edildi. Üç farklı *Salmonella* suşundan elde edilen süspansiyonlardan 1'er ml alınarak ayrı bir steril tüpte birleştirildi ve tüp steril MRD ile 10 ml'ye tamamlanarak *Salmonella* spp. bakteri miksi hazırlandı. Aynı işlem *L. monocytogenes*, *E. coli* O157 ve *B. melitensis* bakterileri için de tekrarlandı. Hazırlanan bakteri solüsyonlarından 1 ml alındı ve ml'sinde yaklaşık 10^6 civarında bakteri olacak şekilde dilue edildi. Mililitresinde yaklaşık 10^6 bakteri içeren her bir solüsyondan 1 ml alınarak plaklara ilave edildi ve üzerine Muller Hinton Agar (MHA) eklendi. Besiyerinin katılaşmasından sonra agar üzerinde belirli yerlere steril pipetin küt ucu

yardımıyla 8 mm çapında kuyucuklar açıldı. Bu kuyucukların her birine daha önce hazırlanmış olan steril LAB postbiyotiklerinden 150 µl olacak şekilde ilave edildi ve plaklar 35 ˚C'de 24 saat inkübasyona bırakıldı. Kontol kuyucuğuna 150 µl steril distile su konuldu. İnkübasyon sonunda kuyucukların çevresinde oluşan inhibisyon zon çapları milimetre biriminden ölçülerek postbiyotiklerin antimikrobiyal etkinlikleri değerlendirildi (Serter ve ark. 2024).

Postbiyotiklerin antimikrobiyal etkilerinin içerdikleri organik asitlerden mi yoksa içerdikleri diğer bileşiklerden mi kaynaklandığını anlamak için postbiyotiklerin pH'ları 5 N NaOH solusyonu ile pH 6.0'a ayarlandı. pH değerleri 6.0'a ayarlanan postbiyotikler yukarıda anlatıldığı şekilde agar kuyucuk difüzyon yöntemiyle patojen bakterilere karşı denenerek inhibisyon zonu oluşturup oluşturmadıkları gözlendi (Serter ve ark. 2024).

İstatistiksel analiz

Çalışmanın tüm aşamaları üç tekrar olacak şekilde gerçekleştirildi. Her bir LAB postbiyotiğinin pH, titre edilebilir asit miktarları ve patojen bakterilere karşı agar kuyucukta oluşturdukları inhibisyon zon çapları *çalışma tekrarı* ^{x} *inkübasyon süresi* ^{x} *postbiyotik konsantrasyonu* olacak şekilde ana etkiler ve değişkenler arası interaksiyonlar yönünden varyans analizine (ANOVA) tabii tutuldu. İstatistiksel önem seviyesi $p<0.05$ olarak belirlendi.

Bulgular

MRS Broth'da farklı sürelerde (24, 48 ve 96 saat) inkübe edilen LAB'lardan elde edilen farklı konsantrasyonlardaki (*x*, *2x* ve *4x*) postbiyotiklerin pH değerleri, laktik asit cinsinden titre edilebilir asit miktarları ve gıda patojenleri üzerine agar kuyucuk yöntemi ile göstermiş oldukları inhibisyon zon çapları Tablo 2-4'de verilmiştir.

LAB postbiyotiklerinin pH değerleri incelendiğinde, bakterilerin inkübasyon süresinin 24 saatten 48 veya 96 saate kadar uzatılmasının postbiyotiklerin pH değerlerinde önemli bir değişiklik meydana getirmediği görüldü ($p>0.05$). Ayrıca, elde edilen postbiyotikler iki veya dört kat yoğunlaştırıldıklarında da pH değerlerinde önemli bir değişim gözlenmedi ($p>0.05$). Postbiyotiklerin pH değerleri bakteri türlerine göre değişiklik göstermesine rağmen birbirlerine yakın değerlerdeydi. Yoğunlaştırılmamış postbiyotikler arasında en düşük pH değerine 96 saat inkübasyona bırakılmış *L. curvatus* bakterisi (pH 3.69) sahipken (Tablo 2), *2x* ve *4x* postbiyotiklerde en düşük pH değerine 96 saat inkübasyona bırakılmış *L. sakei* bakterisinin (sırasıyla pH 3.64 ve 3.61) sahip olduğu görüldü (Tablo 2). Postbiyotikler arasında (*x*, 2x, 4x) en yüksek pH değerine ise inkübasyon süresinden bağımsız olarak *L. rhamnosus* bakterisinin sahip olduğu görüldü (sırasıyla pH 4.12, 4.08 ve 4.05) (Tablo 4).

LAB'ların inkübasyon süreleri 24 saatten 48 veya 96 saate uzatıldığında yoğunlaştırılmamış postbiyotiklerin titre edilebilir asit oranlarında önemli bir artış gözlenmedi ($p>0.05$). Ancak, *L. sakei* (Tablo 2) ve *L. reuteri 2x* postbiyotiklerinin inkübasyon süresinin artmasına paralel olarak içerdikleri titre edilebilir asit miktarlarının da önemli derecede artış olduğu tespit edildi ($p<0.05$) (Tablo 4). Benzer şekilde, *L. rhamnosus* ve *L. reuteri 4x* postbiyotiklerinde inkübasyon süreleri uzadıkça titre edilebilir asit miktarında önemli artış gözlendi ($p<0.05$) (Tablo 4). Yoğunlaştırılmamış postbiyotikler arasında en düşük titre edilebilir asit miktarına %1.41 ile 24 saat inkübe edilen *L. rhamnosus* postbiyotiği sahipken (Tablo 4), en yüksek asit içeriğine %2.19 ile 48 ve 96 saat inkübe edilen *L. plantarum* ve *L. sakei* (Tablo 2) postbiyotiğinin sahip olduğu görüldü. *2x* ve *4x* postbiyotikler arasında en düşük asit içeriğine 24 saat inkübe edilen *L. reuteri* postbiyotiğinin (*2x* için %2.1 ve *4x* için %4.11), en yüksek asit içeriğine de *2x* postbiyotiklerde 96 saat inkübe edilen *L. sakei* (%3.66), *4x* postbiyotiklerde de *L. plantarum*'un (%7.08) sahip olduğu tespit edildi.

Salmonella spp. üzerine en güçlü antimikrobiyal etkiyi *x* ve *2x* postbiyotiklerde sırasıyla 96 ve 24 saat inkübasyona bırakılmış *L. curvatus* (23.7 ve 28.7 mm) gösterirken (Tablo 2), *4x* postbiyotiklerde en güçlü antimikrobiyal etkiyi 29.3 mm zon çaplarıyla 48 saat inkübe edilmiş *L. curvatus* (Tablo 2) ve *L. sakei* (Tablo 2) postbiyotikleri gösterdi (Tablo 3). *L. rhamnosus* ve *L. reuteri* (Tablo 4) suşları MRS Broth içerisinde 24 saatten 96 saate kadar inkübe edildiklerinde *Salmonella* spp. üzerine gösterdikleri inhibisyon zonlarında önemli bir artış görülmedi ($p>0.05$). Benzer şekilde, *L. curvatus*'un *2x* yoğunlaştırılmış postbiyotiği hariç (Tablo 2), çalışmadaki diğer LAB'ların MRS Broth'daki inkübasyon süreleri uzamasına rağmen *Salmonella* spp. üzerine oluşturdukları inhibisyon zonlarında bir artış gözlenmedi ($p>0.05$).

LAB'ların (*P. acidilactici*'nin *4x* postbiyotiği hariç) inkübasyon süreleri 24 saatten 48 veya 96 saate uzatılmasına rağmen *L. monocytogenes* üzerine antimikrobiyal etkileri arasında farklılık görülmedi ($p>0.05$). Yoğunlaştırılmamış postbiyotikler arasında *L. monocytogenes* üzerine en zayıf etkiyi 48 saat inkübe edilmiş *L. fermentum* (17.7 mm) (Tablo 3), en güçlü etkiyi ise 48 saat inkübe edilmiş *L. curva-*

tus (22.0 mm) postbiyotiğinin gösterdiği görüldü (p<0.05) (Tablo 2). *2x* ve *4x* postbiyotikler arasında *L. monocytogenes* üzerine en güçlü antimikrobiyal etkiyi sırasıyla 24 mm ve 29 mm inhibisyon zon çapıyla 24 saat inkübe edilmiş *L. plantarum* postbiyotikleri gösterdi (Tablo 2).

E. coli O157 üzerine en güçlü antimikrobiyal etkiyi yoğunlaştırılmamış postbiyotikler arasında 96 saat inkübe edilmiş *L. plantarum* (22.7) (Tablo 2), *2x* postbiyotikler arasında ise 96 saat inkübe edilmiş *P. acidilactici* (24.7 mm) postbiyotiği gösterdi (Tablo 3). *P. acidilactici*'nin *4x* postbiyotiği hariç, diğer LAB'ların inkübasyon süreleri 24 saatten 96 saate uzatılmasına rağmen *E. coli* O157 üzerine antimikrobiyal etkileri arasında farklılık görülmedi (p>0.05). Postbiyotiklerden *4x* grubunda hepsinin *E. coli* O157 üzerine etkilerinin arttığı, en güçlü etkiyi ise 30 mm zon çapı ile 96 saat inkübe edilmiş *L. plantarum* postbiyotiğinin oluşturduğu görüldü.

Metisilin dirençli *Staphylococcus aureus* (MRSA) üzerine en güçlü antimikrobiyal etkiyi *x* ve *4x* postbiyotikler arasında MRS Broth'da 24 saat inkübe edilmiş *L. plantarum* (sırasıyla 22.7 ve 30.7 mm) gösterirken, *2x* postbiyotiklerde yine 96 saat inkübe edilmiş *L. plantarum* postbiyotiği (25.0 mm) göstermiştir (Tablo 2). *4x* postbiyotiklerin hepsinde antibakteriyel etkinlikte artış belirlendi. Ancak bu artışlar bazı LAB'larda istatistiksel olarak önemli iken bazılarında önemsiz seviyede gözlendi. Özellikle *L. plantarum* ve *L. sakei*'den elde edilen *4x* postbiyotiklerin MRSA üzerine en güçlü etkiyi gösterdikleri tespit edildi. *P. acidilactici*'nin 4x postbiyotiği hariç (Tablo 3), diğer LAB'ların inkübasyon süreleri 24 saatten 96 saate uzatılmasına rağmen MRSA üzerine antimikrobiyal etkileri arasında farklılık görülmedi (p>0.05).

Tablo 2: MRS Broth içerisinde farklı sürelerde inkübe edilen *L. plantarum, L. curvatus* ve *L. sakei*'den elde edilen postbiyotiklerin iki (*2x*) ile dört kat (*4x*) yoğunlaştırılmasından sonra sahip olduğu pH, titre edilebilir asit miktarı (%g laktik asit cinsinden) ve agar kuyucuk yönteminde patojen bakteriler üzerine gösterdikleri inhibisyon zon çapları (mm)

		L. plantarum			***L. curvatus***			***L. sakei***		
	Postbiyotik yoğunluğu	**İnkübasyon süresi (saat)**			**İnkübasyon süresi (saat)**			**İnkübasyon süresi (saat)**		
		24	**48**	**96**	**24**	**48**	**96**	**24**	**48**	**96**
pH	***x***	3.83±0.11	3.72±0.08	3.74±0.12	3.90±0.16	3.73±0.05	3.69±0.12	3.94±0.17	3.77±0.09	3.74±0.06
	2x	3.87±0.06	3.75±0.14	3.76±0.06	3.88±0.08	3,86±0.09	3.75±0.05	3.83±0.06	3.75±0.09	3.64±0.04
	4x	3.80±0.07	3.67 ±0.11	3.67±0.04	3.83±0.12	3.71±0.08	3.62±0.11	3.68±0.11	3.73±0.09	3.61±0.15
Titre edilebilir asitlik	x	1.97^{x}±0.02	2.19^{y}±0.10	2.19^{x}±0.14	1.77^{x}±0.14	2.16^{x}±0.16	2.16^{x}±0.09	1.86^{x}±0.14	2.19^{x}±0.14	2.19^{x}±0.10
	2x	3.63^{y}±0.14	3.62^{y}±0.11	3.63^{y}±0.19	3.33^{y}±0.24	3.36^{y}±0.10	3.59^{y}±0.10	3.30^{Ay}±0.09	3.45^{ABy}±0.14	3.66^{By}±0.19
	4x	6.78^{z}±0.65	7.05^{z}±0.53	7.08^{z}±0.29	6.21^{z}±0.59	6.78^{z}±0.43	6.98^{z}±0.12	6.56^{z}±0.31	6.87^{z}±0.32	6.93^{z}±0.24
Patojen bakteriler		**İnhibisyon zon çapları**			**İnhibisyon zon çapları**			**İnhibisyon zon çapları**		
Salmonella spp.	x	21.7^{x}±1.5	20.3^{x}±2.1	23.3^{x}±2.0	21.0^{x}±1.0	22.0^{x}±2.5	23.7^{xy}±2.0	20.7^{x}±2.1	21.7^{x}±1.0	20.9^{x}±1.0
	2x	26.3^{y}±1.5	22.3^{xy}±2.3	27.3^{xy}±2.3	28.7^{Ay}±1.9	24.7^{ABx}±1.7	21.7^{Bx}±1.1	24.7^{xy}±2.1	24.7^{x}±1.9	22.0^{x}±1.7
	4x	27.4^{y}±1.9	26.3^{y}±2.1	28.3^{y}±0.6	27.7^{y}±0.6	29.3^{y}±1.1	27.0^{y}±0.1	29.0^{y}±1.0	29.3^{y}±0.6	27.0^{y}±1.0
L. monocytogenes	x	20.7^{x}±2.1	20.7^{x}±1.5	20.0^{x}±1.0	19.3^{x}±2.0	22.0^{x}±1.0	19.7^{x}±1.5	20.7^{x}±1.2	20.7^{x}±1.2	20.7^{x}±1.5
	2x	24.0^{x}±1.7	23.3^{xy}±1.5	23.7^{y}±1.2	21.7^{xy}±1.5	22.7^{x}±0.6	22.7^{x}±0.6	24.0^{x}±1.5	23.7^{x}±1.2	23.0^{xy}±2.0
	4x	29.0^{y}±1.0	26.7^{y}±2.1	28.7^{z}±1.5	25.3^{y}±0.6	27.3^{y}±1.5	27.3^{y}±1.5	25.3^{y}±1.2	23.3^{x}±1.2	26.3^{y}±1.5
E. coli O157	x	21.0^{x}±1.5	22.6^{x}±2.1	22.7^{x}±2.1	21.0^{x}±1.6	21.7^{x}±0.6	21.0^{x}±2.0	21.3^{x}±2.3	22.3^{x}±1.2	21.7^{x}±1.5
	2x	20.0^{x}±1.0	21.3^{x}±2.3	23.7^{x}±0.6	20.7^{x}±2.1	22.0^{x}±1.0	19.3^{x}±1.5	22.7^{x}±2.1	22.3^{x}±1.5	21.7^{x}±0.6
	4x	27.7^{y}±1.2	27.0^{x}±2.6	30.0^{y}±1.0	26.7^{y}±1.5	29.0^{y}±1.0	26.3^{y}±1.5	25.7^{x}±1.5	26.7^{y}±1.6	27.0^{y}±1.0
*S. aureus**	x	22.7^{x}±1.2	20.0^{x}±2.0	21.3^{x}±2.1	20.3^{x}±0.6	20.3^{x}±2.5	21.3^{x}±2.1	19.7^{x}±1.5	22.0^{x}±1.0	22.3^{x}±1.2
	2x	22.7^{x}±1.2	21.3^{x}±0.6	25.0^{xy}±1.0	23.3^{xy}±2.3	23.3^{x}±1.5	23.7^{xy}±0.6	22.7^{xy}±1.2	23.7^{x}±1.2	22.3^{xy}±2.3
	4x	30.7^{y}±1.2	26.3^{y}±1.2	29.3^{y}±3.0	23.7^{y}±0.6	25.0^{x}±2.0	27.7^{y}±2.5	26.3^{y}±2.1	26.7^{y}±1.5	29.3^{y}±2.1
B. melitensis	x	19.0^{x}±1.0	21.0^{xy}±2.0	18.7^{x}±0.6	19.7^{x}±0.6	19.3^{x}±1.2	20.3^{x}±2.5	20.7^{x}±2.1	18.7^{x}±1.5	20.3^{x}±1.5
	2x	24.0^{xy}±1.7	19.3^{x}±1.2	20.0^{x}±2.6	22.7^{xy}±1.5	20.0^{x}±1.7	21.3^{xy}±0.6	18.7^{x}±0.6	20.0^{x}±1.7	19.3^{x}±1.2
	4x	25.7^{y}±1.2	23.6^{y}±0.6	27.3^{y}±1.2	22.7^{y}±0.6	24.0^{y}±2.5	24.0^{y}±1.7	24.0^{y}±1.7	25.7^{y}±2.3	26.7^{y}±1.8

Her bir patojen bakteri kendi içerisinde değerlendirilmiştir. $^{x-z}$: Aynı sütunda farklı harfleri taşıyan ortalamalar farklıdır (P<0.05).

$^{A-B}$: Aynı satırda farklı harfleri taşıyan ortalamalar farklıdır (P<0.05).*metisilin dirençli S. *aureus*

Tablo 3. MRS Broth içerisinde farklı sürelerde inkübe edilen *P. acidilactici, L. fermentum* ve *L. delbrueckii*'den elde edilen postbiyotiklerin iki (2x) ile dört kat (4x) yoğunlaştırılmasından sonra sahip olduğu pH, titre edilebilir asit miktarı (%g laktik asit cinsinden) ve agar kuyucuk yönteminde patojen bakteriler üzerine gösterdikleri inhibisyon zon çapları (mm)

		P. acidilactici			***L. fermentum***			***L. delbrueckii***		
	Postbiyotik yoğunluğu	**İnkübasyon süresi (saat)**			**İnkübasyon süresi (saat)**			**İnkübasyon süresi (saat)**		
		24	**48**	**96**	**24**	**48**	**96**	**24**	**48**	**96**
pH	***x***	3.96±0.10	3.87±0.08	3.83±0.11	4.10±0.05	4.04±0.12	4.10±0.07	3.99±0.10	3.94±0.14	3.92±0.11
	2x	3.93±0.10	3.84±0.12	3.82±0.12	4.03±0.07	3.99±0.12	4.03±0.15	3.97±0.12	3.90±0.17	3.87±0.10
	4x	3.77±0.15	3.80±0.09	3.77±0.15	3.94±0.06	3.92±0.08	3.89±0.10	3.93±0.07	3.85±0.09	3.81±0.13
Titre edilebilir asitlik	x	1.74^{x}±0.27	1.98^{x}±0.18	1.95^{x}±0.21	1.50^{x}±0.14	1.71^{x}±0.16	1.68^{x}±0.05	1.65^{x}±0.05	1.80^{x}±0.18	1.80^{x}±0.09
	2x	3.21^{y}±0.14	3.33^{y}±0.18	3.42^{y}±0.09	2.79^{y}±0.16	2.94^{y}±0.19	2.76^{y}±0.14	2.97^{y}±0.16	3.09^{y}±0.27	3.09^{y}±0.19
	4x	6.33^{z}±0.19	6.30^{z}±0.27	6.45^{z}±0.36	5.49^{z}±0.24	5.34^{z}±0.19	5.31^{z}±0.18	5.52^{z}±0.19	5.67^{z}±0.24	5.88^{z}±0.19
Patojen bakteriler		**İnhibisyon zon çapları**			**İnhibisyon zon çapları**			**İnhibisyon zon çapları**		
Salmonella spp.	x	20.0^{x}±2.1	21.7^{x}±2.1	22.7^{x}±0.6	17.3^{x}±1.1	18.0±2.0	20.0±2.0	19.7^{x}±2.1	19.0^{x}±1.7	20.0^{x}±0.6
	2x	24.0^{x}±1.7	23.0^{xy}±2.0	20.7^{x}±0.6	19.7^{xy}±0.6	20.0±1.2	20.0±0.6	18.7^{x}±0.6	20.0^{x}±1.5	22.0^{xy}±2.1
	4x	24.0^{x}±2.3	28.0^{y}±1.7	26.0^{y}±1.5	22.7^{y}±2.5	22.0±1.7	22.0±2.0	27.0^{y}±2.0	23.0^{x}±2.3	25.0^{y}±0.6
L. monocytogenes	x	21.3^{x}±2.2	21.0^{x}±1.7	20.3^{x}±1.8	18.7^{x}±2.1	17.7^{x}±1.5	18.7^{x}±0.6	20.0^{x}±1.0	19.0^{x}±1.0	19.7^{x}±2.1
	2x	23.3^{x}±1.5	23.0^{x}±2.0	23.3^{x}±1.5	20.0^{x}±2.0	20.7^{xy}±2.1	20.7^{xy}± 1.5	19.7^{x}±2.1	20.0^{x}±1.0	20.7^{xy}±2.3
	4x	23.3^{Bx}±1.2	24.3^{Bx}±0.6	28.9^{Ay}±0.6	23.7^{x}±2.1	23.3^{y}±2.0	23.3^{y}±1.2	23.7^{x}±2.1	23.7^{y}±0.6	24.7^{y}±0.6
E. coli O157	x	20.3^{x}±2.5	19.0^{x}±1.0	22.0^{x}±1.0	18.7±1.2	19.3±1.2	18.7±1.2	20.3^{x}±2.5	17.0^{x}±1.0	19.0^{x}±1.7
	2x	24.3^{x}±1.2	21.3^{x}±1.5	24.7^{x}±2.1	18.7±1.2	18.3±0.6	17.7±0.6	19.3^{x}±1.5	21.0^{y}±1.0	19.3^{x}±0.6
	4x	24.7^{ABx}±2.1	22.3^{Bx}±2.3	28.7^{Ay}±0.6	21.7±2.1	21.3±2.3	22.3±2.5	21.7^{x}±1.5	23.7^{y}±1.2	23.0^{y}±1.0
*S. aureus**	x	19.0^{x}±1.0	19.7^{x}±1.5	21.0^{x}±1.0	18.3^{x}±0.6	18.3^{x}±1.5	19.0^{x}±1.0	17.3^{x}±2.1	18.3^{x}±0.6	19.7^{x}±0.6
	2x	21.7^{xy}±0.6	24.0^{y}±1.0	22.7^{x}±2.1	17.3^{x}±0.6	19.0^{x}±1.0	19.3^{x}±2.3	16.7^{Bx}±1.5	23.0^{Ay}±2.0	19.7^{ABx}±0.6
	4x	24.3^{ABz}±1.2	22.3^{Bxy}±2.1	28.7^{Ay}±1.5	23.3^{y}±2.3	23.7^{y}±1.2	22.0^{x}±2.0	23.3^{y}±2.1	24.0^{y}±1.7	23.7^{y}±1.5
B. melitensis	x	19.3^{x}±2.3	19.0^{x}±1.0	19.7^{x}±1.5	17.3^{x}±0.6	17.7^{x}±0.6	17.0^{x}±1.7	17.7^{x}±0.6	19.7^{xy}±2.5	17.3^{x}±0.6
	2x	20.3^{x}±2.5	22.0^{xy}±1.7	20.7^{x}±1.2	17.7^{x}±1.2	17.7^{x}±0.6	18.7^{xy}±1.2	18.0^{x}±0.0	19.3^{x}±1.2	17.3^{x}±1.2
	4x	22.0^{x}±1.7	24.0^{y}±1.7	24.3^{y}±0.6	23.7^{Ay}±0.6	20.0^{ABy}±2.0	20.7^{By}±1.2	23.7^{y}±1.2	22.0^{y}±1.7	21.3^{y}±1.5

Her bir patojen bakteri kendi içerisinde değerlendirilmiştir. $^{x-z}$: Aynı sütunda farklı harfleri taşıyan ortalamalar farklıdır ($P<0.05$).

$^{A-B}$: Aynı satırda farklı harfleri taşıyan ortalamalar farklıdır ($P<0.05$).*metisilin dirençli *S. aureus*

B. melitensis üzerine yoğunlaştırılmamış postbiyotikler arasında en zayıf etkiyi 48 saat inkübe edilmiş *L. reuteri* (16.3 mm) (Tablo 4), en güçlü etkiyi ise 48 saat inkübe edilmiş *L. plantarum* (21.0 mm) postbiyotiğinin gösterdiği görüldü ($p<0.05$) (Tablo 2). *2x* postbiyotikler arasında en güçlü antimikrobiyal etki 24 saat inkübe edilmiş *L. plantarum* postbiyotiğinde (24 mm), yine *4x* postbiyotikler arasında da 96 saat inkübe edilmiş *L. plantarum* postbiyotiğinde (27.3 mm) görüldü. *L. reuteri*'nin *4x* postbiyotiği hariç (Tablo 3), diğer LAB'ların inkübasyon süreleri 24 saatten 96 saate uzatılmasına rağmen *B. melitensis* üzerine antimikrobiyal etkileri arasında farklılık görülmedi ($p>0.05$).

Postbiyotiklerin içerdikleri asit 5N NaOH ile nötralize edildikten sonra tekrar MHA'da yapılan agar kuyucuk testlerinde patojen mikroorganizmalara karşı kuyucuklar çevresinde zon tespit edilmedi (veri gösterilmedi). Dolayısıyla postbiyotiklerin sergiledikleri antimikrobiyal etkinin içerdikleri organik asitlerden kaynaklandığı kanaatine varıldı.

Tartışma ve Sonuç

Organik asitler postbiyotikler içerisindeki en önemli antimikrobiyal etkiye sahip bileşikler olarak bilinir. LAB'lar, MRS Broth besiyerinde gelişimleri esnasında besiyeri içerisindeki glikozu (20 g/L) kullanarak organik asit üretirler (Zalán ve ark. 2010). Organik asitler ortamın pH değerini önemli ölçüde düşürerek patojenleri inhibe eder (Mani-López ve ark. 2012). Mevcut çalışmada, kullanılan LAB'lara bireysel olarak bakıldığında MRS Broth içerisinde inkübasyon süreleri uzamasına (24, 48 ve 96 saat) rağmen postbiyotiklerinin pH değerleri arasında önemli

bir farklılık görülmedi (p>0.05) (Tablo 2-4). Örneğin, *L. plantarum* MRS Broth içerisinde 24, 48 ve 96 saat inkübe edildiğinde elde edilen postbiyotiğin pH değerlerinin sırasıyla 3.83, 3.72 ve 3.74 olduğu (Tablo 2) ve aralarında önemli bir farklılık bulunmadığı tespit edildi (p>0.05). Bu durumun kullanılan diğer tüm LAB'lar için de geçerli olduğu görüldü. Ancak, postbiyotiklerin pH değerleri açısından bakteri türleri arasında farklılıklar olduğu ve genel olarak *L. fermentum*'dan elde edilen postbiyotiğin pH değerinin diğer postbiyotiklerden daha yüksek olduğu tespit edildi (p<0.05).

Tablo 4: MRS Broth içerisinde farklı sürelerde inkübe edilen *L. paracasei*, *L. rhamnosus* ve *L. reuterii*'den elde edilen postbiyotiklerin iki (2x) ile dört kat (4x) yoğunlaştırılmasından sonra sahip olduğu pH, titre edilebilir asit miktarı (%g laktik asit cinsinden) ve agar kuyucuk yönteminde patojen bakteriler üzerine gösterdikleri inhibisyon zon çapları (mm)

		L. paracasei			***L. rhamnosus***			***L. reuterii***		
	Postbiyotik yoğunluğu	**İnkübasyon süresi (saat)**			**İnkübasyon süresi (saat)**			**İnkübasyon süresi (saat)**		
		24	**48**	**96**	**24**	**48**	**96**	**24**	**48**	**96**
pH	***x***	3.95±0.08	3.89±0.10	3.94±0.08	4.12±0.14	3.98±0.10	3.95±0.09	3.98±0.04	3.92±0.08	3.93±0.20
	2x	3.91±0.06	3.86±0.05	3.88±0.10	4.08±0.13	3.92±0.06	3.91±0.09	3.94±0.05	3.91±0.06	3.91±0.17
	4x	3.89±0.08	3.88±0.07	3.83±0.12	4.05±0.09	3.96±0.10	3.89±0.08	3.89±0.04	3.90±0.06	3.86±0.14
Titre edilebilir asitlik	x	1.83^{x}±0.05	1.98^{x}±0.16	1.74^{x}±0.14	1.41^{x}±0.14	1.65^{x}±0.10	1.74^{x}±0.19	1.59^{x}±0.27	1.62^{x}±0.16	1.80^{x}±0.09
	2x	3.06^{y}±0.09	3.45^{y}±0.23	3.03^{y}±0.29	2.88^{y}±0.18	2.97^{y}±0.18	2.88^{y}±0.24	2.10^{Ay}±0.10	2.37^{Ay}±0.05	2.94^{By}±0.19
	4x	5.73^{z}±0.29	5.58^{z}±0.24	5.89^{z}±0.19	4.62^{Az}±0.19	5.34^{Bz}±0.19	5.58^{Bz}±0.16	4.11^{Az}±0.10	4.71^{ABz}±0.29	5.46^{Bz}±0.23
Patojen bakteriler		**İnhibisyon zon çapları**			**İnhibisyon zon çapları**			**İnhibisyon zon çapları**		
Salmonella spp.	x	21.1^{xy}±1.5	20.7^{xy}±0.6	19.0^{x}±1.0	19.3±2.0	19.7±0.6	18.7±2.3	19.3±1.5	20.7±2.1	20.3±0.6
	2x	18.7^{x}±1.5	18.7^{x}±1.5	23.7^{xy}±1.5	19.7±0.6	22.0±1.0	23.0±2.5	20.7±0.6	21.0±2.0	22.3±2.1
	4x	23.0^{y}±1.7	22.7^{y}±2.1	22.7^{y}±0.6	22.7±3.2	22.7±2.5	24.0±2.6	21.0±3.0	21.0±1.7	23.3±2.1
L. monocytogenes	x	19.0^{x}±1.0	17.8^{x}±1.5	19.3^{x}±0.6	20.0^{x}±1.6	18.7^{x}±1.5	19.0^{x}±2.0	19.0^{x}±1.0	20.7^{x}±1.9	19.3^{x}±1.9
	2x	21.0^{x}±2.0	20.5^{xy}±2.1	23.3^{xy}±2.1	20.3^{x}±2.1	19.7^{x}±0.6	19.0^{x}±1.0	19.0^{x}±1.0	19.7^{x}±0.6	20.0^{x}±2.0
	4x	24.3^{y}±0.6	24.2^{y}±1.5	24.7^{y} ±2.1	23.0^{x}±2.0	25.0^{y}±1.7	23.3^{x}±2.5	22.3^{y}±1.1	20.7^{x}±1.1	23.3^{x}±2.5
E. coli O157	x	18.3^{x}±0.6	19.3^{x}±1.2	19.0^{x}±1.0	19.3^{x}±2.5	19.7^{x}±2.1	18.0^{x}±0.0	20.3^{x}±2.1	20.7^{x}±2.1	19.3^{x}±0.6
	2x	20.3^{x}±0.6	17.0^{x}±1.7	20.0^{xy}±2.0	18.3^{x}±1.2	17.0^{x}±1.7	20.3^{y}±0.6	19.0^{x}±1.7	17.3^{x}±1.2	21.0^{x}±1.0
	4x	23.3^{x}±1.5	24.7^{y}±1.1	24.0^{y}±1.7	22.0^{x}±2.0	24.3^{y}±1.5	24.0^{z}±0.0	22.7^{x}±2.1	19.3^{x}±1.5	25.0^{y}±1.7
*S. aureus**	x	16.7^{x}±0.6	19.3^{x}±1.5	16.7^{x}±2.1	17.0^{x}±1.7	17.7^{x}±1.2	18.7^{x}±1.5	16.0^{x}±1.0	18.0^{x}±2.6	19.3^{x}±0.6
	2x	15.3^{x}±1.2	18.7^{x}±1.5	19.0^{xy}±2.6	16.7^{x}±1.5	16.3^{x}±0.6	18.3^{x}±0.6	15.0^{x}±1.0	18.0^{x}±1.7	19.0^{x}±1.0
	4x	24.7^{y}±0.6	23.3^{x}±2.3	23.7^{y}±1.5	20.0^{x}±2.0	23.0^{y}±2.0	24.0^{y}±2.6	21.7^{y}±0.6	20.3^{x}±2.5	24.7^{y}±2.5
B. melitensis	x	17.3^{x}±0.6	19.7^{x}±2.5	17.3^{x}±1.2	16.7^{x}±1.2	19.0^{x}±1.0	17.7^{x}±0.6	16.7^{x}±1.5	16.3^{x}±1.5	18.7^{x}±0.6
	2x	17.7^{x}±0.6	19.3^{x}±1.2	19.3^{xy}±0.6	18.3^{xy}±2.5	18.7^{x}±1.2	20.3^{y}±1.5	16.7^{x}±1.5	19.3^{y}±1.5	18.3^{x}±1.2
	4x	23.7^{y}±1.2	24.0^{y}±1.0	22.3^{y}±0.6	20.7^{y}±1.5	22.3^{x}±2.0	24.0^{z}±2.0	19.3^{Ax}±1.2	20.7^{ABy}±0.6	23.3^{By}±1.5

Her bir patojen bakteri kendi içerisinde değerlendirilmiştir. $^{x-z}$: Aynı sütunda farklı harfleri taşıyan ortalamalar farklıdır (P<0.05).
$^{A-B}$: Aynı satırda farklı harfleri taşıyan ortalamalar farklıdır (P<0.05).*metisilin dirençli *S. aureus*

Postbiyotiklerin karakteristikleri elde edildikleri bakteri türlerine ve bakterilerin geliştiği kültür ortamına göre değişkenlik göstermektedir (Moradi ve ark. 2020; Sabahi ve ark. 2022). Elde edilen postbiyotiklerin pH durumunun LAB'ların metabolizmaları sonucu ürettikleri organik asitlerle birlikte değerlendirilmesi daha uygun olacaktır. Postbiyotikler titre edilebilir toplam asitlikleri bakımından da incelendiğinde, bakterilerin inkübasyon süresinin uzamasının üretilen organik asit miktarında önemli bir farklılık oluşturmadığı tespit edildi (p>0.05) (Tablo 2-4). Genel olarak *L. plantarum*, *L. curvatus* ve *L. sakei* postbiyotiklerinin titre edilebilir asitlik değerlerinin çalışmada kullanılan diğer LAB'lardan daha yüksek olduğu tespit edildi. Bilindiği gibi, homofermentatif LAB'lar glikozdan %90-95 oranında laktik asit sentezlerken, heterofermentatif LAB'lar asetik asit, aseton, CO_2, diasetil ve etanol ile birlikte laktik asidin %50'sini üretebilmektedir (Gunkova ve ark. 2021). Mevcut çalışmada postbiyotiklerdeki % laktik asit miktarı postbiyotiklerin asit-baz titrasyonu sonucunda harcanan NaOH miktarına göre hesaplandı.

Dolayısıyla, hesaplamada baskın organik asit (laktik asit) dikkate alındığından laktik asit bakterilerinin ürettiği diğer organik asitlerin miktarı hakkında fikir vermemektedir.

Düşük pH (ya da yüksek laktik asit miktarı) laktik asit bakterileri için gelişmeyi kısıtlayıcı bir faktördür. Laktobasillerin düşük pH değerlerinde metabolik bozulmaya yatkın oldukları, stres seviyesine bağlı olarak hücrelerin ana metabolik aktivitelerini azalttıkları, bu durumun enerji sentezinin, proton hareket gücünün, büyümenin ve canlılığın azalmasına neden olduğu belirtilmiştir (De Angelis ve Gobbetti 2011). Bundan dolayı, MRS Broth içerisinde inkübasyon süresi uzamasına rağmen laktik asit bakterileri laktik asit üretimini durdurmuş olabilir. Bu yüzden, postbiyotiklerin titre edilebilir asit miktarlarında önemli bir farklılık görülmemiş olabilir.

Elde edilen postbiyotikler *2x* ve *4x* yoğunlaştırıldıklarında titre edilebilir asit miktarları önemli oranlarda artış gösterdi ($p<0.05$). Ancak, *2x* ve *4x* postbiyotiklerin asit miktarları artmasına rağmen pH değerlerinde önemli bir düşüş görülmedi ($p>0.05$) (Tablo 2-4). Bilindiği gibi titre edilebilir asitlik bir çözeltideki toplam ayrışmış ve ayrışmamış asitleri ölçer. pH ile titre edilebilir asitlik arasında doğrudan veya öngörülebilir bir ilişki olmadığı, ancak pH'nın asitlerin ayrışma yeteneklerinden etkilendiği belirtilmiştir (Tyl ve Sadler 2017). Mevcut bu çalışmada da, postbiyotikler konsantre edildiklerinde içlerinde bulunan tüm ayrışmış ve ayrışmamış özellikte bulunan asitlerin miktarı artsa da aralarındaki orantı değişmediğinden pH değerlerinde önemli bir değişiklik görülmemiş olabilir.

Postbiyotiklerin patojen bakteriler üzerine inhibisyon özellikleri değerlendirildiğinde, genel olarak en güçlü antimikrobiyal etkiyi *L. plantarum*, *L. sakei* ve *L. curvatus*'tan elde edilen postbiyotiklerin gösterdiği tespit edildi. *L. plantarum*, *L. sakei* ve *L. curvatus* fakültatif heterofermentatif bakterilerdir. Fakültatif heterofermentatif LAB'lar heksozları ya tamamen laktik aside ya da laktik asit, asetik asit, etanol ve formik aside fermente ederler (De Angelis ve Gobbetti 2011). Mun ve ark. (2019) *L. plantarum*'un antimikrobiyal ve antifungal etkisini araştırdıkları çalışmalarında, *L. plantarum*'un organik asit olarak laktik asit (%75.2), asetik asit (%17.9), sitrik asit (%6.5) ve fenillaktik asit (%0.3) oluşturduğunu tespit etmişlerdir. Heterofermentatif karakterdeki *L. curvatus*, *L. sakei* ve *L. plantarum* postbiyotiklerinin homofermentatif *P. acidilactici* ve *L. delbrueckii* subsp. *bulgaricus* postbiyotiklerinden daha güçlü antibakteriyal etki göstermesinin sebebi laktik asit ve asetik asidin birlikte sinerjistik etki göstermesinden kaynaklanmış olabilir. Habeeb ve ark (2021) laktik asit ve asetik asidin birlikte kullanıldıklarında sinerjistik antibakteriyal etkiye sahip olduğunu belirtmişlerdir. Mevcut çalışmada *L. paracasei*, *L. rhamnosus*, *L. fermentum* ve *L. reuteri* heterofermentatif karakterde LAB'lar olsa da ürettikleri postbiyotiklerin titre edilebilir asit miktarları düşük kalmıştır (Tablo 3-4). Bu yüzden antimikrobiyal etkilerinin de *L. plantarum*, *L. curvatus* ve *L. sakei* postbiyotiklerine nazaran düşük kalması beklenebilir. Çalışmamızda % laktik asit miktarı asit-baz titrasyonu sonucu harcanan NaOH miktarına göre hesaplanmıştır. Formülde, materyaldeki hakim organik asit (laktik asit) dikkate alınarak hesaplama yapıldığı için laktik asit bakterilerinin ürettiği diğer organik asitler de laktik asit hesabına dahil olmakta ve diğer organik asit miktarları hakkında fikir vermemektedir.

Bakteriyosinler, *Lactobacillus* ve bifidobakteriler gibi çeşitli bakteriler tarafından üretilen antibakteriyel aktiviteye sahip peptitler veya proteinlerdir. Bakteriyosinlerin patojenik bakterilerin büyümesini ve gelişmesini engellemek gibi birçok olumlu etkiye sahip oldukları, ayrıca ısıya ve pH'ya dayanıklı oldukları bildirilmiştir (O'Connor ve ark. 2020). Postbiyotiklerin antimikrobiyal etkilerinin organik asitlerden veya organik asit dışı bileşiklerden ileri gelip gelmediğini anlamak için, postbiyotik bir alkali ile (genellikle 5 N NaOH) pH değeri 6.0-7.0'ye ayarlanır ve antimikrobiyal testler uygulanır (Moradi ve ark. 2021). Bu çalışmada, postbiyotiklerin pH değerleri 5 N NaOH ile pH 6.0'a ayarlandıktan sonra yapılan testlerde kuyucukların çevresinde inhibisyon zonu oluşmadığından antimikrobiyal etkinin postbiyotiklerin içerdikleri organik asitlerden kaynaklandığı, bakteriyosin ve diğer bileşiklerin (bakteriyosin benzeri inhibitör bileşikler, hidrojen peroksit, diasetil, kısa ve uzun zincirli yağ asitleri ve etanol) antimikrobiyal etki oluşturacak düzeyde bulunmadıkları kanaatine varılmıştır. LAB'lar tarafından sentezlenen en önemli antimikrobiyallerin laktik asit ve asetik asit olduğu, bunların yanı sıra düşük konsantrasyonlarda çok çeşitli bileşiklerde sentezledikleri bildirilmiştir (Mani-López ve ark. 2022). Sentezlenen bu bileşiklerin bir kısmının kendi minimum inhibisyon konsantrasyonlarının altında olabileceği (Nasrollahzadeh ve ark. 2022), ancak çok düşük konsantrasyonlarda dahi olsa da sentezlenen bu bileşiklerin laktik asit ve asetik asitle sinerjistik etki göstermelerinin mümkün olduğu ileri sürülmüştür (García-Díez ve ark. 2021).

Arena ve ark. (2016) şarap ve şıradan izole ettikleri 79 adet *L. plantarum* suşundan elde ettikleri postbiyotiklerin pH'ları nötralize edildikten sonra *L. monocytogenes*, *E. coli* O157:H7, *S.* Enteritidis ve

*S.*aureus patojenlerine karşı çok az inhibisyon etkisi gözlediklerini ya da hiç gözlemleyemediklerini belirtmişlerdir. Divyashree ve ark. (2021) yaptıkları çalışmada laktik asit bakteri süpernatantlarının *S*. Paratyhpi'ye karşı inhibe edici etkilerinin organik asitlerden kaynaklanabileceği sonucuna varmışlardır. Araştırmacıların aldıkları sonuç yapılan bu çalışmayla uyumludur. LAB' ların bazı suşlarının bakteriyosin sentezi yaptıkları bildirilmiş olsa da, bakteriyosin üretiminin besiyeri ortamına, inkübasyon koşullarına, biyokütleye, pH, sıcaklık ve üreme fazlarına bağlı olduğu, bakterilerin gelişmesi için optimum olan koşulların bakteriyosin üretimi için ideal olmayabileceği bildirilmiştir (Zhou ve ark. 2015).

Bildiğimiz kadarıyla, laktik asit bakteri postbiyotiklerinin *Brucella* spp.'ye karşı antimikrobiyal etkisini araştıran bir çalışma literatürde bulunmamaktadır. Elde edilen veriler incelendiğinde, çalışmada kullanılan postbiyotiklerin *B. melitensis* tip III üzerinde antimikrobiyal etkiye sahip oldukları, ancak oluşturdukları zon çaplarının *L. monocytogenes*, *Salmonella* spp., *E. coli* O157 ve metisilin dirençli *S. aureus* üzerinde oluşturdukları zon çaplarına göre daha düşük olduğu görüldü. Sonuçlar değerlendirildiğinde, *B. melitensis* tip III'ün organik asitlere karşı nispeten dirençli olduğu ileri sürülebilir. *Brucella* spp.'lerin minimum 4.1 pH'da hayatta kalabildiği belirtilmektedir (Jansen ve ark. 2019). El-Khawas ve Elbauomy (2015) tarafından yapılan ve düşük asitli yumuşak bir peynir (Karish peyniri) türünde *B. melitensis* üzerine organik asitlerin etkisinin incelendiği bir çalışmada, *B. melitensis*'in %1 asetik asit veya %1.5 sitrik asitten etkilenmedikleri, %1.5 asetik asit ve %2 sitrik asidin ise inhibe edici etkilerinin olduğu bildirilmiştir.

Sonuç olarak, bu çalışmada kullanılan LAB postbiyotiklerinin antimikrobiyal etkilerinin içerdikleri organik asitlerden kaynaklandığı tespit edilmiştir. Laktik asit bakterisinin MRS Broth besiyeri içerisinde inkübasyon süresinin 24 saatten 96 saate uzatılmasının organik asit üretimini önemli seviyede artırmadığı ($p>0.05$) ve postbiyotiğin pH değerini önemli seviyede değiştirmediği görülmüştür ($p>0.05$). İnkübasyon sürelerinden bağımsız olarak, laktik asit bakterilerinden elde edilen postbiyotiklerin iki veya dört kat konsantre edilmelerinin içerdikleri titre edilebilir organik asit miktarlarını önemli seviyede artırdığı ($p<0.05$), ancak pH değerlerinde önemli bir değişikliğe sebep olmadığı tespit edilmiştir ($p>0.05$). Postbiyotikler iki veya dört kat konsantre edildiklerinde içerdikleri organik asit miktarlarındaki artışa paralel olarak patojen bakteriler üzerinde gösterdikleri inhibisyon zon alanlarının arttığı ve daha güçlü antimikrobiyal etki gösterdikleri görülmüştür. Çalışılan laktik asit bakterileri arasında patojen bakteriler üzerine en güçlü antimikrobiyal etkiyi *L. plantarum*, *L. sakei*, *L. curvatus* ve *P. acidilactici* bakterilerinden elde edilen postbiyotiklerin gösterdiği tespit edilmiştir.

Çalışmada elde edilen bulguların laktik asit bakterilerinden elde edilen postbiyotiklerin gıdalarda raf ömrünü artırmak veya mikrobiyal riskleri azaltmak açısından yapılacak olan ilerideki çalışmalara faydalı veriler sağlayacağı düşünülmektedir. Postbiyotiklerin içermiş oldukları organik asitler ile antimikrobiyal diğer bileşiklerin çeşit ve miktarlarına yönelik daha ayrıntılı çalışmalar yapılması, bu organik ürünlerin gıda ve sağlık alanlarında kullanılma olanaklarına dair daha güçlü veriler ortaya koyacaktır.

Etik kurul kararı: Çalışma canlı örnek materyali içermemesi nedeniyle etik kurul izni gerektirmeyen çalışmalar arasında yer almaktadır.

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Özgün Araştırma
Original Article

Ege Bölgesi'ndeki sığırlarda paratüberküloz seroprevalansının belirlenmesi

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Özet: Sığır paratüberkülozu (PTB) sığırların granulomatoz gastroenteritle karakterize kronik seytirli, bulaşıcı, zoonoz bir hastalığıdır. Süt sığırcılığı sektöründe önemli ekonomik kayıplara sebep olmakla birlikte zoonotik karakterli olması insanlarda görülen Chron's hastalığının temelini oluşturabilme olasılığını düşündürmektedir. Bu çalışmada paratuberküloz hastalığının Ege Bölgesi'nde yetiştirilen süt sığırlarındaki seroprevalansının belirlenmesi amaçlanmıştır. 2022 yılı içerisinde İzmir, Muğla, Denizli, Aydın, Kütahya ve Uşak illerinden tesadüfi örnekleme ile seçilip Bornova Veteriner Kontrol Enstitüsü'ne gönderilen 2 yaşından büyük aşılanmamış süt sığırlarına ait 1000 adet kan serumu üzerinde çalışılmıştır. Bu amaçla diğer tanı yöntemlerinden avantajlı olduğu belirlenen ELISA yöntemi kullanılmıştır. Çalışma materyalinin ELISA ile incelenmesi sonucunda toplamda 84 (%8,4) örnek pozitif; 28 (%2,8) örnek şüpheli bulundu. 888 (%88,8) serum örneği ise negatif olarak tespit edildi. Elde edilen veriler ışığında serumlarda seropozitifliğin görülmesi hastalığın bölgede halen varlığını koruduğunu, koruma, kontrol ve eradikasyon yöntemlerinin belirlenerek uygulanmasının gerekli olduğunu düşündürmektedir.

Anahtar kelimeler: Ege Bölgesi, ELISA, paratuberculosis**,** paratüberküloz, sığır

Determination of paratuberculosis seroprevalence in cattle in the Aegean Region

Abstract: Bovine paratuberculosis (PTB) is a chronic, infectious, zoonotic disease of cattle characterized by granulomatous gastroenteritis. Although it causes significant economic losses in the dairy cattle industry, its zoonotic character suggests the possibility that it may form the basis of Chron's disease seen in humans. This study aimed to determine the seroprevalence of paratuberculosis disease in dairy cattle raised in the Aegean Region. In 2022, 1000 blood serum samples of unvaccinated dairy cattle over 2 years old, selected by random sampling from the provinces of Izmir, Muğla, Denizli, Aydın, Kütahya and Uşak and sent to Bornova Veterinary Control Institute, were studied. For this purpose, the ELISA method, which was determined to be advantageous over other diagnostic methods, was used. As a result of examining the study material with ELISA, a total of 84 (8.4%) samples were positive; 28 (2.8%) samples were found suspicious. 888 (88.8%) serum samples were detected as negative. In the light of the data obtained, the presence of seropositivity in serum suggests that the disease still exists in the region and that prevention, control and eradication methods should be determined and implemented.Key Words: Aegean Region, cattle, ELISA, paratuberculosis

Keywords: Yok

Giriş

Paratüberküloz (PTB), *Mycobacterium avium subsp. paratuberculosis*'in geviş getiren hayvan türlerinde ilerleyici granülomatöz enterit sonucu oluşan kronik enfeksiyöz bir sürü hastalığıdır (Beard ve ark. 2001; Manning ve ark. 2001; Gilardoni ve ark. 2012). Hastalık, kronik gastroenteritis, ishal ve canlı ağırlık kaybı ile karakterize olup hastalığa genç sığırlar daha duyarlıdır (Liu ve ark. 2017; Fawzy ve ark. 2018). Hastalık verim düşüklüğü, ciddi ölçüde kilo kaybı, tedavi masrafları gibi giderler yönünden dünya çapında önemli ekonomik sorunlara neden olabilmektedir (Selim ve ark. 2013). Etkenin insanlarda görülen Crohn's hastalığının etiyolojisinde rol oynaması gerekçesiyle paratüberküloz, halk sağlığı yönünden dikkate alınması gereken önemli hastalıklardandır.

Paratüberkülozun teşhisinde, mikroorganizma kültürü başta olmak üzere, kültür dışında bakteriyolojik inceleme, serolojik testler, immünolojik test yöntemleri kullanılmaktadır. Kültür yönteminin spesifitesinin yüksek, sensitivitesinin düşük olması ve aynı zamanda laboratuvar sonuçların üç aya kadar uzaması önemli dezavantajlardır (Ayele ve ark. 2005; Öztürk Kalın ve ark. 2019; Whitlock ve ark. 2000). Bu sebeple paratuberküloz hastalığının teşhişinde serolojik yöntemler (Komplament Fikzasyon Testi (CFT),

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Agar Gel Immunodiffusion (AGID), Enzyme-linked Immunosorbent assays (ELISA), moleküler metodlar (PCR, Real Time-PCR vb.) ve hücresel immun yanıt testlerinden (gama interferon, alerjik deri testi vb.) faydalanılmaktadır (Ayele ve ark. 2001; Harris ve Barletta 2001; Singh ve ark. 2007). Strick Enzim Bağlantılı İmmüno-Sorbent Testi (ELISA), MAP*(Mycobacterium avium subsp. paratuberculosis)* enfeksiyonunun subklinik aşamasında ve sonrasında serum ve sütteki MAP antikorlarını tespit etmek için tercih edilen ve yaygın olarak kullanılan bir serolojik testtir. Kan serumunda MAP'a karşı oluşan antikorların tespitinde ELISA'ya sıkça başvurulmaktadır. Bunun nedeni olarak ELISA yönteminin diğer serolojik yöntemlerden daha kolay uygulanabilir ve tekrarlanabilir olması, maliyet olarak daha ucuz olması, çok sayıda örneğin bir arada çalışılabilmesi ve elde edilen verilerin tarafsız bir şekilde yorumlanabilmesi gibi avantajları gösterilebilir. Özellikle subklinik enfekte hayvanların belirlenmesinde diğer testlere göre daha güvenilir bir metot olduğu bildirilmiştir (Strickland ve ark. 2005; Mecitoğlu ve ark. 2012).

Bu testlerden başka immunite testleri de tanı yöntemleri arasında bulunmaktadır. Enfekte hayvanlarda gelişen humoral ve hücresel yanıtlar esas alınarak *M. paratuberculosis* ekstraklarının enjekte edildiği hayvanlarda gecikmiş tip aşırı duyarlılık reaksiyonlarının değerlendirildiği intradermal testlerdir ancak son yıllarda düşük spesifite ve sensitivite gibi dezavantajları nedeniyle önerilmemektedir (Mecitoğlu ve ark. 2012).

Bu çalışmada ciddi ekonomik kayıplara neden olan paratüberküloz hastalığının Ege Bölgesi'nde yetiştirilen sığırlardaki seroprevalansının belirlenmesi amaçlanmıştır.

Materyal ve Metot

Bu çalışmada 2022 yılı içerisinde Ege Bölgesi'nde bulunan İzmir, Muğla, Denizli, Aydın, Kütahya ve Uşak illerinden tesadüfi örnekleme ile seçilen 2 yaşından büyük süt sığırlarına ait kan serumlarından 1000 adet çalışıldı. Çalışılan kan serumları paratuberküloz yönünden aşılanmamış hayvanlardan seçildi. Antikoagülansız jelli tüplerde gelen kan örnekleri 3000 rpm'de 5 dk santrifüj edilerek serumları ayrıldı ve serumlar çalışma için -20 °C'de saklandı. Anti-MAP antikorların belirlenmesi amacıyla kan serumu örneklerinden ticari ELISA kiti (IDEXX Paratuberculosis Screening Ab Test, Netherlands) kullanıldı. Test üretici firmanın gösteriği biçimde uygulandı. Pozitif ve negatif kontroller ile serum örnekleri mikropleyt kuyucuklarına 1/19 oranında dilusyon buffer ile sulandırılarak 10'ar µl olacak şekilde konuldu. Mikropleytler oda ısısında (18-26 °C) 15 dakika tutulduktan sonra kuyucuklarda bulunan solüsyonlar antijen kaplı pleytlere aktarıldı. Oda ısısında ısıda 45 dakika boyunca inkube edildi. İnkübasyondan sonra tüm kuyucuklar 3 kez 300 µl yıkama solüsyonu ile yıkandı ve her birine 100 µl konjugat ilave edildi. Mikropleytler oda ısısında 30 dakika inkübasyona bırakıldı. Süre sonunda 300 µl yıkama sıvısı ile 3 kez yıkama işlemi uygulandıktan sonra, tüm kuyucuklara 100 µl TMB substrat ilave edildi. Daha sonra mikropleytler oda ısısında karanlık ortamda 10 dakika inkübasyona bırakıldı ve kuyucuklara 100 µl stop solüsyonu konularak 450 nm'de kuyucuklardaki solüsyonların absorbans değerleri ELISA Reader (Thermo Scientific™ Multiskan™ FC, Germany) ile ölçüldü. Çıkan sonuçlar ELISA kitine uygun olacak şekilde değerlendirildi.

Bulgular

Çalışma materyalinin ELISA yöntemi ile incelenmesi sonucunda toplamda 84 (%8,4) örnek pozitif; 28 (%2,8) örnek şüpheli bulunmuştur. 888 (%88,8) serum örneği ise negatif olarak tespit edilmiştir. Çalışmada kan örneklerinin gönderildiği illere göre seropozitiflik oranları İzmir ilinde %16, Muğla ilinde %16, Aydın ilinde %16 ve Manisa ilinde %16 iken; Denizli, Kütahya ve Uşak illerinde bu oranlar %12 olarak saptanmıştır (Tablo 1).

Tablo 1. Paratüberküloz hastalığının Ege Bölgesi illerine göre seroprevalansı

Yerleşim yeri	İncelenen serum sayısı n (%)	Pozitif serum sayısı n (%)	Şüpheli serum sayısı n (%)	Negatif serum sayısı n (%)
İzmir	160 (16)	16 (10)	4 (2,5)	140 (87,5)
Muğla	160 (16)	16 (10)	-	144 (90)
Denizli	120 (12)	8 (6,6)	8 (6,6)	104 (86,6)
Aydın	160 (16)	4 (2,5)	4 (2,5)	152 (95)
Kütahya	120 (12)	20 (16,6)	12 (10)	88 (73,3)
Uşak	120 (12)	16 (13,3)	-	104 (86,6)
Manisa	160 (16)	4 (2,5)	-	156 (97,5)
Toplam	1000	84	28	888

Tartışma ve Sonuç

Bu çalışmada Ege Bölgesi'nde yetiştiriciği yapılan sığırlarda paratuberküloz hastalığı seroprevalansı ELISA yöntemi ile araştırıldı. Paratüberkuloz hastalığı kronik zayıflıkla karakterize gastroenterik bir hastalık olup ciddi ekonomik kayıplara neden olmaktadır (Abdellrazeq ve ark. 2014; Selim ve ark. 2021a). Paratüberküloz kronik seyirli bir enfeksiyon olduğundan diğer seroprevalans çalışmalarında olduğu gibi bu çalışmada da 2 yaş üstü hayvanlar tercih edildi. 2 yaşından küçük sığırlarda ELISA'nın sensitivitesi ve spesifitesinin düşük olduğu yapılan çalışmalarda ortaya konmuştur (Çetinkaya ve ark. 2000; Diequez ve ark. 2009; Nielsen ve Toft 2009; Öztürk ve ark. 2010). Enzimin antikora bağlanma özelliğinden yararlanılarak hastalığın teşhisinde kullanılan ve serolojik bir ölçüm yöntemi olan ELISA tekniği kullanıldı. Bu teknik ile hayvanların serumunda MAP'a karşı oluşan antikorlar araştırıldı.

Dünya genelinde paratüberkulozun seroprevalansı ile ilgili pek çok çalışma yapılmış olması hastalığın güncelliğini koruduğunun ve problem olarak görüldüğünün göstergesidir. Makav ve ark. (2013), Kars yöresinde sığırlarda subklinik paratüberkülozun prevalansını belirlemek amacıyla rastgele 13 odak seçmiş ve bu odaklardaki 24 işletmeden 2 yaş ve üzeri toplam 400 sığır üzerinde çalışmışlardır. Çalışmalarının sonunda subklinik paratüberkülozun seroprevalansını %3.5 (14/400), çiftlik prevelansını ise %46.1 (10/24) olarak bulmuşlardır. Elde edilen bu farkın Kars bölgesinin içinde ve bölgeden başka yerlere hayvan transferinin sık olması nedeniyle olabileceği, yüksek çiftlik prevalansının (%46.1) hastalığın yayılmasında ciddi bir risk faktörü olması açısından önemli olduğu sonucuna varmışlardır. Başka bir çalışmada Karatay ve ark. (2020), Ardahan çevresinde yetiştirilen süt sığırlarında paratüberkülozun prevalansının belirlenmesi amacıyla 400 adet sığır kan serumunu araştırmışlardır. Çalışmada, ELISA yöntemini kullanmışlar ve uyguladıkları test sonucunda 400 hayvandan 17'sinde MAP yönünden pozitiflik tespit etmişlerdir. Karakaş ve ark. (2018), Kırklareli ve yöresinde yaptıkları bir çalışmada yaşları 3-4 arasında değişen 400 adet süt sığırına ait kan serumunda, seroprevalans oranlarını sırasıyla %1.5 pozitif, %1.5 şüpheli ve %97 negatif olarak tespit etmişlerdir. Bir diğer araştırmada Çelik ve ark. (2022) Şanlıurfa ve yöresinde tesadüfi örnekleme yöntemi ile Şanlıurfa'nın merkez ilçelerinden, 465 sığırdan alınan kan serumlarını ELISA yöntemini kullanarak incelemişlerdir. Serumdaki anti-MAP antikorlarını tespit etmek amacıyla çalıştıkları 465 serumun 21'ini (%4,51) pozitif, 16'sını (%3,4) şüpheli ve 428'ini (%92) negatif bulmuşlardır. Manisa ilinde Berberoğlu ve ark. (2016) yaptıkları bir çalışmada 442 adet Holstein süt sığırına ait kan serumlarını ELISA yöntemi ile paratuberkuloz hastalığı yönünden taramış, çalışmalarının sonucunda hastalığın seroprevalansını %21.72 olarak belirlemişlerdir.

Dünyada yapılan çalışmalara bakıldığında Good ve ark. (2009), İrlanda'da paratüberkülozun ELISA seroprevalansını belirlemek için 639 sürüden rastgele seçtikleri 12 aylıktan büyük 20.322 süt sığırının tamamından serum örnekleri toplamışlardır. Tüm numuneleri ELISA ile test etmişler, en az bir ELISA pozitif hayvanın varlığına dayalı olarak enfekte sürülerin genel prevalansını %21,4 bulmuşlardır. Belçika'da yapılan Aralık 1997'den Mart 1998'e kadar yürütülen bir serolojik araştırmada Boelaert ve ark. (2000) rastgele yaptıkları bir örneklemede 24 aylık veya daha büyük sığırların serumlarını antikorların varlığı açısından ELISA kiti kullanılarak test etmişlerdir. PTB sürü içi seroprevalansını (seropozitif sürüler içindeki tespit edilen hayvanların oranı) ve PTB bireysel hayvan seroprevalansını (tespit edilen hayvanların oranı) sırasıyla %2,9 ve %0,87 olarak bulmuşlardır. Park ve ark. (2006), Kore'deki çalışmalarında tüm illerde yetiştirilen 1261 adet süt sığırının kan serumlarını toplamışlar, 2.161 serumun 71'i pozitif çıkmıştır. Ulusal PTB prevalansının %7,1 olduğu tahmin edilmiştir.

Çalışmamızda Ege Bölgesi'nin birbirinden farklı 7 ilinde yetiştirilen sığırların paratüberküloz hastalığı yönünden seroprevalanslarının belirlenmesi amaçlanmıştır. İzmir ilinde 16 (%10), Manisa ilinde 16 (%10), Denizli'de 8 (%6,6), Aydın 4 (%2,5), Kütahya 20 (%16.6), Uşak 16 (13,3), Manisa 4 (%2,5) bireysel seroprevalans saptanmıştır. Kars, Ardahan, Kırıkkale ve Şanlıurfa'da yapılan çalışmalarda bu çalışmadan daha düşük seroprevalas saptansa da Manisa ilinden yapılan çalışmada daha yüksek oranda bir seroprevans tespit edilmiştir. Ülkemizde yapılan bu çalışmalardaki seropozitiflik oranları arasındaki farklılıkların hayvan nakilleri, besleme ve bakım koşulları farklılıkları, çalışma materyali alınırken mevsim dönemi ve iklim gibi farklılıklardan kaynaklandığı düşünülmektedir.

Bu araştırmada Ege Bölgesi illerinde yetiştirilen sığırlarda paratüberküloz seroprevalansı üzerinde çalışılmıştır. Sonuç olarak yapılan çalışma sonucunda Ege bölgesi yöresinde paratüberküloz infeksiyonunun seroprevalansı %8,4 belirlenmiştir. Çalışmanın kapsamı genişletilerek bölge ve ülke çapında yapılacak araştırmalarla hastalık ile mücadele açısından infeksiyonun gerçek boyutlarının düzenli aralıklar ile belirlenmesi ve hastalığın koruma, kontrol ve eradi-

kasyon yöntemlerinin belirlenerek uygulanmasının gerekli olduğu düşünülmektedir.

Etik kurul onayı: Çalışmamızın etik kurul izni gerektirmediğine dair beyan formu ek dosyalar kısmında imzalanıp yüklenmiştir.

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Özgün Araştırma
Original Article

Sütte Glukokortikoidlerin analizi için metot karşılaştırması

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Özet: Betametazon, deksametazon, prednizolon ve metilprednizolon büyükbaş hayvanlarda glukokortikoidlere duyarlı çeşitli akut ve kronik hastalıklarda yaygın olarak kullanılan sentetik glukokortikoidlerdir. Glukokortikoidler sütte kalıntı yapmaları sonucu insanlarda ciddi sağlık sorunlarına yol açabilmektedir. Bu çalışmada, sütte glukokortikoidlerin belirlenmesine yönelik hassas metodun seçilebilmesi için sıvı-sıvı ekstraksiyon (liquid liquid extraction, LLE), katı faz ekstraksiyon (solid phase extraction, SPE) ve dispersif katı faz ekstraksiyon (dispersive solid phase extraction, DSPE) metotları karşılaştırılmış ve en hassas metot seçilerek metodun performans özelliklerinin belirlemek için geçerli kılma çalışmaları yapılmıştır. Kromatografik ayırım, Diyot Dizi Dedektörü (Diode Array Detector, DAD) ile bütünleşik Yüksek Performanslı Sıvı Kromatografi (High Performance Liquid Chromatography, HPLC) ile gerçekleştirilmiştir. Betametazon 0,12 µg/kg, 0,3 µg/kg, 0,45 µg/kg seviyesinde analit eklenmiş örneklerin geri alımı %97 ila %102 arasında, deksametazon 0,12 µg/kg, 0,3 µg/kg, 0,45 µg/kg seviyesinde analit eklenmiş örneklerin geri alımı %97 ila %102 arasında, metilprednizolon 0,2 µg/kg, 2 µg/kg, 3 µg/kg seviyesinde analit eklenmiş örneklerin geri alımı %94 ila %100 arasında, prednizolon 0,6 µg/kg, 6 µg/kg, 9 µg/kg seviyesinde analit eklenmiş örneklerin geri alımı %97 ila %99 arasında değişmiştir. C8 kartuş ile valide edilmiş ekstraksiyon yöntemi, sütte deksametazon, betametazon, prednizolon ve metilprednizolonun kantitatif taraması için güvenilir ve basit bir metot olup günlük laboratuvar kullanımına uygun olabileceği sonucuna varılmıştır.

Anahtar kelimeler: dispersif katı faz ekstraksiyon, HPLC, katı faz ekstraksiyon, sıvı sıvı ekstraksiyon, süt

Comparison of methods for the analysis of Glucocorticoids in milk

Abstract: Betamethasone, dexamethasone, prednisolone and methylprednisolone are synthetic glucocorticoids that are widely used in various acute and chronic diseases sensitive to glucocorticoids in cattles. Glucocorticoids can cause serious health problems in humans as a result of residues in milk. In this study, in order to choose the sensitive method for the determination of glucocorticoids in milk, liquid-liquid extraction(LLE), solid phase extraction(SPE) and dispersive solid phase extraction(DSPE) methods were compared and the most sensitive method was selected and validation studies were carried out to determine the performance specifications of the method. Chromatographic separation was performed by Agilent 1100 series High Performance Liquid Chromatography (HPLC) combined with Diode Array Detector (DAD). The recovery of samples spiked with betamethasone 0.12 µg/kg, 0.3 µg/kg, 0.45 µg/kg ranged from 97% to 102%, the recovery of samples spiked with dexamethasone 0.12 µg/kg, 0.3 µg/kg, 0.45 µg/kg ranged from 97% to 102%, the recovery of samples spiked with methylprednisolone analyte at 0.2 µg/kg, 2 µg/kg, 3 µg/kg ranged from 94% to 100%, and the recovery of samples spiked with prednisolone at 0.6 µg/kg, 6 µg/kg, 9 µg/kg ranged from 97% to 99%. The validated extraction method using the C8 cartridge is a reliable and simple method for the quantitative screening of dexamethasone, betamethasone, prednisolone and methylprednisolone in milk and may be suitable for routine laboratory use.

Keywords: dispersive solid phase extraction, HPLC, liquid liquid extraction, milk, solid phase extraction

Giriş

Glukokortikoidler, adrenal korteks tarafından salgılanan metabolizma ve bağışıklık fonksiyonlarının yürütülmesini düzenleyen steroid hormonlardır (Luo ve ark., 2005; Dirikolu, 2013). Sentetik glukokortikoidler, doğal bir glukokortikoid olan kortizolün kimyasal yapısında yapılan modifikasyonlar ile anti-inflamatuar gücü arttırılıp ve mineralokortikoid etkileri azaltılarak geliştirilmiştir (Buchwald ve Bodor, 2004; Schimmer ve Funder, 2022). Geniş fizyolojik ve farmakolojik etkilerinin bir sonucu olarak, glukokortikoidler en çok kullanılan veteriner ilaç sınıflarından biridir. Sentetik glukokortikoidlerin büyükbaş hayvanlarda enfeksiyöz, metabolik ve inflamatuar hastalıklardaki terapötik uygulamaların yanı sıra; buzağılarda, boğalarda ve üretken döngülerinin sonundaki yaşlı ineklerde büyüme destekleyicileri olarak kullanımları yaygındır (Courtheyn ve ark., 2002). Gluko-

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kortikoidler, insan sağlığını olumsuz yönde etkileyen immunosupresyon, osteoporoz, obezite ve diyabet gibi birçok yan etkiye neden olduklarından süt gibi hayvansal gıdalardaki kalıntılarının tespiti önem arz etmektedir (Schacke, Döcke ve Asadullah, 2002). Terapötik endikasyonları nedeniyle, hayvan yetiştiriciliğinde deksametazon, betametazon, prednizolon ve metilprednizolonun uygulanmasına izin verilmektedir ve Avrupa Birliği düzenlemeleri ile uyumlu olarak Türkiye'de tüketime yönelik süt ve hayvansal dokularda maksimum rezidü limitleri (MRL) belirlenmiştir. Sütte MRL değerleri betametazon ve deksametazon için 0,3 µg/kg, prednizolon ve metilprednizolon için ise sırasıyla 6 µg/kg ve 2 µg/kg'dır (TC Tarım ve Orman Bakanlığı, 2017).

Glukokortikoidlerin sütte tespiti için birçok metot geliştirilmiştir (Guo ve ark., 2024; Caretti ve ark., 2010; Malone, Elliott, Kennedy ve Regan, 2010). Bu çalışmanın amacı glukokortikoidlerin sütte tespiti için tercih edilen metotlardan sıvı-sıvı ekstraksiyon, katı faz ekstraksiyon ve yeni nesil yaklaşımlardan dispersif katı faz ekstraksiyon metotlarının karşılaştırılarak en hassas metodun validasyonunu gerçekleştirmektir.

Katı Faz Ekstraksiyon; analitin, katı faz (sorbent) ile analiti içeren sıvı faz arasında dağılım katsayısına uygun olarak bölünmesi, analitin katı faza adsorpsiyonu ve ardından katı fazdan daha fazla tercih ettiği organik çözücü ile elüe edilmesi esasına dayanır. Van der Waals, hidrojen bağlanması, dipol-dipol veya elektrostatik (iyon değişimi) gibi farklı etkileşimlere dayanan mekanizmalar ekstraksiyonda rol oynar (Ötles ve Kartal, 2016).

Sıvı-sıvı ekstraksiyon; analitin, sulu faz ve organik faz arasında belirli bir oranda kendisini dağıtabilmesi prensibine dayalı olarak çalışan bir yöntemdir. İyonize olmayan analitlerin polar fazdan polar olmayan organik faza aktarılması asit-baz kimyası gereği analitin yüksüz hale geçebilmesi sonucu gerçekleşir. Analitin yüksüz hale geçebilmesi, sulu fazın pH' sının, analit asidik iyonlaşma sabiti (pKa)değerine göre ayarlanması ile sağlanır (Kyle, 2017).

Dispersif katı faz ekstraksiyonu; analiti içeren sıvı fazda, katı fazın (sorbent) dağılmasına prensibine dayalı olarak çalışan bir yöntemdir. Dispersiyon sonrası, yüzeyinde tutunan analitlerle birlikte sorbent, santrifüjleme veya filtreleme gibi mekanik bir işlemle ayrılarak yüzeyinde adsorbe edilmiş analitlerin organik çözücü ile ayrıştırılması amaçlanır. Katı faz ekstraksiyonda olduğu gibi sorbentin yapısal özelliklerine göre elektrostatik etkileşimler, hidrojen bağları, Van der Waals kuvvetleri, hidrofobik ve hidrofilik etkileşimler ve dipol-dipol bağları gibi etkileşimler söz konusudur (Islas, Ibarra, Hernandez, Miranda ve Cepeda 2017).

Metot karşılaştırması için tercih edilen metotlardan geri alımı yüksek olan metot, matrix-matched kalibrasyon yöntemlerinden biri olan standartla güçlendirilmiş matriks yöntemi kullanılarak her bir analit için ayrı ayrı 2021/808/EU yönetmeliğine göre valide edilmiştir (The European Commission, 2021).

Bu çalışmada, sütte glukokortikoidlerin belirlenmesine yönelik hassas metodun seçilebilmesi için sıvı-sıvı ekstraksiyon (liquid liquid extraction, LLE), katı faz ekstraksiyon (solid phase extraction, SPE) ve dispersif katı faz ekstraksiyon (dispersive solid phase extraction, DSPE) metotlarının karşılaştırılması ve en hassas metodun performans özelliklerinin belirlemesi için geçerli kılma çalışmalarının yapılması amaçlanmıştır.

Gereç ve Yöntem

Reaktifler ve Kimyasallar Çalışmada; Metanol (%99, HPLC düzey), asetonitril, diklormetan, sodyum dihidrojen fosfat dihidrat**,** disodyum hidrojen fosfat, formik asit, izopropanol, heptan, etil asetat, trietilamin, asetik asit, sodyum hidroksit (Isolab Chemicals, Almanya), oktil silika (C8) ters faz kartuş(500 mg/6 ml), oktadesil silika (C18) sorbent (United Chemical Technologies, USA) kullanılmıştır.

Standart maddeler Çalışmada; Betametazon, deksametazon, prednizolon ve metilprednizolon referans standartları (Sigma-Aldrich, USA) kullanılmıştır. Standart maddelerin stok çözeltileri metanol (MeOH) içerisinde 1 mg/ml seviyesinde hazırlanmış ve -20°C 'de muhafaza edilmiştir.

Analitik cihazlar Kromatografik analizler Agilent 1100 serisi Diyot Dizi Dedektörü (Diode Array Detector, DAD) ile bütünleşik Yüksek Persformanslı Sıvı Kromatografi (High Performance Liquid Chromatography, HPLC) ile değerlenmiştir. Hedef analitlerin tespiti Agilent XDB-C18 kolon (150 mm x 4,6 mm, 5 µm) ile sağlanmıştır. Mobil faz akış hızı 0.7 ml/dk, akış şekli izokratik ve cihaz metot süresi 4 dk'dır. Cihaz kolonu sıcaklığı 40°C ve enjeksiyon hacmi 10 µl'dir. Mobil faz A: su/formik asit (%0,1), B:metanoldür.

Ekstraksiyon yöntemleri Katı faz ekstraksiyonda kartuş aktivasyonu (şartlandırma) için 3 ml metanol ve 3 ml fosfat tampon ph 6,8 kullanılmıştır. Ardından MRL düzeyinde analit içeren 5 ml süt örneği ve 2 ml fosfat tampon ph 6,8 kartuştan geçirilmiş, en son 1 ml heptan ile kartuştan kirliliğe neden olabilecek

moleküller uzaklaştırılmıştır. Elüsyon 5 ml trietilamin/etilasetat (10:90, h/h) çözeltisi ile gerçekleştirilmiştir. Elüat nitrojen gazı altında 40°C'de tamamen kuruyana kadar bekletilmiş ve 100 µl metanol ile çözülerek HPLC sistemine enjekte edilmiştir.

Sıvı sıvı ekstraksiyonda 5 ml MRL düzeyinde analit ihtiva eden süt örneği 50 ml'lik polipropilen tüpe alınarak üzerine ekstraksiyon çözeltisi olarak diklormetan/izopropanol (75:25, h/h) çözeltisinden 5 ml eklenmiştir. Orbital çalkalayıcıda 30 dk boyunca çalkalama sonrası elüat ve süt örneğinin ayrımı için 5000 rpm de 10 dk santrifüj edilmiş ve supernatant uzaklaştırılmıştır. Elüat nitrojen gazı altında 40°C'de tamamen kuruyana kadar bekletilmiş ve 100 µl metanol ile çözülerek HPLC sistemine enjekte edilmiştir.

Dispersif katı faz ekstraksiyonda MRL düzeyinde analit ihtiva eden 5 ml süt örneği 50 ml'lik bir polipropilen tüpe alınarak üzerine 10 ml etil asetat ilave edilip vorteks yardımıyla karıştırılmış ve 30 dakika boyunca çalkalanmıştır. Örnek karışımı 5000rpm'de 5 dakika boyunca santrifüj edilip ve -20°C'de 6 saat süreyle dondurulmuştur. Ekstrakt bir tüpe aktarılarak nitrojen altında kuruyana kadar buharlaştırıldıktan sonra 1 ml asetonitril/su çözeltisi (20:80, h/h) içerisinde yeniden çözülmüş, karışım, 50 mg C18 sorbenti içeren 10 ml'lik bir santrifüj tüpüne aktarılmıştır. Tüp 1 dk boyunca kuvvetlice çalkalandıktan sonra 5000 rpm'de 5 dakika boyunca santrifüj edilmiştir. Elüat tüpe aktarılarak nitrojen altında kuruyuncaya dek buharlaştırılmış ve 100 µl metanol ile çözülerek HPLC sistemine enjekte edilmiştir.

Bulgular

Yüksek Performanslı Sıvı Kromatografisi Parametreleri

Glukokortikoidlerin kantitatif tespitinde, mobil fazın asidik yapısını ayarlayabildiği ve hedef analitin iyonizasyonunu arttırarak polaritesini değiştirmeyi ve kolon alıkonma süresini değiştirdiği için mobil fazda formik asit kullanılmıştır (Dolan, 2017).

Hedef analitlerin kolon alıkonma zamanlarının ve dalga boylarının tespit edilebilmesi için, MRL değerinin 0,5 ve 1 katı düzeyinde analit eklenerek ekstraksiyonu yapılan boş (kör) süt örnekleri, boş (kör) olarak ektraksiyonu yapılmış cihaz analizinden önce üzerine 0,5 MRL ve 1 MRL düzeyinde analit eklenerek hazırlanan süt örnekleri (matriks eklenmiş standartlar) ve 0,5 MRL ve 1 MRL çalışma seviyelerinde hazırlanan analit saf standartları halinde hazırlanarak aynı koşullar altında HPLC'de analiz edilmiştir. Validasyonlarda matrix-matched kalibrasyon yöntemi kullanıldığı için matriksle güçlendirilmiş standartlar analitik yöntemin performans kriterlerinin araştırılması çalışmalarında yer almıştır. Yapılan bu çalışma sonucu analitlerin, ekstrakttaki alıkonma sürelerinin, ± 0,1 dakikalık bir toleransla kalibrasyon standartlarına, matriksle güçlendirilmiş standartlarına karşılık geldiği 2021/808/EU yönetmeliği gerekliliğini karşıladığı görülmüştür. Daha önce yapılan bir çalışma ile uyumlu olarak, analitlere ait dalga boylarının aynı olduğu görülmüş ve analitlere özgü dalga boyları ve alıkonma zamanları Tablo 1'de sunulmuştur (Anonim, 2011).

Tablo 1. Hedef analitler için alıkonma zamanları ve dalga boyları

Analit	Alıkonma Zamanı (dk*)	Dalga Boyu (nm**)
Betametazon	1.92	254
Deksametazon	1.92	245
Prednizolon	1.92	254
Metilprednizolon	1.92	254

*Dakika **Nanometre

Metot Karşılaştırma Çalışmaları

Çalışmada seçilmiş olan glukokortikoidler nonpolar davranış sergileyen nötr maddelerdir (A. Tölgyesi, L. Tölgyesi, Sharma, Sohn ve Fekete, 2010). Glukokortikoidlerin katı faz ekstraksiyon ve dispersif katı faz ekstraksiyonlarında, hidrofobik etkileşimleri ve yapılarındaki uzun karbon zincirleri nedeniyle polar olmayan analitlerin ekstraksiyonunda tercih edilen C8 ekstraksiyon kartuşları ve C18 sorbentleri kullanılmıştır (Anonim, 2023). Ekstraksiyon solventleri sıvı-sıvı ekstraksyion, katı faz ekstraksiyon ve dispersif katı faz ekstraksiyonları için sırasıyla diklormetan/izopropanol (75:25, h/h), trietilamin/etilasetat (10:90, h/h) ve etil asetattır. MRL düzeyinde analit içeren süt örnekleri üç farklı metotla on tekrar yapılarak analiz edilmiş ve tüm hedef analitler için katı faz ekstraksiyon metodunun en yüksek geri kazanıma ve tekrarlanabilirliğe sahip olduğu belirlenmiştir. Dispersif katı faz ekstraksiyonda her bir analit için MRL düzeyinde yapılan çalışmalarda HPLC-DAD'da sinyal gürültü ayrımı gerçekleşmemiştir. Katı faz ve sıvı faz ekstraksiyonlara ait tekrarlanabilirlik standart sapması ve % geri kazanım değerleri Tablo 2'de yer almaktadır.

Tablo 2. Katı faz ve sıvı-sıvı faz ekstraksiyonlara ait tekrarlanabilirlik standart sapması ve % geri kazanım değerleri

Analit Adı	Katı Faz Ekstraksiyon		Sıvı Sıvı Faz Ekstraksiyon	
	Geri Kazanım (%) (n=10)	Tekrarlanabilirlik RSD* (%) (n=10)	Geri Kazanım (%) (n=10)	Tekrarlanabilirlik RSD* (%) (n=10)
Betametazon	101	7,0	81,3	17,6
Deksametazon	96,3	9,4	82,8	21,8
Prednizolon	98	4,1	79,3	13,8
Metilprednizolon	99,2	7,2	81,7	18,5

RSD*: Bağıl Standart Sapma

Metot Validasyonu

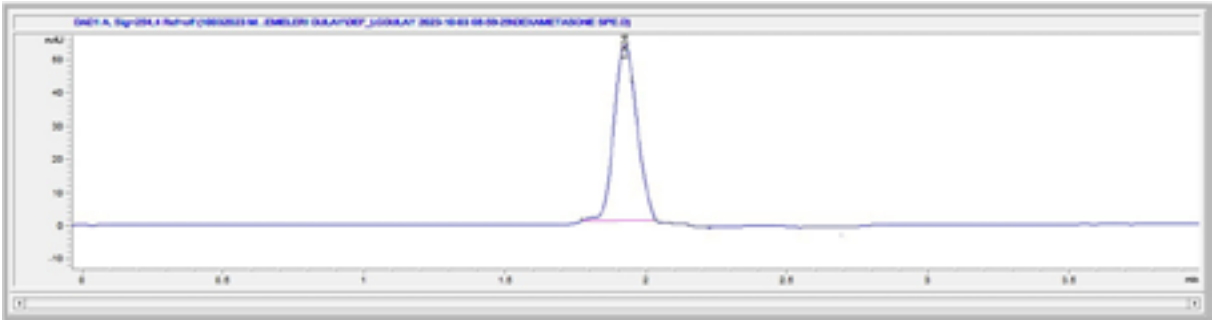
Çalışmada yer alan glukokortikoidlerin HPLC-DAD'da alıkonma zamanları ve UV spektrumundaki absorpsiyon maksimumları (dalga boyu) aynı olduğu için, yöntemin performans özellikleri her bir glukokortikoid için ayrı ayrı validasyon çalışması yapılarak belirlenmiştir. Yöntem sırası ile betametazon-deksametazon için 0 µg/kg (kör), 0,12 µg/kg, 0,3 µg/kg, 0,45 µg/kg, 0,6 µg/kg, prednizolon için 0 µg/kg (kör), 0,6 µg/kg, 6,0 µg/kg, 9,0 µg/kg, 12,0 µg/kg ve metilprednizolon için 0 µg/kg (kör), 0,2 µg/kg, 2,0 µg/kg, 3,0 µg/kg, 4,0 µg/kg doğrusal çalışma aralıklarında standartla güçlendirilmiş matriks yöntemi kullanılarak HPLC-DAD'da kantitatif tarama metodu olarak geçerli kılınmıştır. Farmakolojik aktif madde grubunda yer alan hedef analitlerin kantitatif tarama metodu olarak geçerli kılma çalışmaları 2021/808/EU yönetmeliğinde yer alan kriterler çerçevesinde gerçekleştirilmiştir. 2021/808/EU yönetmeliğinde "Spesifik bir farmakolojik aktif madde için, MRL'nin 0,1 katı bir konsantrasyonun doğrulanmasının makul bir şekilde elde edilemediği durumlarda, MRL'nin 0,1 katı olan konsantrasyonun, makul olarak ulaşılabilir olan MRL'nin 0,1 katı ile 0,5 katı arasındaki en düşük konsantrasyon ile değiştirilebilir" ifadesine uygun olarak deksametazon ve betametazon için 0,4 MRL, prednizolon ve metilprednizolon için 0,1 MRL konsantrasyon düzeyi lineer olarak tespit edilmiştir.

Kalibrasyon eğrileri, analit pik alanlarının analit konsantrasyonlarına karşı oranlarının grafiğinin çizilmesiyle elde edilmiş ve sonuçlar doğrusal regresyon kullanılarak analiz edilmiştir. Kalibrasyon eğrileri R^2 değerleri 0,996-0,997 aralığında tespit edilmiştir.

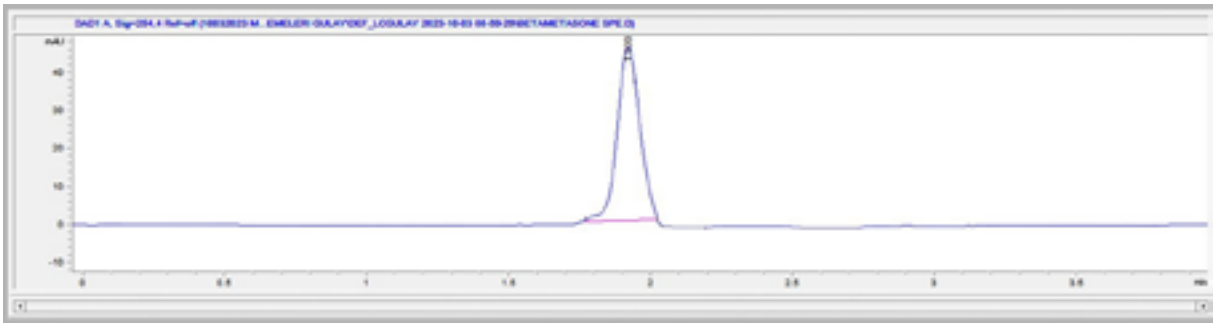
Metodun doğruluğu, gerçeklik (% geri kazanım) ve kesinliği (tekrarlanabilirlik ve laboratuvar içi tekrar üretilebilirlik) belirlenerek değerlendirilmiştir. Geri alımı ve kesinliği (tekrarlanabilirlik ve laboratuvar içi tekrar üretilebilirlik) değerlendirmek için sırası ile betametazon ve deksametazon için 0.12 µg/kg, 0.3 µg/kg, 0.45 µg/kg, prednizolon için 0,6 µg/kg, 6,0 µg/kg, 9,0 µg/kg ve metilprednizolon için 0.2 µg/kg, 2,0 µg/kg ve 3,0 µg/kg konsantrasyon seviyelerinde standart çözeltileri ile güçlendirilmiş boş (kör) süt örnekleri kullanılmıştır. Her konsantrasyon seviyesinde güçlendirilen örnekler, her biri farklı bir günde ve her biri altı tekrar halinde olmak üzere üç seri halinde analiz edilmiştir. Geri kazanımlar, analit eklenen örneklerin belirlenen konsantrasyonlarını, hedef seviyelerle karşılaştırılarak hesaplanmıştır. Betametazon 0,12 µg/kg, 0,3 µg/kg ve 0,45 µg/kg seviyesinde analit eklenmiş örneklerin geri alımı %97 ila %102 arasında, deksametazon 0,12 µg/kg, 0,3 µg/kg ve 0,45 µg/kg seviyesinde analit eklenmiş örneklerin geri alımı %97 ila %102 arasında, metilprednizolon 0,2 µg/kg, 2,0 µg/kg ve 3,0 µg/kg seviyesinde analit eklenmiş örneklerin geri alımı %94 ila %100 arasında, prednizolon 0,6 µg/kg, 6,0 µg/kg ve 9,0 µg/kg seviyesinde analit eklenmiş örneklerin geri alımı %97 ila %99 arasında değişmiştir ve 2021/808/EU yönetmeliğine göre minimum kabul edilebilir % geri kazanım değeri sağlanmıştır (≤ 1 µg/kg için %50-%120 ve > 1 µg/kg - 10 µg/kg için %70-%120). t-testi ile kontrol edilen geri alım sonuçları %95 güven aralığında serbestlik derecesi 54 için 1,42 ve 1,60 arasında değişerek t tablo(α=2,00) değerinden küçük olduğundan gerçek değere yakın olduğu kabul edilmiştir. Tekrarlanabilirlik ve tekrarüretilebilirlik sonuçları Horwitz eşitliğinden hesaplanan $\%RSD_{kritik}$ değerleri kıyaslanarak %RSD ≤ %RSDkritik(< 10 µg/kg için %30) koşulunu sağladığından, sonuçlar uygun olarak değerlendirilmiştir (The European Commission, 2021). Validasyon parametrelerine ait tekrarlanabilirlik, tekrarüretilebilirlik, % geri kazanım, MRL, hedef tarama konsantrasyonu (STC) verileri Tablo 3'te ve MRL düzeyinde güçlendirilmiş örneklere ait kromatogramlar aşağıda verilmiştir.

Tablo 3:Katı faz ekstraksiyon yöntemi validasyon parametreleri

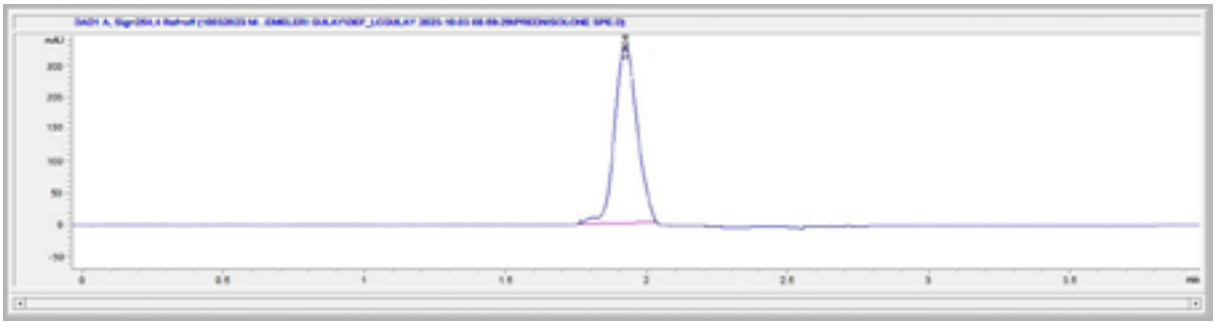
Analit	Konsantrasyon (µg/kg)	Geri Alım (%)	Tekrarlanabilirlik RSD (%)	Tekrar Üretilebilirlik RSD (%)	MRL (µg/kg)	STC (µg/kg)
Betametazon	0,12	96,6	6,6	7,4	0,3	0,12
	0,3	100,5	6,1	8,4		
	0,45	101,6	6,5	9,9		
Deksametazon	0,12	96,6	12,9	13,9	0,3	0,12
	0,3	99,9	10,1	11,2		
	0,45	101,7	9,5	10,7		
Prednizolon	0,6	96,7	3,2	5,8	6,0	0,6
	6,0	97,9	3,4	5,7		
	9,0	99,3	0,8	2,9		
Metilprednizolon	0,2	94,2	6,3	6,6	2,0	0,2
	2,0	99	6,9	7,2		
	3,0	99,5	5,8	6,4		



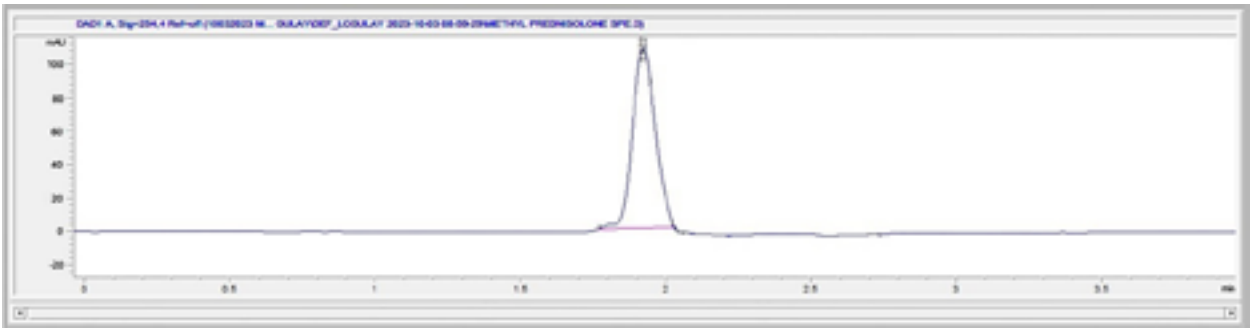
Şekil 1. Deksametazona ait kromatogram



Şekil 2. Betametazona ait kromatogram



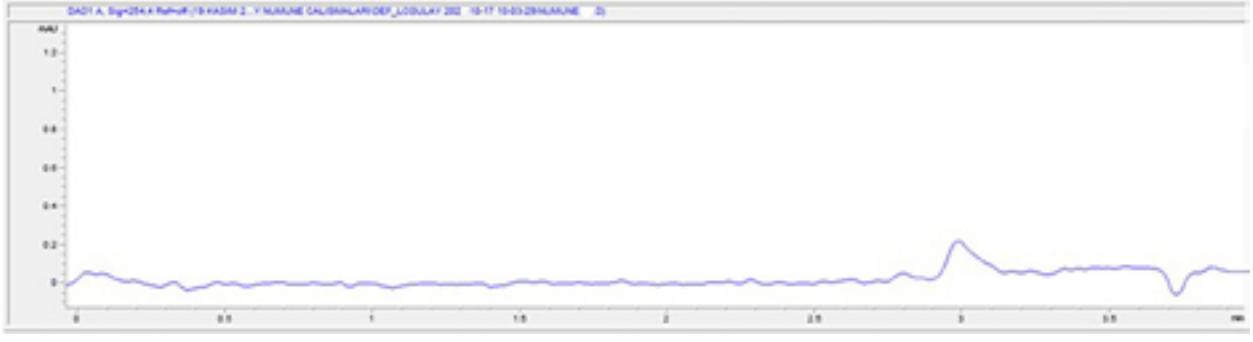
Şekil 3. Prednizolona ait kromatogram



Şekil 4. Metilprednizolona ait kromatogram

Analitlerin matriks içerisindeki stabilitesinin belirlenmesi için, STC düzeyinde güçlendirilmiş süt örnekleri -20°C'de muhafaza edilmiştir. Saklama koşullarının matriks içerisindeki analit stabilitesine etkisini ölçmek için -20°C'de muhafaza edilen STC düzeyinde güçlendirilmiş 5 süt örneği ve yeni hazırlanan STC düzeyinde güçlendirilmiş 5 süt örneği geçerli kılınan metot ile analiz edilmiştir. Stabilite çalışması kısa, orta ve uzun aralıkları için üç kez tekrarlanmış ve her çalışmaya ait % geri kazanım ve RSD'ler hesaplanmıştır. Kısa, orta ve uzun dönem stabilite çalışmaları % geri kazanım verileri betametazon için %95,1 deksametazon için %98,8 prednizolon için %98,6 ve metilprednizolon için %94,8 olarak tespit edilmiştir. %RSD verileri betametazon için 6,7 deksametazon için 13,6 prednizolon için 4,1 ve metilprednizolon için 6,4 olarak tespit edilmiş ve STC düzeyinde hesaplanmış olan metot laboratuvar içi tekrarüretilebilirliği ile anlamlı fark görülmediğinden, saklama koşullarının matriks içerisindeki analit stabilitesini etkilemediği belirlenmiştir (The European Commission, 2021).

2021/808/EU yönetmeliğinde yer alan uygulamalar doğrultusunda seçicilik / spesifiklik parametresinin belirlenmesi için 20 farklı boş (kör) süt örneği analiz edilmiş ve hedef analite ait pikin görülmesinin beklendiği bölgede herhangi bir sinyal, tepe noktası, pik varlığına rastlanmamıştır. Yine 20 farklı boş (kör) süt örneği hedef analitlerin tanımlanmasına ve/veya niceliğine müdahale edebilecek klenbuterol ile hedef analitlerin MRL seviyesinde güçlendirilerek analiz edilmiş ve analitlerin tanımlanmasını engellemediği görülmüştür. Boş (kör) süt örneğine ait kromatogram aşağıda verilmiştir.



Şekil 5. Boş (kör) örneğe ait kromatogram

HPLC-DAD'da kantitatif tarama yöntemi için dedeksiyon kapasitesi (Detection Capacity, CCβ), betametazon için 0,36 µg/kg, deksametazon için 0,58 µg/kg, prednizolon için 0,79 µg/kg ve metilprednizolon için 0,42 µg/kg olarak belirlenmiştir. CCβ 'nın hesaplanma formülü aşağıda yer almaktadır. Birleştirilmiş belirsizlik STC'de hesaplanmış olup birleştirilmiş belirsizliğin bileşenleri laboratuvar içi tekrar üretilebilirlik ve gerçekliktir (The European Commission, 2021).

CCβ = Tarama hedef konsantrasyonu (STC) + k(tek taraflı, %95) × STC'de veya üzerinde (birleşik) standart ölçüm belirsizliği.

k-faktörü MRL düzeyinde izin verilen maddeler için 1,64'tür.

Tartışma ve Sonuç

Çalışma süt örneklerinde katı faz ekstraksiyonun, sıvı-sıvı ekstraksyion ve dispersif katı faz ekstraksiyona göre üstünlüğe sahip olduğunu göstermektedir. Dispersif katı faz ekstraksiyon metodu dondurma basamağı nedeniyle analiz süresini diğer metotlara göre uzatmıştır. Katı faz ekstraksiyonda, sıvı-sıvı ekstraksiyondaki gibi emülsiyon oluşma riski elimine edilmiştir. Katı faz ekstraksiyon manifold sistemi kullanılması nedeniyle aynı anda çoklu örnek analizine avantaj sağlamakta ve zaman kaybının önüne geçmektedir. Katı faz ekstraksiyon daha fazla solvent kullanımını gerektirse de örnekteki istenmeyen bileşikleri ortamdan uzaklaştırması sebebiyle daha temiz analiz sonuçlarına ulaşılmasını sağlamaktadır. Kromatografi sisteminde matriksten gelebilecek bileşiklere ait sinyaller hedef analitin sinyaline girişim yapabilme riski ve dolayısıyla hedef analitin sinyalinde artış ya da baskılamaya sebep olabilmektedir. HPLC sisteminde cihaz metot süresinin kısa olması ve türevlendirme gibi basamaklara ihtiyaç olmaması yöntemin diğer avantajlarındandır (Cun, Yinliang, Ting ve Yan, 2010). Tekrarlanabilirlik ve tekrarüretilebilirlik sonuçları Horwitz eşitliğinden hesaplanan $\%RSD_{kritik}$ değerleri kıyaslanarak %RSD ≤ %RSDkritik(< 10 µg/kg için %30) koşulunu sağladığından, yöntemin tekrarlanabilir ve tekrarüretilebilir olduğunu ve HPLC-DAD'da sütte betametazon, deksametazon, prednizolon ve metilprednizolon için kantitatif tarama yöntemi olarak kullanılabileceğini göstermiştir (The European Commission, 2021). Çalışmada HPLC-DAD'da hedef analitlerin, cihaz kolonunda alıkonma zamanlarının ve dalga boylarının aynı olması, yöntemin tek başına MRL düzeyini aşan hedef analitlerin varlığını ve konsantrasyonunu belirlemek için tarama yöntemi olarak uygulanabileceği, ancak likit kromatografi sıralı kütle spektrometresinde (LC-MS/MS) analitin kendisine özgü ana iyon ve parçalanma iyonlarının identifiye edilerek, doğrulanması gerektiği sonucuna varılmıştır. LC-MS/MS'te kütle/yük (m/z) oranına göre ayrılan moleküller parçalanma sonucu oluşan iyonları üzerinden tespit edilebilmektedir. Betametazon ve deksametazon için ana iyon 437 ve parçalanma iyonları 361 ve 307, metilprednizolon için ana iyon 419 ve parçalanma iyonları 343 ve 309, prednizolon için ana iyon 405 ve parçalanma iyonları 329 ve 295 olup, bahsi geçen iyonlar üzerinden LC-MS/MS'te ayırımları mümkün olabilmektedir (Arioli ve ark., 2022). Betametazon ve deksametazon birbirlerinin izomeri olup, kütle spektometrede aynı ana iyon ve parçalanma iyonlarını verdiğinden çeşitli kolonlarla (Hypercarb kolon gibi) ayırımı gerçekleştirilebilmektedir (Cun, Yinliang, Ting ve Yan, 2010). Kütle spektrometrede, C18 kolon kullanımında betametazon ve deksametazonun kolonda alıkonma süreleri aynı olduğundan ayırımları iyon oranı (ion ratio) farkı ile yapılabilmektedir (Turhan, Kabil, Dudaklı ve Dirikolu, 2018). Sütte glukokortikoidlerin tespiti için yapılan çalışma daha önce yapılan çalışmalarla analitik limitler üzerinden karşılaştırılmış ve Tablo 4'te sunulmuştur.

Tablo 4. Sütte glukokortikoidlerin belirlenmesi için önerilen yöntemin diğer bazı yöntemlerle karşılaştırılması

Analit	Matriks	Analiz Yöntemi	Analitik seviye (µg/kg)	Kaynak
Deksametazon	Süt	Likit Kromatografi Sıralı Kütle Spektrometri (Katı Faz Ekstraksiyon, Doğrulama Yöntemi)	CCβ: 0,76	(Cherlet, De Baere ve De Backer, 2004)
Deksametazon, Prednizolon, Betametazon	Süt	Sıralı Kütle Spektrometri (Katı Faz Ekstraksiyon, Doğrulama Yöntemi)	CCβ: 0,45-8,67	Mcdonald, Granelli ve Sjöberg, 2007)
Betametazon, Deksametazon, Prednizolon, Metil Prednizolon	Süt	Yüksek Performanslı Sıvı Kromatografi Diyot Dizi Dedektörü (Katı Faz Ekstraksiyon, Tarama Yöntemi)	CCβ:0,36-0,58-0,79-0,42	Çalışma verileri

En iyi sonuçları gösteren C8 kartuş ile valide edilmiş ekstraksiyon yöntemi basit ve güvenilir olmakla birlikte sütte deksametazon, betametazon, prednizolon ve metilprednizolonun kantitatif tarama metodu olarak günlük laboratuvar uygulamalarında uygulanabileceği ancak HPLC-DAD'da hedef analitlerin kolondaki alıkonma zamanları aynı olduğundan, HPLC-DAD'ın analitlerin identifikasyonu için tek başına yeterli olmayacağı, bu nedenle ileri doğrulama yöntemi olarak LC –MS/MS'te analitlerin kendilerine özgü ana iyon ve parçalanma iyonlarının identifiye edilerek, doğrulanması gerektiği sonucuna varılmıştır.

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Review Article
Derleme

Effects of bacterial biodosimeters against radiation: A review study

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Abstract: The bacterial biodosimeter is a type of biodosimeter that utilizes bacteria as the challenging organism to measure the effective dose of a reactor. These biodosimeters capitalize on the unique responses of bacterial systems to ionizing radiation, providing valuable insights into the biological effects of radiation, and enabling accurate dose estimation, and the potential health risks for living organisms. This review covers the details of the advantages and disadvantages of using bacteria for area monitoring of radiation and the current state of knowledge regarding bacterial biodosimeters. Additionally, methods for the detection of bacteria, protocols for radiation exposure, and factors that could influence culture conditions have been examined. This review aims to consolidate the existing knowledge on bacterial biodosimeters and stimulate further research to harness their full potential in radiation monitoring and protection.

Keywords: Biodosimeter; Bacterial biodosimeter; Exposure; Radiation; Optimization

Bakteriyel biyodozimetrelerin radyasyona karşı etkileri: Bir derleme çalışması

Özet: Bakteriyel biyodozimetre, reaktörün etkin dozunu ölçmek için bakterileri meydan okuma organizması olarak kullanan bir tür biyodozimetredir. Bu biyodozimetreler, bakteri sistemlerinin iyonlaştırıcı radyasyona karşı benzersiz tepkilerinden faydalanarak, radyasyonun biyolojik etkileri hakkında değerli bilgiler sunmakta ve doğru doz tahminini sağlamaktadır, aynı zamanda canlı organizmalar için potansiyel sağlık risklerini belirlemektedir. Bu derleme, radyasyonun alan izlemesi için bakterilerin kullanılmasının avantajları ve dezavantajlarının detaylarını ve bakteriyel biyodozimetreler hakkındaki mevcut bilgi durumunu kapsamaktadır. Ayrıca, bakterilerin tespit yöntemleri, radyasyon maruziyeti protokolleri ve kültür koşullarını etkileyebilecek faktörler incelenmiştir. Bu derleme, bakteriyel biyodozimetrelerin var olan bilgisini bir araya getirme ve radyasyon izleme ve koruma konularında potansiyellerini tam olarak kullanmak için daha fazla araştırmayı teşvik etmeyi amaçlamaktadır.

Anahtar kelimeler: Bakteriyel Biyodozimetre; Biyodozimetre; Maruziyet; Optimizasyon; Radyasyon

Introduction

According to the International Atomic Energy Agency (IAEA), some 23 million workers worldwide are are subjected to ionizing radiation as part of their job responsibilities (*Workers*, n.d.). In order to protect them against such an exposure, some specific steps as; regular monitoring, protective equipment, or countermeasures such as shielding can be followed (Boice et al., 2020). Additionally; training, information exchange, and consistent health surveillance are also important factors for an efficient occupational radiation protection regime (Albander, 2021). In the modern era of radiation exposure assessment and radiation protection, biodosimetry has emerged as a critical tool for accurately evaluating radiation doses and understanding their biological effects. Among the diverse array of biodosimetric approaches, bacterial biodosimeters have garnered increasing attention for their unique ability to provide reliable and versatile assessments of ionizing radiation exposure. These biodosensors capitalize on the distinct and specific responses of bacterial systems to radiation-induced damage, offering valuable insights into the radiation-induced biological effects and facilitating precise dose estimations(Heron et al., 2010).

The IAEA Occupational Radiation Protection Programme aims to promote and assist in establishing an internationally harmonized approach for optimizing occupational radiation protection(*International Conference on Occupational Radiation Protection – Strengthening Radiation Protection of Workers – Twenty Years of Progress and the Way Forward*, n.d.) . It offers direction to member nations of the International Atomic Energy Agency (IAEA) by means of a structured set of

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safety standards, categorized into three levels: Safety Fundamentals, Safety Requirements, and Safety Guides(*International Conference on Occupational Radiation Protection – Strengthening Radiation Protection of Workers – Twenty Years of Progress and the Way Forward*, n.d.). In addition to that it also helps Member States to apply these standards and guidelines in practice through various activities, such as training, information exchange, technical cooperation, and peer review services. In the year of 2014, the IAEA developed the Occupational Radiation Protection Call-for-Action with several other international organizations, which includes nine key areas that require global attention in the field of radiation protection of workers (*Workers*, n.d.). These actions have significance on the safe use of ionizing radiation. The IAEA also releases e-learning courses on radiation protection of workers to enhance national capabilities and awareness (Suárez et al., 2001).

In 2014, the (IAEA), in collaboration with various international organizations, created the Occupational Radiation Protection Call-for-Action. This document was designed to tackle the deficiencies, obstacles, and advancements within the domain of protecting workers from radiation exposure(*Workers*, n.d.). It states nine key areas that require global attention, which are:

1. Implementing existing safety standards and guidance
2. Strengthening assistance to countries with less developed programs for occupational radiation protection
3. Improving safety culture among exposed workers and their management
4. Enhancing the protection of workers in medicine
5. Enhancing the protection of workers exposed to natural sources.
6. Enhancing the protection of workers in the nuclear fuel cycle and decommissioning activities
7. Enhancing the protection of workers in emergency exposure situations and existing exposure situations
8. Convening international forum for information exchange
9. Strengthening education, training, and qualification programs for occupational radiation protection professionals

In this regard, these actions plays a crucial role and are taken for the safe utilization of ionizing radiation and ensuring that workers receive sufficient protection from potential hazards.

Dosimeters and biodosimeters

A dosimeter is an instrument employed to measure an individual's exposure to ionizing radiation. Typically, dosimeters provide a report in the form of a dose, expressed as the absorbed radiation energy measured in grays (Gy) or the equivalent dose measured in sieverts (Sv). There are different types of dosimeters, such as film badge, thermoluminescent, electronic or ion-chamber dosimeters. Dosimeters possess the capability to offer a quantitative and reproducible measurement of absorbed dose by inducing alterations in one or more of their physical characteristics when subjected to ionizing radiation energy. Dosimeters are worn by people who work with or near sources of radiation, such as radiographers, nuclear power plant workers, doctors using radiotherapy, or HAZMAT workers (Yukihara et al., 2022).

Biodosimetry is a measurement of biological response as a surrogate for radiation dose. Biodosimetry serves the purpose of estimating the dose and, when possible, forecasting or reflecting the medically significant reaction, which refers to the biological outcomes resulting from that dose(De Deene, 2022). Biodosimetry can utilize changes induced in the individual by ionizing radiation, such as cytogenetic damage, gene expression, protein or metabolite levels, or physiological alterations (Macchione et al., 2022). Ideally, the changes should be specific for ionizing radiation, and the response should be unaffected by other factors (Sproull et al., 2017). Biodosimetry can be useful for medical management of radiological emergencies, such as mass casualty incidents or accidental exposures (Swartz et al., 2014).

Popular types of biodosimetry methods can be listed as;

1. Dicentric chromosome assay: This method measures the frequency of dicentric chromosomes, which are abnormal chromosomes with two centromeres, in peripheral blood lymphocytes. Dicentric chromosomes are specific for ionizing radiation and correlate well with radiation dose.
2. Cytokinesis-block micronucleus assay: This method measures the frequency of micronuclei, which are small fragments of chromosomes that are not incorporated into the daughter nuclei during cell division, in binucleated lymphocytes. Micronuclei can be induced by ionizing

radiation and other genotoxic agents (Wilkins et al., 2017).

3. γH2AX marker of DNA damage: This method measures the level of phosphorylated histone H2AX (γH2AX), which is a marker of DNA double-strand breaks, in lymphocytes or other cell types. γH2AX foci can be detected by immunofluorescence staining and microscopy or flow cytometry (Wilkins et al., 2017).
4. Electron paramagnetic resonance (EPR): This technique quantifies the levels of persistent free radicals in biological substances like tooth enamel, nail clippings, or bone, which are generated as a result of exposure to ionizing radiation. EPR can provide an estimate of cumulative radiation exposure over a long period of time (Swartz et al., 2014).

Biodosimetry methods can be standardized and validated by following the guidance and recommendations from relevant authorities, such as the IAEA or the Food and Drug Administration (FDA) (Sholom et al., 2022). These authorities provide criteria and procedures for the performance, quality assurance, calibration, and interpretation of biodosimetry methods. Biodosimetry methods can also be standardized and validated by comparing them with physical dosimetry methods, such as thermoluminescent dosimeters or mobile phone components (*Radiation Biodosimetry Medical Countermeasure Devices Guidance for Industry and Food and Drug Administration Staff*, 2016). Such comparisons can help verify and validate the dose reconstruction accuracy and reliability of biodosimetry methods. Biodosimetry methods can also be standardized and validated by participating in inter-laboratory comparisons or proficiency testing programmes, such as the WHO BioDoseNet, which can help evaluate and improve the technical competence and performance of biodosimetry laboratories (Sproull et al., 2017).

Bacterial biodosimetry

Bacterial biodosimetry which is a form of biodosimetry that employs bacteria as an organism of challenge to assess the radiation dose of a reactor by evaluating its capacity to render them nonfunctional (Sperle et al., 2023). In another term bacterial biodosimetry is a type of biodosimetry that uses bacteria as a challenge organism to measure the fluence of a reactor by determining its ability to inactivate them. Bacterial biodosimetry can be used to evaluate the performance of lab-scale flow-through ultraviolet water disinfection reactors, which are used to control biofouling. Bacterial biodosimetry can be standardized and validated by following the same principles and procedures as other biodosimetry methods, such as using appropriate challenge organisms, avoiding photo repair, reducing protractions, and minimizing cell absorption on labware (*Workers*, n.d.). Bacterial biodosimetry can also be standardized and validated by comparing it with physical dosimetry methods, such as thermoluminescent dosimeters or mobile phone components (Sproull et al., 2017). Bacterial biodosimetry can also be standardized and validated by participating in inter-laboratory comparisons or proficiency testing programs.

Widespreadly used bacteria as challenge organisms for biodosimetry are:

1. *Escherichia coli*: This is a common gram-negative bacterium that can be found in the human gut and in various environments. It is widely used as a model organism for molecular biology and biotechnology. It is also sensitive to UV-C irradiation and can be easily cultured and quantified (Nocker et al., 2018).
2. *Bacillus subtilis:* This is a gram-positive bacterium that can form endospores, and are highly resistant to environmental stresses, including UV radiation. It is often used as a reference organism for biodosimetry of UV disinfection of water. It can also be used to study the mechanisms of DNA repair and spore formation (Sperle et al., 2023).
3. *Aquabacterium citratiphilum*: This is a gram-negative bacterium that can form biofilms on various surfaces, such as pipes or membranes. It is relevant for biofouling control by UV disinfection of water. It can also be used to study the effects of UV irradiation on biofilm formation and detachment (Sperle et al., 2023).
4. *Deinococcus radiodurans:* This is a bacterium known for its remarkable ability to withstand ionizing radiation, ultraviolet radiation, oxidation, desiccation, and various other environmental pressures (Farci et al., 2022). *Deinococcus radiodurans* is a crucial model for studying the mechanisms of DNA damage and repair, redox regulation, and survival strategies in response to high-dose ionizing radiation (Farci et al., 2022). *Deinococcus radiodurans* can also be used for bioremediation of radioactive waste or heavy metal pollution, as it can degrade organic compounds and transform toxic metals (Liu et al., 2023). *Deinococcus radiodurans* can also be

genetically manipulated to enhance its biotechnological applications (Ghosal et al., 2005).

Bacterial biodosimetry for area monitoring

Bacteria can be used for area monitoring of radiation by measuring their survival or inactivation rate after exposure to different doses of radiation. The same situation is also valid if their DNA damage or repair is measured after the exposure. Bacteria can also be used for area monitoring of radiation by measuring their growth rate or metabolic activity under different environmental conditions (Zhang et al., 2018). Bacteria can provide a biological indicator of the radiation level and the potential health effects on living organisms. Bacteria can also be used for bioremediation of radioactive waste or heavy metal pollution by degrading organic compounds or transforming toxic metals (*Workplace Monitoring - Home*, n.d.).

There are significant advantages of using bacteria for area monitoring. Bacteria are easy to culture, handle, and transport. Bacteria have a fast growth rate and a short generation time, which allows for rapid and repeated measurements. Bacteria have a high sensitivity and specificity to radiation, which enables accurate and reliable dose estimation. Bacteria have a wide range of radiation resistance and metabolic diversity, which allows for the selection of suitable strains for different radiation sources and environmental conditions. Bacteria can provide information on the biological effects of radiation, such as DNA damage, repair, mutation, and cell death.

On the contrary; disadvantages of using bacteria for area monitoring of radiation also exists. Bacteria may have different responses to radiation depending on their physiological state, growth phase, culture medium, and environmental factors. Bacteria may have different responses to radiation depending on the type, energy, dose rate, and quality of radiation. Bacteria may have different responses to radiation depending on the presence of other stressors, such as temperature, pH, oxygen, nutrients, or chemicals. Bacteria may have different responses to radiation depending on the interaction with other microorganisms or host cells. Bacteria may have limitations in detecting low doses or chronic exposures of radiation.

The technic to be applied for bacterial biodosimetry

To highlight the technic of standard culture conditions, irradiation protocols, and detection methods for bacteria some parameters should be taken into account.

Culture conditions; bacteria can be cultured in liquid or solid media with appropriate nutrients, pH, temperature, and oxygen levels (Wang & Salazar, 2015). The culture conditions should be optimized for the growth and survival of the selected bacterial strain and consistent for each experiment (Wang & Salazar, 2015). The culture conditions can be affected by the physiological state, growth phase, culture medium, and environmental factors of the bacteria. For example, the bacterial growth rate, metabolism, and resistance to radiation may vary depending on the nutrient availability, pH, temperature, oxygen level, and presence of other microorganisms or chemicals in the culture medium (Wang & Salazar, 2015).

Irradiation protocols; bacteria can be irradiated with different sources and doses of radiation, such as gamma rays, X-rays, or ultraviolet rays. The irradiation protocols should be standardized for the type, energy, dose rate, and quality of radiation and calibrated with physical dosimeters. The irradiation protocols should also minimize the exposure time and the variation of radiation dose across the sample. The irradiation protocols can be affected by the type, energy, dose rate, and quality of radiation and the sample matrix. For example, the radiation dose and the biological effects may vary depending on the source and energy of radiation, the exposure time and distance, the shielding and attenuation of radiation, and the composition and geometry of the sample.

Detection methods; bacteria can be detected and quantified by different methods, such as nucleic acid-based methods (e.g., PCR, hybridization), immunological methods (e.g., ELISA, immunofluorescence), biosensor methods (e.g., electrochemical, optical), or phenotypic methods (e.g., colony counting, turbidity). The detection methods should be sensitive, specific, accurate, and reliable for the target bacteria and compatible with the sample matrix. The detection methods can be affected by the sensitivity, specificity, accuracy, and reliability of the method and the sample matrix. For example, the detection limit, signal-to-noise ratio, false positive or negative results, and reproducibility may vary depending on the target bacteria, the detection principle and technique, the calibration and quality control of the method, and the interference or inhibition of the sample (Wise, 2006).

Conclusion

The conclusion of this review is that bacterial biodosimetry is a useful and promising technique for area monitoring of radiation, but it also has some challenges and limitations that need to be addressed. Bacterial biodosimetry requires standardization and validation of the culture conditions, irradiation protocols, and detection methods for bacteria, as well as the consideration of the factors that can influence the bacterial responses to radiation. Bacterial biodosimetry can provide information on the biological effects of radiation and the potential health risks for living organisms. Bacterial biodosimetry can also be integrated with other methods for radiation detection and bioremediation. There are some possible future directions for bacterial biodosimetry including, developing novel and improved culture conditions, irradiation protocols, and detection methods for bacteria that are more sensitive, specific, accurate, and reliable. The knowledge of exploring the molecular and cellular mechanisms of bacterial responses to radiation, such as DNA damage and repair, redox regulation, and survival strategies not only enhances our comprehension of the biological effects of radiation but also holds potential for the development of advanced radiation protection strategies. As we advance towards a deeper comprehension of bacterial biododosimeters, these invaluable tools will continue to play a pivotal role in safeguarding human health and enhancing radiation protection measures in various fields, including healthcare, space exploration, and emergency preparedness. Establishing and expanding the collaboration and communication among researchers, regulators, and stakeholders in the field of bacterial biodosimetry will require continued research and interdisciplinary collaboration.

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Review Article
Derleme

Enhancing biosafety and biosecurity: Quality management in high-containment laboratories

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Abstract: Quality Management in high-containment laboratories plays a pivotal role in ensuring the safehandling of biological agents and toxins, thereby mitigating potential biorisks. This paper provides a comprehensive exploration of the key aspects of Quality Management Systems (QMS) tailored to the unique challenges of high-containment laboratories. It delves into the significance of QMS in enhancing biosafety and biosecurity measures, safeguarding laboratorypersonnel, the community, and the environment. The paper also discusses the integration of international standards, risk management strategies, and the role of top management in fostering a culture of safety. Through this examination, it becomes evident that a robust QMS not only ensures compliance but also promotes continual improvement and innovation in high- containment laboratory operations, ultimately advancing the field of biosafety and biosecurity.

Keywords: Biological agents, biological risks, biosafety, Quality Management System

Biyogüvenlik ve biyogüvenliğin geliştirilmesi: Yüksek korumalı laboratuvarlardakalite yönetimi

Özet: Yüksek korumalı laboratuvarlarda Kalite Yönetimi, biyolojik ajanların ve toksinlerin güvenli bir şekilde ele alınmasını sağlamada ve böylece potansiyel biyolojik riskleri azaltmada çok önemli bir rol oynamaktadır. Bu makale, yüksek korumalı laboratuvarların kendine özgü zorluklarına göre uyarlanmış Kalite Yönetim Sistemlerinin (KYS) temel yönlerinin kapsamlı bir incelemesini sunmuştur. Biyogüvenlik ve biyogüvenlik önlemlerinin geliştirilmesinde, laboratuvar personelinin, toplumun ve çevrenin korunmasında KYS'nin önemini ele almıştır. Çalışmada ayrıca uluslararası standartların entegrasyonu, risk yönetimi stratejileri ve üst yönetimin güvenlik kültürünü teşvik etmedeki rolü de tartışılmıştır. Bu inceleme sayesinde, sağlam bir KYS'nin yalnızca uyumluluğu sağlamakla kalmayıp aynı zamanda yüksek muhafazalı laboratuvar operasyonlarında sürekli iyileştirme ve yeniliği teşvik ettiği ve nihayetinde biyogüvenlik ve biyogüvenlik alanını ilerlettiği anlaşılmıştır.

Anahtar kelimeler: Biyolojik ajanlar, biyolojik riskler, biyogüvenlik, Kalite Yönetim Sistemi

Introduction

The introduction to this paper lays the foundation for comprehending the intricate dynamics of Quality Management in high-containment laboratories concerning biorisks. High-containment laboratories serve as critical environments where the handling of biological agents and toxins demands an elevated degree of safety and security. In these specialized facilities, the management of biorisks, including those posed by pathogens of epidemic potential anddeliberate misuse, requires meticulous attention. This section of the paper initiates the discourse by emphasizing the indispensable role of Quality Management Systems (QMS) in orchestratingan effective response to these challenges (Coelho and Garcia Diez 2015; Peng et al. 2018).

Within high-containment laboratories, the paramount objective is to prevent laboratory- acquired infections, safeguard public health, and protect the environment from potential hazards associated with biological agents. Achieving this objective necessitates the systematic implementation of QMS protocols and principles. The paper undertakes a rigorous exploration of the multifaceted dimensions of QMS, underscoring its centrality in fortifying biosafety and biosecurity measures within the context of high-containment laboratories. As the paper progresses, it will delve into various facets of QMS, elucidating their intricate interplay within high-containment laboratory settings. Furthermore, it will elucidate the manner in which QMS harmonizes with international standards, affords a structured framework for risk management,and fosters a culture of safety and

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compliance. Ultimately, this section primes the reader for anin-depth examination of the critical components and implications of Quality Management in high-containment laboratories, subsequently contributing to the broader understanding of biorisk management in these specialized research environments (Zaki 2010; Allen 2013).

Regulatory Framework and International Standards

In the realm of high-containment laboratories, the regulatory landscape is characterized by a complex tapestry of guidelines, standards, and international regulations. These multifarious directives are instrumental in shaping the Quality Management practices within such facilities. This section of the paper will elucidate the intricate web of regulatory frameworks and international standards that exert a profound influence on the operations and protocols of high-containment laboratories (Hou et al. 2019).

Regulatory Oversight

Beyond the United States, high-containment laboratories operate within a global regulatory landscape that prioritizes safety, security, and ethical responsibility. Organizations such as the North Atlantic Treaty Organization (NATO) play a pivotal role in strengthening global biosecurity. Global approach to biorisks continues to be widely utilized in regulatory documents, amplifying the authoritative and non-negotiable nature of these guidelines. At the international level, the World Health Organization (WHO) provides guidance and recommendations for the safe and secure operation of high-containment laboratories. Global approach to biorisks remains prominent in these international standards, serving as a constant reminder of the stringent expectations for biosafety and biosecurity (Aravind and Christmann 2011). Moreover, various regional and national defense organizations collaborate to enhance global security against biological threats. NATO, as a defensive alliance, has been at the forefront of efforts to bolster biorisk management practices worldwide. In its directives and agreements, the global approach to biorisks is employed extensively to emphasize the collective commitment to preventing and mitigating potential risks associated with high-containment laboratories (Bchner et al. 1994; Bremond and Plebani 2001). The table 1. provides a general overview of the status of high-containment laboratories in selected countries:

Table 1. General overview of the status of high-containment laboratories in selected countries

Country	Status
United States	The US has a well-established regulatory framework for high-containment laboratories, overseen by the Centers for Disease Control and Prevention (CDC) and the US Department ofAgriculture (USDA).
United Kingdom	The UK also has a robust regulatory framework for high-containment laboratories, overseen by the Health and Safety Executive (HSE).
Canada	Canada has a well-developed regulatory framework for high-containment laboratories, overseen by the Public Health Agency of Canada (PHAC).
Australia	Australia has a well-established regulatory framework for high-containment laboratories, overseen by the Office of the Gene Technology Regulator (OGTR).
Japan	Japan has a well-developed regulatory framework for high-containment laboratories, overseen by the Ministry of Health, Labour and Welfare (MHLW).
China	China has a rapidly developing regulatory framework for high-containment laboratories, overseen by the National Health Commission (NHC).
India	India has a well-developed regulatory framework for high-containment laboratories, overseen by the Indian Council of Medical Research (ICMR).
Brazil	Brazil has a well-developed regulatory framework for high-containment laboratories, overseen by the National Health Surveillance Agency (ANVISA).
South Africa	South Africa has a well-developed regulatory framework for high-containment laboratories, overseen by the National Health Research Ethics Council (NHREC).

International Standards

Internationally recognized bodies such as the World Health Organization (WHO) and the International Organization for Standardization (ISO) play pivotal roles in shaping the regulatory landscape for high-containment laboratories. The ISO/IEC 17025:2017 standard, designed for the recognition of laboratory competence, serves as a cornerstone for many laboratories seeking accreditation. Additionally, the CEN Workshop Agreement (CWA)

15793:2011, renowned forits focus on biosafety and biosecurity, complements the accreditation process, reinforcing ethical provisions and technical aspects (Vlachos et al.2002; Castka and Balzarova 2008; Heires 2008). The standards for biorisks are given in table 2.

Table 2. The standards for biorisks

Standard	Date of Publication	Organization
Biosafety in microbiological and biomedicallaboratories	2020	Centers for Disease Control and Prevention(CDC) and National Institutes of Health (NIH)
Laboratory biosafety manual	2020	World Health Organization (WHO)
AS/NZS 2243.3:2019 safety in laboratories -part 3: microbiological safety and containment	2019	Standards Australia and Standards NewZealand
BS 5728:2014 code of practice formicrobiological safety in laboratories	2014	British Standards Institution (BSI)
CAN/CSA-Z316.10-19 biological safety cabinets - design, construction, performance, and testing requirements	2019	Canadian Standards Association (CSA)
ANSI/ASSE Z358.1-2014 american nationalstandard for emergency eyewash and shower equipment	2014	American National Standards Institute(ANSI)
NFPA 45 standard on fire protection forlaboratories using chemicals	2021	National Fire Protection Association(NFPA)

These instances represent only a subset of the numerous biorisk standards disseminated by diverse organizations worldwide. It is crucial to underscore that the precise set of standards applicable to a specific laboratory can exhibit variations contingent upon its geographical location, falling within the purview of distinct countries or regions (Aller 1996; Casey and Souvignet 2020).

Importance of Compliance

It is crucial to underscore that adherence to these regulatory frameworks and international standards is paramount for high-containment laboratories. Compliance with these standards is not only a matter of legal obligation but also a fundamental component of risk mitigation (Altenstetter 2012). Global approach to biorisks is intentionally utilized within these regulatory documents to establish clear guidelines and expectations, ensuring that laboratory activities align with the principles of biorisk management (Wijkström and McDaniels 2013).

Harmonization Challenges

Throughout this section, we will explore the nuances of these regulatory frameworks, shedding light on their overarching significance in shaping Quality Management practices. Moreover, wewill delve into the challenges and complexities associated with harmonizing diverse international standards and regulations, ultimately providing a comprehensive overview of theregulatory landscape that governs high-containment laboratories (Casey and Souvignet 2020).

COVID-19 and the Wuhan Laboratory

The emergence of COVID-19, caused by the novel coronavirus SARS-CoV-2, brought unprecedented global attention to laboratory safety, especially in high-containment facilities. The Wuhan Institute of Virology (WIV) in Wuhan, China, where the virus was first identified, became a focal point of discussions and investigations regarding the origins of the virus (Barman et al. 2020; Chan et al. 2020; Singh et al. 2020).

Amid the initial confusion and uncertainty surrounding the outbreak, questions arose about whether the virus could have accidentally leaked from a laboratory. This speculation fueled debates and underscored the paramount importance of rigorous biorisk management in high- containment laboratories (Cai et al.2020; Elfiky 2020).

Origins of the Controversy

The controversy surrounding the Wuhan laboratory primarily revolved around two hypotheses:zoonotic spillover and laboratory escape. The former suggests that the virus naturally transferred from animals to humans, possibly through a seafood market in Wuhan where live animals were also sold. The latter hypothesis raised concerns that the virus accidentally escaped from the laboratory due to lapses

in safety protocols (Nishiura et al.2020; Ruiz-Medina et al. 2022).

The Role of Laboratory Biosafety

The Wuhan laboratory incident, whether as the origin of the virus or not, brought laboratory biosafety and biosecurity into the global spotlight. It prompted discussions about the need for stricter adherence to established biorisk management protocols, stringent safety measures, and international collaboration in assessing laboratory safety (Kreuder Johnson et al. 2015; Domingo 2022).

Strengthening Biorisk Management

In response to the Wuhan laboratory incident, the international scientific community called for a comprehensive review of laboratory safety standards and practices. This included revisiting biosafety guidelines, enhancing transparency, and reinforcing international cooperation in monitoring high-containment laboratories. The Wuhan laboratory incident serves as a stark reminder that even the most advanced high-containment laboratories are not immune to potential risks. It underscores the critical importance of maintaining the highest standards of biorisk management, transparency, and accountability to prevent future incidents (Zhu and Cai 2020; Zhu et al.2020).

Lessons Learned

The global response to the COVID-19 pandemic and the scrutiny of laboratory safety in its wake have provided valuable lessons. These lessons emphasize the need for a global approach to biorisk management, stringent adherence to safety protocols, and open collaboration among nations to ensure the safe operation of high-containment laboratories (Coelho and García Díez2015; Munson 2018).

In conclusion, the Wuhan laboratory incident, regardless of its origins, has underscored the significance of biorisk management in high-containment laboratories worldwide. It serves as a catalyst for strengthening global efforts to enhance laboratory safety, protect public health, and advance scientific knowledge while minimizing the potential risks associated with infectious disease research (Su et al. 2020).

Future Directions

The evolving nature of biological research and the emergence of novel pathogens necessitate a dynamic and adaptive regulatory framework. In the coming years, international organizations, governments, and scientific communities must collaborate to refine and update these standards to address emerging biosecurity and biosafety challenges. This section will also touch upon the future directions in regulatory oversight, emphasizing the need for agility and responsiveness in the face of evolving biorisks (Filonchyk et al.2021).

Quality Management Systems in High-Containment Laboratories

In high-containment laboratories, the implementation of robust Quality Management Systems (QMS) is paramount for ensuring the safe and secure handling of biological agents and toxins.This section delves into the intricacies of QMS within the context of high-containment laboratories, shedding light on the global approach to biorisks used extensively to emphasize the systematic and controlled nature of these management systems (Coelho and García Díez 2015). The global distribution of BSL-4 laboratories are given in table 3 (Global Biolabs 2023).

Table 3. The global distribution of BSL-4 laboratories.

Region	Number of BSL-4 Laboratories
North America	15
Europe	26
Asia	20
South America	1
Africa	3
Australia	4
Total	**69**

Development and Implementation of QMS

Global approach to biorisks is often employed in describing the development and implementation of QMS to underscore the importance of rigorous planning and systematic execution. Quality managers oversee the establishment of QMS, with a primary objective of integrating biorisk management seamlessly into laboratory operations (Dirnagl et al. 2018).

Role of ISO/IEC 17025:2017 Standard

The international standard for laboratories conducting testing and calibration activities worldwide is ISO/IEC 17025:2017, General standards for the competence of testing and calibration laboratories. The ISO/IEC 17025:2017 standard, recognized internationally for evaluating laboratory competence, serves as the backbone of many QMS in high-containment

laboratories. The ISO/IEC 17025:2017 standard enables laboratories to demonstrate their expertise and produce accurate and reliable results by using a superior method. The globalapproach to biorisks is strategically used to highlight its role as a cornerstone document for QMS. Laboratories seek recognition of their competence through compliance with this standard, demonstrating their commitment to quality and safety (Dirnagl et al. 2018; Ghernaout et al. 2018).

Integration of CEN Workshop Agreement (CWA) 15793:2011

In parallel, CEN Workshop Agreement (CWA) 15793:2011, notable for its emphasis on biosafety and biosecurity, finds its place within the QMS. Global approach to biorisks is employed to emphasize its association with the accreditation process and its specific focus on ethical provisions and technical aspects.

Ensuring Compliance

Within high-containment laboratories, compliance with QMS is a critical factor in ensuring thesystematic management of biorisks. The global approach to biorisks is intentionally used to highlight that QMS is designed to be adhered to comprehensively, with clear procedures and guidelines in place to facilitate compliance. Continuous improvement is a core principle of QMS in high-containment laboratories. Global approach to biorisks is effectively used to convey the iterative nature of improvement processes. These laboratories adhere to the Plan- Do-Check-Act (PDCA) principle, where each phase is rigorously monitored and assessed. Establishing resource constraints before acting is an essential aspect of QMS. Global approach to biorisks underscores the need for meticulous planning and allocation of resources (Gill and Jones 1997). Quality managers serve as facilitators, ensuring that QMS does not hinder laboratory activities but instead enhances them. The implementation of QMS in high- containment laboratories is a dynamic process, evolving in response to emerging biorisks and advancements in biological research. This section provides insights into the future prospects ofQMS in high-containment laboratories, emphasizing the need for adaptability and integration with evolving international standards and regulations. By delving into the development, integration, and compliance aspects of QMS within high-containment laboratories, this section aims to underscore the critical role these management systems play in biorisk management and overall laboratory safety. The global approach to biorisks effectively conveys the structured and systematic nature of QMS within this context (Audu et al. 2012; Chua et al. 2013).

ISO 35001:2019 Standard

The ISO 35001 standard offers the principles of biorisk management by applying ISO's management system approach through a constant improvement model and accounting for the organization's context, leadership, planning, support, operations, performance evaluation, and improvement. Plan-Do-Check-Act (PDCA) is a systematic approach to track, adjust, and evaluate each principle's progress toward "continuous improvement of processes and products." Reducing workplace biosafety and biosecurity risks is the primary goal of ISO 35001, as this lowers the risk of infections linked to laboratories, accidental releases, and other accidents (Callihan et al. 2021).

Challenges and Complexities of Quality Management Systems in High-Containment Laboratories

In the intricate landscape of high-containment laboratories, the development and sustenance ofQuality Management Systems (QMS) pose formidable challenges and complexities. This section, approached from an academic and global perspective on biorisks, delves deeper into these multifaceted aspects, unveiling the intricacies of managing biorisks within such environments. One of the foremost challenges in implementing QMS within high-containment laboratories is the inherent variability in regulatory frameworks across different countries and regions. While international guidelines exist, the interpretation and enforcement of these guidelines often differ, leading to ambiguity and inconsistencies in compliance. Navigating thisregulatory maze requires a nuanced understanding of local, national, and international regulations, demanding meticulous attention to detail (Hauschild et al. 2021).

High-containment laboratories operate under stringent resource constraints, both in terms of financial investments and human capital. Developing and maintaining a robust QMS necessitates substantial financial allocations for infrastructure, equipment, and training. Moreover, the recruitment and retention of highly skilled personnel proficient in biorisk management can be a significant challenge, given the specialized nature of the work. Balancing these resource limitations while upholding QMS standards remains an ongoing struggle. The ever-e-

volving landscape of biorisks adds another layer of complexity. Pathogens mutate, new infectious agents emerge, and our understanding of potential hazards continually expands. High-containment laboratories must adapt swiftly to these changes, updating risk assessments,safety protocols, and training regimens. This dynamic environment requires a proactive approach to risk management, with the flexibility to address unforeseen challenges (Gill and Jones 1997; Dirnagl et al. 2018).

Cultural and ethical factors play a pivotal role in shaping QMS within high-containment laboratories. Different cultures perceive risk, safety, and accountability in distinct ways.Bridging these cultural gaps and fostering a culture of safety and responsibility is an ongoing endeavor. Moreover, ethical dilemmas, such as dual-use research concerns, demand careful deliberation and ethical frameworks that transcend geographical boundaries. In an interconnected world, where pathogens know no borders, global collaboration is imperative. High-containment laboratories must engage in international partnerships to share best practices, harmonize standards, and collectively address emerging threats. However, collaboration itself can introduce complexities related to intellectual property, data sharing, and the equitable distribution of benefits and responsibilities. While technological advancements offer innovative solutions for biorisk management, they also introduce new challenges. Laboratories must continually invest in state-of-the-art equipment and systems for pathogen detection, containment, and surveillance. Keeping pace with rapidly evolving technologies demands not only financial investments but also the agility to integrate new tools seamlessly into existing QMS (Trinchero et al. 2019; Moreira et al. 2021).

The education and training of personnel are at the heart of effective QMS. Ensuring that scientists, technicians, and support staff are well-versed in biorisk management protocols is a perpetual undertaking. Moreover, developing standardized training programs that can be applied globally, considering linguistic and cultural diversity, is a formidable task. In conclusion, the management of biorisks within high-containment laboratories is a multifaceted endeavor, rife with challenges and complexities (Lee et al. 2017). The global perspective presented in this section underscores the need for harmonized regulations, resource allocation, adaptability, cultural sensitivity, international collaboration, technological integration, and comprehensive education and training. Overcoming these challenges is essential to ensure the continued safety, security, and ethical responsibility of high-containment laboratories on a global scale (Ausher et al. 1996; Huang et al.2019).

Continuous Improvement and Future Directions

Global approach to biorisks is employed to underscore the ongoing nature of improvement initiatives within high-containment laboratories. These initiatives encompass iterative cycles of assessment, action, and enhancement in the pursuit of enhanced biosafety and biosecurity. The integration of advanced technologies remains a pivotal aspect of future directions (Bakanidze et al. 2010). Global approach to biorisks emphasizes the need for laboratories to adopt state-of- the-art instrumentation and digital systems, facilitating real-time monitoring, data analysis, and decision-making processes. The global approach to biorisks highlights the significance of intensified training and skill development programs. Laboratories must prioritize continuous learning to ensure that personnel remain well-versed in evolving biosafety and biosecurity practices (Kimman et al. 2008; Evans et al. 2020).

The importance of global collaboration and knowledge sharing is accentuated through global approach to biorisks. High-containment laboratories must engage in collaborative networks to exchange best practices, lessons learned, and emerging strategies for biorisk management. Global approach to biorisks is aptly used to convey the ethical responsibilities of laboratories. Laboratories should engage in transparent and ethical practices while actively participating in public engagement efforts to foster understanding and trust. The global approach to biorisks emphasizes the need for regulatory alignment and harmonization at national, regional, and international levels. Laboratories should actively contribute to efforts aimed at standardizing biosafety and biosecurity regulations. The global approach to biorisks effectively underscores the laboratory's need to bolster resilience and preparedness for unforeseen biological threats. Laboratories must remain vigilant, adapt to changing circumstances, and develop robust contingency plans (Kimman et al. 2008; Bakanidze et al. 2010).

The global approach to biorisks effectively conveys the importance of ethical responsibility and public engagement. Laboratories must adhere to ethical principles and actively engage with thepublic to promote transparency and societal safety. Global approach to biorisks construction is well-suited to discuss emerging threats and anticipatory measu-

res. Laboratories must proactively anticipate potential risks, conduct scenario planning, and implement preemptive measures to safeguard against novel biological threats. In conclusion, the necessity of continuous improvement and outlines future directions for high-containment laboratories in therealm of biorisk management. Global approach to biorisks construction effectively conveys the ongoing nature of improvement efforts and the proactive stance laboratories must adopt to navigate evolving challenges and opportunities (Zaki 2010; Moritz et al. 2020).

Conclusion

In conclusion, this paper has delved into the intricate domain of Quality Management in High-Containment Laboratories concerning Biorisks, employing a global approach to biorisks to emphasize the gravity of biorisk management within these specialized facilities. Through an exploration of the global approach to biorisks, this paper underscored the essential elements of a robust quality management system (QMS), commencing with the establishment of a QMS framework and followed by the meticulous identification, assessment, and mitigation of biorisks. The discussion illuminated the significance of standardized international guidelines, such as ISO 35001: Biorisk Management for Laboratories, and their potential to provide a comprehensive foundation for laboratory biosafety and biosecurity. Global approach to biorisks usage throughout this section served to accentuate the critical role played by laboratory leadership in fostering a culture of biosafety, instigating commitment, and furnishing adequate resources to drive continual improvement. Moreover, the adoption of a global approach effectively conveyed the ongoing necessity for unwavering vigilance amid continually evolving biological threats, underscoring the laboratories' essential need for adaptability and resilience. The section concluded by looking ahead to prospects, emphasizing the critical importance of a global approach, integration of cutting-edge technologies, ethical responsibility, and proactive measures to anticipate emerging threats.

Throughout this paper, the global risk approach has added depth and gravity to the discussion regarding biorisk management in high-containment laboratories, reinforcing the core tenets of biosafety and biosecurity. The future trajectory of high-containment laboratories hinges on their steadfast commitment to quality management, thereby ensuring the safety of personnel, communities, and the environment, all while pushing the boundaries of scientific knowledge.

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