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Özet:Özet 300 kelimeyi aşmamalıdır ve kaynak kullanımından kaçınılmalıdır. Türkçe sunulan makalelerde ayrıca İngilizce Özet (Abstract) bölümü hazırlanmalıdır.

Giriş:Bu bölümde sonuçların anlaşılabilmesi ve yorumlanabilmesi için o konu ile ilgili (daha önceden) yapılmış olan çalışmalar hakkında bilgilere yer verilmelidir. Giriş'te çalışmanın hipotezi belirtilmelidir. Çalışmanın amacı bu bölümün en sonunda açık olarak yazılmalıdır. Sadece gerekli olan kaynaklara atıf yapılmalıdır.

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RESEARCH ARTICLE

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Ameliorative Effect of Topical Clinoptilolite on 2,4- Dinitrofluorobenzene Induced Atopic Dermatitis Model in Mice

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Abstract

Atopic dermatitis is a multifactorial disease process. It is defined as "a genetically predisposed inflammatory and itchy allergic skin disease associated with the production of immunoglobulin E against environmental allergens". Experimental models are considered important in the evaluation of therapeutic agents for the treatment of atopic dermatitis. This study aimed to reveal the effects of clinoptilolite and tacrolimus on atopic dermatitis lesions in the atopic dermatitis model in mice induced with 2,4-dinitrofluorobenzene. For inducing the atopic dermatitis model, mice were administered topically on the back with 0.15% 2,4-dinitrofluorobenzene twice a week for 5 weeks. For the next 4 weeks, 0.15% 2,4-dinitrofluorobenzene was applied once a week to maintain inflammation. Afterward, topical tacrolimus cream (0.1%) and topical clinoptilolite powder were used for 4 weeks. Clinical score, serum thymus and activation-regulated chemokine, histopathology, and thymic stromal lymphopoietin (TSLP) immunostainings were evaluated between groups. While clinoptilolite treatment was found to be effective in the normalization of clinical scores, serum thymus and activation regulated chemokine levels were found to be variable and insignificant. Histopathologically, clinoptilolite had an ameliorative effect on epidermal thickness and inflammation yet there was no significant difference of mast cells and fibrosis between groups. Furthermore, clinoptilolite had an inhibitory effect of TSLP immunostaining on epidermal tissue. In conclusion, clinoptilolite could be an alternative treatment of atopic dermatitis with its effects similar to tacrolimus.

Keywords: Atopic dermatitis, clinoptilolite, tacrolimus, TARC, TSLP

Introduction

Atopic Dermatitis (AD), a prevalent chronic inflammatory skin condition, is marked by recurring eczema, dryness, and severe itching. Its global prevalence reaches up to 20% in children and 5% in adults. Notably, about 80% of cases start in early childhood or infancy, while the remaining 20% manifest during adulthood, as highlighted by Siegels et al. (1) and Bieber (2). The disruption of the skin's barrier function increases susceptibility to irritation and sensitization to various external stimuli. Inflammation and itchiness are key factors in the onset and exacerbation of AD (3). Alleviating itch is crucial for enhancing the quality of life of AD patients. Local or systemic corticosteroids have been the cornerstone of AD treatment, primarily due to their effectiveness in reducing inflammation. Despite their status as the primary treatment choice, the frequent occurrence of side effects with systemic steroids raises concerns (4). Consequently, there is an urgent need for the development of novel and more effective treatment modalities for AD.

Animal models play a pivotal role in understanding the etiopathogenesis of AD. These models have revealed that skin lesions in AD are linked with immunological shifts, notably Th2 immune responses and elevated serum IgE levels, making them crucial for evaluating potential AD treatments (5). In mice, it has been observed that a 0.15% concentration of 2,4-dinitrofluorobenzene in acetone, when applied topically and repeatedly, can cause eczema-

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tous changes. These changes include significant infiltration of neutrophils and eosinophils, as well as epidermal hypertrophy, leading to chronic itching (6).

Tacrolimus, an effective calcineurin inhibitor first isolated from Streptomyces tsukubaensis in Japan (7,8), was initially developed as an oral medication to prevent transplant rejection (9). It is believed to bind to the FK506 binding protein within cells and suppress calcineurin activity (6,9). Tacrolimus reduces inflammation by inhibiting T helper cell activity and the production of proinflammatory cytokines like IL-2, IL-3, and IL-4. It has also been demonstrated to lower the expression of high-affinity IgE receptors in epidermal cells from AD-affected skin (10). These properties have positioned topical calcineurin inhibitors, particularly tacrolimus, as primary anti-inflammatory agents in AD treatment (9). Additionally, tacrolimus cream has shown promise in animal models for mitigating inflammatory skin reactions in allergic contact dermatitis, acute irritant dermatitis, and delayed-type hypersensitivity (10). Clinoptilolite zeolite, a type of natural zeolite, is a microporous tuff stone composed of ions and crystalline water. Structurally, it is an aluminum silicate featuring channels in its crystal lattice that measure approximately 0.4 nanometers. The fundamental structure of clinoptilolite zeolites includes a crystal lattice with cavities approximately 4 Ångströms in size (1 Ångström = 10^-10 m = 0.1 nm). To date, over 34 minerals have been identified in natural zeolites, but only clinoptilolite zeolite is deemed suitable for human and animal treatments. No long-term adverse effects have been observed in humans or animals when administered in acceptable doses (11). Clinoptilolite powder is known for its antiviral, antibacterial, antifungal, and absorbent properties (12).

However, so far, there have been no reports on the effectiveness of clinoptilolite in treating atopic dermatitis-like lesions. Our study explores clinoptilolite's potential clinical and pathological benefits in 2,4-dinitrofluorobenzene (DNFB) induced dermatitis. The goal was to compare the efficacy of clinoptilolite with tacrolimus in a mouse model of AD induced by DNFB. To achieve this, we assessed effects of both clinoptilolite and tacrolimus clinically, biochemically, and pathologically.

Materials and methods

Animals

CD1 type female mice used for the research were obtained from Burdur Mehmet Akif Ersoy University Experimental Animal Production and Experimental Research Center. During the study, the principles of Burdur Mehmet Akif Ersoy University Experimental Animals Ethics Committee were followed. Thirty-two female CD1 mice, 24 weeks old, were kept in standard housing cages until the day of the experiment. There were 8 mice in each cage for each group (control, DNFB, Tacrolimus, and Clinoptilolite). Mice were provided with standard pellet feed and water ad libitum throughout the experiment. Drinking water was changed daily, and standard cage cleaning was performed throughout the experiment. Mice were housed at 21°C room temperature, in ventilated rooms with 12-hour light-dark cycles.

Chemicals

The lumbar regions of the mice in all groups were shaved, and the mice were kept for one day to allow for the amelioration of any microtraumas that might have occured. To create atopic dermatitis-like lesions, DNFB (Sigma, D1529) was used. DNFB was dissolved in a mixture of acetone and olive oil (3:1). For sensitization, 100 µL of 0.15% DNFB was applied to the shaved lumbal regions of mice (DNFB, Tacrolimus, Clinoptilolite group) twice a week for 5 weeks (13). Skin lesions formed after 5 weeks. The mice continued to be administered 100 µL of 0.15% DNFB once a week to maintain inflammation between weeks 6 and 9 (14). The mice in the control group were applied 100 µL of 3:1 acetone-olive oil solution to the lumbar regions once a week for 9 weeks. For treatment, topical tacrolimus (tacrolin 0.1% ointment) and clinoptilolite (froximun toxaprevent powder) were applied to the lumbar region once a day as a thin layer from weeks 6 to 9 weeks in the tacrolimus and clinoptilolite groups, respectively.

Measurement of clinical skin score

In the mouse atopic dermatitis model, clinical assessment and damage grading of skin lesions were performed for each mouse. According to this, lesions were graded as 0 (absent), 1 (mild), 2 (moderate), and 3 (severe) in terms of erythema, edema, abrasion, lichenification, oozing/scabbing, and dryness findings twice a week. The total score from the 6 symptoms of each mouse was counted as the score for that mouse (15). Evaluation was done by a researcher who was blinded to the grouping of the animals.

Pathological analyses

For histopathological and immunohistochemical examination, an area of 2 cm2 containing epidermis and dermis was excised from the lumbar regions of the animals with lesions. Skin samples were placed in 10% buffered formaldehyde and fixed for 24 hours. Following routine histolog-

ical procedures, tissues were embedded in paraffin blocks and 4 μm sections were taken. Sections were stained with hematoxylin & eosin (H&E) for histopathological analysis. Toluidine Blue staining was performed to reveal mast cells. In the histopathological examination, epidermis thickness and dermal inflammation levels were evaluated. Epidermal thickness level was determined by taking the average of 3 different measurements from a randomly selected area with 100x magnification. For dermal inflammation, a score of 0-3 was given by counting the inflammatory cells (mononuclear cells) in four 400x magnification fields. Accordingly, scores given the lesions similar to previous research (16,17) as follows: 0 - no inflammation, 1 - mild inflammation, 2 - moderate inflammation, 3 - severe inflammation. Additionally, in terms of fibrosis scores given in four 400x magnification field as follows: 0 - no fibrosis, 1 - mild fibrosis, 2 - moderate fibrosis and 3 - severe fibrosis. Mast cells were counted in four randomly selected 400x magnification fields from the slides stained with toluidine blue.

ImmPRESS® Excel Amplified Polymer Staining Kit (Vector Lab, Anti-Rabbit IgG, Peroxidase, MP-7601) was used for immunohistochemical analysis. All procedures were performed according to the manufacturer datasheet. Primary antibody for TSLP (Novus, NB110-55234) was diluted 1:1000 and applied overnight. DAB (ImmPACT EqV Reagent) was used as chromogen. The sections, which were counterstained with Mayer's hematoxylin for 20 seconds, were left to dry and covered with entellan. Mouse spleen and liver tissue were used for TSLP as positive control. For negative control, PBS was used instead of the primary antibody after protein blocking (Supp. Fig 1-3).

Quantitative Pathology & Bioimage Analysis (QuPath version 0.2.3) program was used to evaluate the staining intensity of immunohistochemically stained sections with anti-TSL*P.* For this purpose, DAB-positive cell numbers were determined with standard program settings by selecting areas of equal size at 100x microscopic magnification for each section.

Statistical analyses

Evaluation of clinical scores of mice in all groups was performed using the Repeated Anova test with the IBM SPSS program (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp). Statistical evaluation of histopathological, histochemical, and immunohistochemical findings between groups was performed in the Minitab™ 16.1.1 program with One Way Anova and Tukey test following the Ryan-Joiner normality test.

Results

Effect of tacrolimus and clinoptilolite treatment on DNFB-induced AD-like skin lesions

To evaluate the efficacy of treatment, tacrolimus cream and clinoptilolite powder were applied topically once a day to mice with DNFB-induced atopic-like dermatitis between weeks 6 and 9. For clinical scoring, lesions were scored twice a week for 28 days. Repeated application of DNFB increased bleeding, crusting, edema, itching, and erosion on the dorsal skin of mice (Figure 1A-D). At the end of the treatment, a significant difference was found when the mean clinical scores of the mice in the DNFB group were compared with the mean clinical scores of the control, tacrolimus, and clinoptilolite groups ($p < 0.001$) (Figure 1E-H). However, there was no statistically significant difference between tacrolimus and clinoptilolite groups ($p > 0.05$) (Table 1).

Table 1. Clinical scores (between 5-9 weeks) and serum TARC levels of mice in groups

	Control	DNFB	Tacrolimus	Clinoptilolite
Clinical Score	0.0720±0.0294^{c}	15.048±0.212^{a}	9.07±1.55^{b}	9.77±1.25^{b}
TARC (ng/L)	265,9±15,9	230,3±10,0	252,1±13,5	249,9 ± 14,4

^{a,b,c}: The statistical difference between the means with different letters in the same row is significant. Each value reveals mean ± standard error.

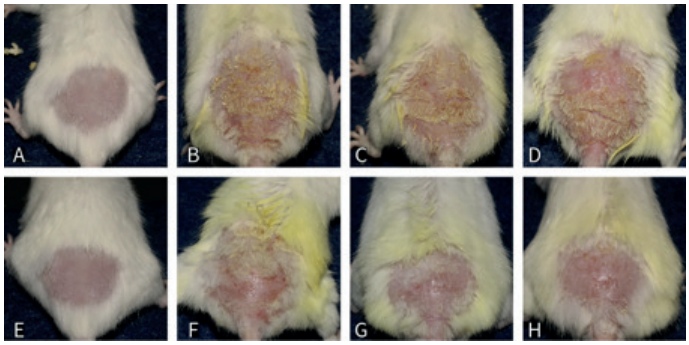

Figure 1. Atopic dermatitis-like lesions occurring at the end of the 5th week in the groups. Control group (A), DNFB group (B), clinoptilolite (C), tacrolimus (D). Appearance of skin lesions after 9th week in the groups. Control group (E), DNFB group (F), clinoptilolite (G), tacrolimus (H).

In the DNFB group, edema and erythema emerged at the application site in the first week. Itching and scratching due to skin irritation from the second week, and lichenification and crusting in the subsequent weeks were the most significant clinical observations. Three mice developed crusting in week five. While the group's mean clinical score at the end of the fifth week was 14.87±0.75, no significant increase or decrease in mean clinical scoring was seen with the lowering of the DNFB application dosage beginning in the sixth week. In the tacrolimus group, only two mice developed erythema at the application site in the first week. Scratch-

ing, abrasion due to irritation, and lichenification and crusting in the following weeks were the most significant clinical symptoms beginning in the second week. At week 5, eight mice exhibited crusting, but two had severe crusting. While the group's mean clinical score was 15.00±0.75 at the end of the fifth week, a substantial reduction in the weekly mean clinical score was observed with the lowering of the DNFB application dosage and the start of therapy in the sixth week. The group's mean clinical score at the end of the ninth week was 7.00±0.64. In the clioptilolite group, mice developed erythema at the experimental site in the first week, but only three animals developed edema. Scratching, abrasion due to itching, and lichenification and crusting in the following weeks were the most significant clinical symptoms beginning in the second week. Crust development was detected in 8 animals at week 5 and was more severe in 2 mice. While the group's mean clinical score was 14.37±0.75 at the end of the fifth week, a substantial drop in the weekly mean clinical score was noted with the lowering of the DNFB application dosage and the start of therapy in the sixth week. The group's mean clinical score at the end of the ninth week was 6.50±0.64 ($p < 0.05$).

Effect of Tacrolimus and Clinoptilolite on Serum TARC Level

At the end of the study, mean serum TARC levels in the treatment groups, tacrolimus and clinoptilolite, were 252.1±13.5 ng/L and 249.9 ± 14.4 ng/L, respectively. These values were lower than the mean serum TARC levels of the control group (265.9±15.9 ng/L) and higher than the mean serum TARC levels of the DNFB group (230.3±10.0 ng/L). In the statistical evaluation between the groups, no significant difference was found in terms of serum TARC levels ($p > 0.05$) (Table 1).

Effect of Tacrolimus and Clinoptilolite on Histopathological Changes

In the evaluations made in terms of epidermal thickness levels, a significant increase was observed in the DNFB group compared to the control group (18.75±0.52 μm) ($p < 0.001$). Epidermal thickness levels were significantly lower in the tacrolimus (107.25±6.470 μm) and clinoptilolite groups (83.25±4.95 μm) compared to the DNFB (179.00±9.02 μm) group ($p < 0.001$). In addition, epidermal thickness level was significantly lower in the clinoptilolite group compared to the tacrolimus group ($p < 0.001$) (Figure 2A-D).

In the evaluation of dermal inflammation, DNFB group (2.75±0.25) was found to be significantly higher than the control group (0.00±0.00) ($p < 0.001$). The dermal inflammation value of the tacrolimus group (2.25±0.25) was found to be lower numerically than the DNFB group, while it was found to be significantly lower in the clinoptilolite group (1.75±0.25) compared to the DNFB group ($p < 0.001$). While no significant difference was observed between the tacrolimus and clinoptilolite groups, a significant increase of inflammation was detected in these groups compared to the control group ($p < 0.001$).

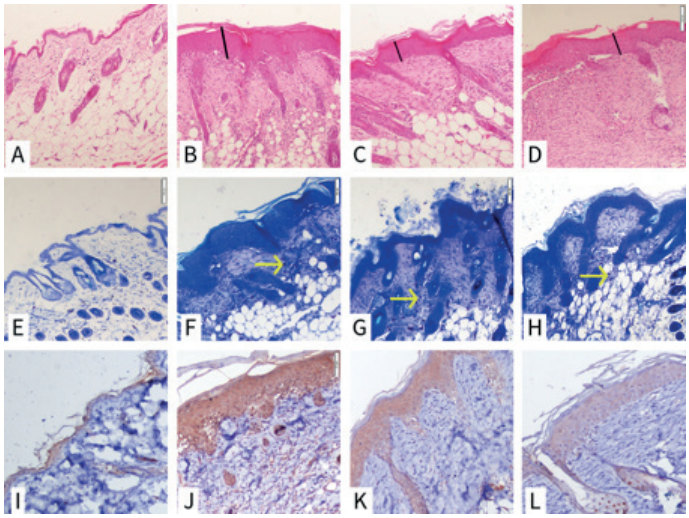

Figure 2. Comparative views of dermal lesions between groups. Compared to control (A) significant increase in epidermal thickness level in DNFB group (B), decrease in epidermal thickness levels (black line) in clinoptilolite (C) and tacrolimus (D) groups, 100x, H&E. Compared to control group (E), increased mast cells (yellow arrows) in DNFB (F), clinoptilolite (G) and tacrolimus (H) groups, 100x, Toluidine Blue. Immunohistochemically, low TSLP positivity in the control (I) group, high positivity in the DNFB (J) group and decreased TSLP positivity in the tacrolimus (K) and clinoptilolite (L) groups, 200x, DAB.

In terms of dermal fibrosis, a significant increase was observed DNFB (2.50±0.26), tacrolimus (2.37±0.37) and clinoptilolite (2.00±0.18) groups compared to the control (0.00±0.00) group ($p < 0.001$). There was no difference between the DNFB, tacrolimus and clinoptilolite groups themselves.

It was noted that mast cells showed a significant increase in the DNFB group (25.75±3.10) compared to the control group (7.88±1.32) in the counts performed on the sections stained with Toluidine Blue ($p < 0.001$). However, there was no significant difference between the tacrolimus (26.13±2.99), clinoptilolite (24.13±3.40) and DNFB groups ($p > 0.05$), while a significant increase was observed compared to the control group ($p < 0.001$) (Figure 2E-H). All histopathological score values are given in Table 2.

Table 2. Histopathological and immunohistochemical findings of mice in the groups.

Group	Epidermal thickness (µm)	Dermal inflammation	Dermal fibrosis	Number of mast cells	TSLP positivity
Control	18,75±0,52[a]	0,00±0,00[a]	0,00±0,00[a]	7,88±1,32[a]	77,80±14,40[b]
DNFB	179,00±9,02[b]	2,75±0,250[c]	2,50±0,260[b]	25,75±3,10[b]	291,6±45,60[a]
Tacrolimus	107,25±6,47[d]	2,25±0,25 [bc]	2,37±0,37[b]	26,13±2,99[b]	124,9±46,30[b]
Clinoptilolite	83,25±4,95[c]	1,75±0,25 [b]	2,00±0,18[b]	24,13±3,40[b]	132,40±42,20[b]

[a,b,c]: The statistical difference between the means with different letters in the same row is significant. Each value reveals mean ± standard error.

Effect of Tacrolimus and Clinoptilolite on Tissue TSLP in DNFB-Sensitized Mice

As a result of the statistical analyses performed on the positive cell numbers obtained from the immunohistochemically stained sections with anti-TSLP, a significant increase in positive cells was determined in the DNFB group (291.6±45.6) compared to the control group (77.80±14.4) ($p < 0.01$). Although TSLP positivity was found to be higher in both the tacrolimus (124.90±46.30) and clinoptilolite (132.4±42.2) groups compared to the control group, the difference was not statistically significant and it was significantly lower than the DNFB group ($p < 0.01$). The difference between tacrolimus and clinoptilolite groups was not significant (Figure 2I-L) (Table 2).

Discussion

Various factors play a role in the pathogenesis of AD, including immunological abnormalities (18). The spontaneous clinical symptoms of atopic dermatitis typically begin with itching, redness, bleeding, flaking, dryness, and hair loss (5). The severity of dermatitis induced by haptens can range widely, from mild and temporary skin inflammation characterized by itching and redness to more severe and lasting exudative dermatitis (19). Studies involving DNFB application in mice (15,20), have shown that the average clinical score was higher in the DNFB-treated group compared to control and vehicle groups. These mice exhibited symptoms including significant redness/bleeding, swelling, erosion, and dryness/scaling of the back skin. In similar experiments using DNCB on mice, the dermatitis score, based on factors like redness, dryness, swelling, and abrasion, was significantly higher than in control and vehicle groups (18,21). Our study, aligning with these findings, showed that the average clinical score was lower for mice that did not develop atopic dermatitis-like lesions after only vehicle administration. The clinical scores in the DNFB group were higher compared to the control and other groups. The clinical manifestations of dermatitis in mice, which include the inflammatory process marked by the infiltration of neutrophils and eosinophils, and the hyperplasia of the epidermis, lead to eczematous changes in the skin and itching, likely due to repeated topical applications of DNFB.

Inagaki and colleagues (22) assessed the effectiveness of tacrolimus and dexamethasone in a mouse model of AD using 0.15% DNFB in a 50 µl acetone solution. Histopathological evaluation of the untreated group's skin 24 hours post-final DNFB application showed pronounced inflammatory responses including epidermal and dermal thickening and an increase in neutrophils, eosinophils, lymphocytes, and mast cells. They also observed epidermal crusting, swelling, single-cell necrosis, and dermal fibrosis. Dexamethasone mitigated these alterations effectively, reducing lymphocyte and eosinophil levels significantly - effects not mirrored by tacrolimus, which did not influence mast cell counts or scratching behavior. Conversely, a 0.1% topical tacrolimus application, while not significantly anti-inflammatory, greatly reduced itching. Aksoy et al. (12) noted that clinoptilolite activates MHC class II and mononuclear phagocytes, elevating T cell numbers through the release of proinflammatory cytokines like IL-1 and TNF-alpha, and transcription factors, thereby promoting wound healing and granulation tissue formation. In our research, the DNFB group showed a significant increase in epidermal thickness compared to the control group, but this increase was markedly lower in the tacrolimus and clinoptilolite groups. The clinoptilolite group showed statistically lower epidermal thickness than the tacrolimus group. Furthermore, while the tacrolimus group did not show a significant reduction in dermal inflammation, a substantial decrease was observed in the clinoptilolite group, suggesting, in contrast to previous reports, that clinoptilolite may have an anti-inflammatory effect.

Mast cells, known as key players in allergic reactions, are involved in the formation of skin lesions in AD. During chronic skin inflammation, these cells release a variety of cytokines, contributing to allergic responses, as explained by Han et al. (23). In a study conducted by Yamashita et al. (6), the effectiveness of 0.1% tacrolimus, cyclosporine (Cys) -A at 30 mg/kg, and 0.05% dexamethasone was assessed in a mouse model of AD induced by 2,4-dinitrofluorobenzene (DNFB). The assessment included counting the number of mast cells and the rate of degranulated mast cells in three toluidine blue-stained areas chosen at random. The results indicated that neither tacrolimus nor Cys A had an impact on the mast cell count. In our own research, we effectively induced AD using DNFB. Following this, we treated the condition with tacrolimus as a control and observed that it mitigated some of the histopatholog-

ical features of AD, such as epidermal thickening and dermal inflammation. However, we noted that tacrolimus did not decrease the number of mast cells. Furthermore, the clinoptilolite group's mast cell count showed no significant difference compared to the DNFB group.

Despite the absence of histopathological markers exclusively identifying AD, skin biopsies often reveal acute, subacute, or chronic dermatitis patterns. Especially in chronic AD stages, a notable increase in collagen within healed and healing lesions has been observed. Lee et al. (24) highlighted that dermal fibrosis is a significant characteristic of AD in its chronic stage. While acute AD lesions histopathologically exhibit an increase in eosinophils and their dermal infiltration, chronic lesions are more defined by collagen accumulation and dermal thickening, as Jin et al. (25) reported. A study evaluating five different wound healing agents found that clinoptilolite enhanced the healing process notably. This research parallels ours in that clinoptilolite was shown to reduce inflammation microscopically, yet it also notably increased collagen formation and fibroblast proliferation, as noted by Uraloğlu et al. (26). In our research, clinoptilolite did not show effectiveness in reducing fibrosis; however, it seemed to primarily exert its healing effects by dampening inflammation. Şentürk Demirel et al. (27) conducted a study on rats, by adding varying doses of clinoptilolite to their diet. They reported that incorporating 6% clinoptilolite increased collagen density and epidermal thickness. Additionally, researchers like Hubner et al. (28) have found that gelatin-based clinoptilolite and silver-infused preparations, used as antimicrobial dressings, can aid in wound healing by inhibiting the growth of pathogens like Escherichia coli and Staphylococcus aureus in wounds. While our study did not assess antibacterial and antioxidative properties, the observed reduction in epithelial thickness, alongside clinoptilolite's anti-inflammatory action, might be attributed to these unexplored effects of the substance.

Tacrolimus acts on T cells in affected skin by binding to the cytosolic FK506 binding protein-12, thereby inhibiting calcineurin enzyme activity. This action prevents T cell activation and suppresses the release of proinflammatory cytokines, making tacrolimus particularly effective against immune cells involved in AD pathogenesis. It blocks cytokine production in mast cells, eosinophils, and basophils, inhibits T cell activation by antigen-presenting dendritic cells, and reduces the number of inflammatory dendritic epidermal cells, as detailed by Rustin (29). In our research, the average clinical score for the clinoptilolite-treated group was lower than that of the tacrolimus-treated group from the first week of treatment, though the difference was not statistically significant. Upon histopathological and immunohistochemical examination, we observed that both clinoptilolite and tacrolimus reduced epidermal thickness, with clinoptilolite showing a more significant reduction than tacrolimus. Only the clinoptilolite group demonstrated a considerable reduction in dermal inflammation compared to the DNFB group. However, no difference was observed in fibrosis and mast cell count between the clinoptilolite and tacrolimus groups relative to the DNFB group. Regarding the count of TSLP-positive cells, both the tacrolimus and clinoptilolite groups had lower counts than the DNFB group, but no significant difference was noted between the two treatment groups.

TSLP, a cytokine resembling interleukin 7, is produced by activated mast cells and plays a crucial role in triggering allergic inflammation, through dendritic cell-mediated Th2 responses. TSLP is overexpressed in both acute and chronic AD skin lesions, as observed by Han et al. (23). This cytokine, produced by epithelial cells located on barrier surfaces such as the skin, lungs, and intestines in both mice and humans shows significantly elevated expression AD-affected epidermis compared to non-allergic dermatitis or healthy skin. Genetic mutations that compromise the barrier functions of skin in mouse models have been linked to increased TSLP expression in the epidermis, contributing to Th2-type inflammation and AD as noted by Indra (30). Additionally, Yoou et al. (31) found elevated levels of TSLP, IL-6, and TNF-α, alongside a higher count of inflammatory cells in DNFB-induced AD mice compared to controls. In our study, TSLP expression was notably higher in the DNFB group versus the control group. However, in both the tacrolimus and clinoptilolite groups, TSLP positivity was found to be similar to the control and significantly lower than in the DNFB group.

TARC, part of the CC chemokine family, found naturally in the thymus, is produced by dendritic cells, endothelial cells, keratinocytes, bronchial epithelial cells, and fibroblasts. Acting as a ligand for CCR4, which is primarily found in Th2 lymphocytes, basophils, and natural killer cells, TARC plays a pivotal role in AD, particularly in its acute phase where Th2 cells are predominant (32,33). Using the NC/Nga mouse model, similar to human AD, Vestergaard et al. (34) revealed that TARC significantly expressed in lesioned skin but not in healthy skin. Further studies demonstrated that NC/Nga mice with DNFB-induced AD-like lesions had higher serum TARC levels compared to controls (18), a pattern also seen in humans where AD patients show markedly higher serum TARC levels, especially in severe

cases. Notably, TARC levels decreased following treatment, correlating with clinical improvement (33). Thijs et al. (35) proposed serum TARC as a reliable marker for AD. Similarly, tissue TARC levels were also significantly higher in AD-like lesion groups (36,37).

However, our study found no significant difference in serum TARC levels between AD-like lesion groups and controls, diverging from established literature. It's known that serum TARC levels are considerably higher in infants than adults and decrease significantly with age (38). Additionally, thymus cellularity in mice notably decreases with age, almost halving by 12 months (39). Given that our mice were, on average, 24 weeks old at the start and around 33 weeks or 8 months old at the end, this age-related reduction in thymic size and TARC level could explain the lack of significant increase in TARC in our AD mice, suggesting that TARC may not be a reliable marker for diagnosing AD in adult mice.

Conclusion

This study aimed to evaluate the efficacy of tacrolimus cream and clinoptilolite, which has been used for a long time in the treatment of atopic dermatitis model in mice. Although it seems to contribute to the improvement of some local clinical and histopathological parameters, it has been observed that it is better in terms of correcting some parameters when compared to tacrolimus. It is thought that this mouse model can contribute to atopic dermatitis and its treatment, which is an important problem in both human and veterinary medicine. As a result, it was concluded that clinoptilolite may be a safe alternative in the treatment of atopic dermatitis with local lesions. However, further studies are needed to elucidate the therapeutic potential and molecular mechanism of clinoptilolite in the treatment of AD.

Ethics statements

Before the study, the ethics committee approval was obtained of Burdur Mehmet Akif Ersoy University Experimental Animals Local Ethics Committee (17.02.2021; decision number:727).

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Sıçan İleumunda Kapsaisinin Neden Olduğu Morfometrik Değişikliklerin, IGF-I ve IGF-IR Ekspresyonlarının İncelenmesi

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Özet

Bu çalışma kapsaisin uygulaması sonrası ileumdaki histomorfolojik değişiklik ve endokrin, parakrin, otokrin etkiler yoluyla hücresel büyümeyi, farklılaşmayı ve apoptozu düzenlemede rol alan IGFI ve onun reseptörü olan IGFI-R'nün ifadesi araştırılmıştır. 21 günlük Sprague-Dawley sıçan Kontrol gurubu ve Kapsaisin alan tedavi grubu olarak 2'ye ayrıldı. Tedavi grubuna 20 gün boyunca deri altında 0,5mg/kg Kapsaisin uygulandı Deney sonunda ileumlar toplanarak dokular immünohistokimya (IGF-I-IGF-IR) ve morfometrik analizlerle değerlendirildi. Tedavi gruplarının sindirimi destekleyen parametrelerinden villus yüksekliği, kript derinliği, total mukozal kalınlığı, villus/kript oranı, yüzey emilim alanı istatistiki olarak arttı. Ayrıca kriptlerde ve düz kas tabakasında IGF-I ve IGFI-R'nün ifadesinde artış belirlendi. Ancak yüzey epitelinde kontrol grubuna göre herhangi bir istatistiki önem tespit edilmedi. Histomorfolojik parametrelerdeki artış ve hücrelerin mitotik olarak en yoğun olduğu kript bezlerinde görülen IGF-I ve reseptörünün ifadesinde artış Kapsaisinin IGF-I ve IGF-IR etki mekanizmasını etkilediği ve tedavinin sindirim sisteminde besin emilimini ve yararlanımını destekleyerek bağırsak morfolojisini olumlu yönde etkilediği sonucuna vardık.

Anahtar kelimeler: İleum, IGF-I, IGF-IR, Kapsaisin.

Investigation of Capsaicin-Induced Morphometric Changes, IGF-I and IGF-IR Expressions in Rat Ileum

Abstract

This study investigated the histomorphological changes in the ileum after capsaicin application and the expression of IGFI and its receptor IGFI-R, which play a role in regulating cellular growth, differentiation and apoptosis through endocrine, paracrine and autocrine effects. 21-day-old Sprague-Dawley rats were divided into 2 groups as the Control group and the Capsaicin-receiving treatment group. 0.5mg/kg Capsaicin was applied subcutaneously to the treatment group for 20 days. At the end of the experiment, the ileums were collected and the tissues were evaluated with immunohistochemistry (IGF-I, IGF-IR) and morphometric analyses. The parameters supporting digestion in the treatment groups, such as villus height, crypt depth, total mucosal thickness, villus/crypt ratio, and surface absorption area, increased statistically. In addition, an increase in the expression of IGF-I and IGF-IR was determined in the crypts and smooth muscle layer. However, no statistical significance was detected in the surface epithelium compared to the control group. The increase in histomorphological parameters and the increase in the expression of IGF-I and its receptor seen in the crypt glands where the cells are mitotically densest lead to the conclusion that capsaicin affects the mechanism of action of IGF-I and IGF-IR and that the treatment positively affects intestinal morphology by supporting nutrient absorption and utilization in the digestive system.

Keywords: Ileum, IGF-I, IGF-IR, Capsaicin.

Giriş

Acı biberin, MÖ 7000'den beri dünya çapında gıda ve ilaçları tatlandırma, koruma konusunda uzun bir geçmişi vardır. Acı biberin başlıca aktif bileşiği olan kapsaisin (KAP), baharatlılık hissi verir ve ağrı, iltihap ve kanser tedavisi de

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dahil olmak üzere insan organizmasında sayısız faydalı rolü belirlenmiştir (1).

KAP, antioksidan, obezite karşıtı, ağrıyı hafifletici ve iltihap önleyici etkiler de dahil olmak üzere geniş kapsamlı güçlü biyolojik özellikler göstermiştir (2). Bununla birlikte, son çalışmalar KAP'nin düşük dozlarda aktivite gösterdiğini ancak yüksek dozlarda yan etkilere sahip olma eğiliminde olduğunu ortaya koymaktadır. KAP sürekli olarak yüksek seviyelerde tüketildiğinde, insanlar günlük yaşamlarında mide ekşimesi, ishal, ağrı ve diğer semptomlar gibi bazı rahatsız edici gastrointestinal (GI) semptomlar hissedebilirler (3). Bu nedenle, KAP alımının GI etkileri giderek daha fazla ilgi görmektedir.

Keskinliği nedeniyle, yüksek doz KAP gastrik asit üretimini engelleyebilir, gastrik iltihaplanmaya neden olarak GI mukozasına zarar verebilir, bağırsak bariyerinde yapısal değişikliklere neden olabilir ve ayrıca bunun gibi pek çok GI sistem semptomlarına neden olabilir (4-12). Bununla birlikte, KAP üzerine yapılan çalışmalardan elde edilen sonuçlar, KAP'nin dozajı ve süresi ve araştırma deneklerinin türleri ve karakterizasyonu nedeniyle farklılık göstermektedir. Bu da KAP'nin spesifik GI etkilerini doğrulamayı zorlaştırmaktadır. Bu nedenle, KAP alımının GI sağlığı üzerindeki etkilerini belirlemek için tek tip ve net deneysel modeller ve değerlendirme kriterleri bulunmamaktadır.

Buna karşın mevcut araştırmalar KAP'nin GI sağlığı üzerindeki etkilerini açıklamayı amaçlamaktadır. Farelerde doku iltihabı, histopatolojik hasar ve serum GI nöropeptit seviyelerinin KAP'ın farklı dozlarıyla ilişkili olduğu belirtilmiştir. Ayrıca, KAP'nin kısa zincirli yağ asitlerini (SCFA'lar) ve bağırsak mikrobiyotasının bileşimini düzenleyip düzenlemediğini belirlemek için bir korelasyon analizi yapılmıştır ve bu KAP tüketimini GI sağlığı ile ilişkilendirmiştir. Çalışmada KAP'nin GI sağlığı üzerindeki etkilerini değerlendirmek için farelere üç dozda intragastrik olarak CAP uygulanmış ve 40 mg/kg KAP uygulamasının farelerde GI sistemi üzerinde önemli olumsuz etkilere sahip olmadığını, 60 ve 80 mg/kg KAP'nİn ise GI dokularına zarar vererek ve anti-inflamatuar sitokin (IL-10) seviyelerini azaltarak GI hasarına neden olduğunu bildirilmiştir (13).
Sindirim sistemi ile ilişkili büyüme faktörlerinden IGF-I (Insülin benzeri büyüme faktörü -I), endokrin, parakrin ve otokrin etkiler yoluyla hücresel büyümeyi, farklılaşmayı ve apoptozu düzenlemede önemli bir rol oynar. IGF-I etkisi, IGF-I peptit hormonu, hücre yüzeyi IGF-I reseptörleri (IGF-IR)ve IGF-I'in reseptörüne bağlanmasını düzenleyen altı, iyi karakterize edilmiş IGF bağlayıcı protein (IGFBPs 1-6) içeren karmaşık bir sistem tarafından kontrol edilir (14,15). Sunulan çalışmalarda farelere intraperitoneal IGF-I uygulamasını bağırsak kript derinliğini arttırdığı (34) sıçanlara 14 günlük parenteral IGF-I uygulamasının ise, kript derinliğini ve villus yüksekliğini %30 oranında artırdığı (35), ve oral yolla IGF-I alımının ince bağırsak ağırlığı, DNA içeriği, protein içeriği ve ince bağırsaktaki villus yüksekliği endekslerini artırdığı bildirilmiştir (33).

Son çalışmalar, 14 gün boyunca 20 mg/kg KAP dozunda oral uygulamanın sıçanlarda gastrik ve jejunal mukozal dokularda belirgin inflamatuar hücre infiltrasyonuna ve hücre vakuollerine neden olduğunu ortaya koymuştur (23). Bununla birlikte, KAP'nin diğer GI dokularını etkileyip etkilemediği bilinmemektedir. KAP alımının GI sağlığı üzerindeki etkileri için hala net bir deneysel model ve değerlendirme kriteri yoktur.

Bu çalışmanın amacı, düşük doz KAP uyguşamasının ileum mukoza epitelinde neden olabileceği morfometrik değişikliklerin belirlenmesini yanı sıra IGFI sinyal yolağının düşük doz KAP tedavisi alan sıçan ileumu üzerindeki etki mekanizmasında herhangi bir rolü olup olmadığını araştırmaktır

Materyal ve Metot

Çalışmada Uludağ Üniversitesi Deney Hayvanları Yetiştirme ve Araştırma Merkezinden alınan 21 günlük Sprague-Dawley dişi sıçanlar kullanıldı. Hayvanlar çalışma boyunca kontrollü laboratuvar koşullarında tutuldu.

Etik Standartlara Uygunluk

Deney protokolleri Uludağ Üniversitesi Hayvan Bakım ve Kullanım Komitesi tarafından onaylandı ve Ulusal Sağlık Enstitüsü Laboratuvar Hayvanlarının Bakımı ve Kullanımı Kılavuzu'na (karar no: 08.06.2005/2) uygun olarak ilerlendi.

Deneysel Prosedür

Sıçanlar 10 hayvandan oluşan 2 gruba ayrıldı. 1. grup Kontrol grubu İkinci grup ise; KAP deney grubu (0,5 mg/kg/gün-20gün sc.) (10% etanol, 10% Tween 80 ve 80% distile sudan oluşan bir çözücüde hazırlanan KAP'ın (Sigma Chemical Co. Solventi). 20 günlük uygulama sonunda hayvanlar sodyum pentobarbital enjeksiyonu ile ötenazi edildi ve karın duvarları açıldı. İleumun 2 cm'lik bir kısmı çıkarıldı ve %10 tamponlu formaldehit ile fikse edildi. Doku örnekleri rutin histolojik prosedürlere göre parafin bloklara gömüldü. Beş mikrometre kalınlığında kesitler kesildi ve bir kısmı mormometrik analiz için bir kısmı ise IGF-I ve IGF-IR lokalizasyonu belirlemek üzere ayrıldı.

Morfometrik Analizler

Morfometrik analiz için alınan kesitler Crossman'ın modifiye edilen üçlü boyasıyla boyandı (16). Villus yüksekliği, kript derinliği, villus/kript oranı, toplam mukozal kalınlık, villus yüzeyi emilim alanı ölçüldü ve mikrograflandı (17). Villus emici yüzey alanı şu formül kullanılarak hesaplandı: Villus emici yüzey alanı = (2 π) x (ortalama villus genişliği/2) x villus yüksekliği (18).

Immunohistokimya

Standart streptavidin-biyotin peroksidaz kompleksi tekniği ImPRESS reaktif kiti (MP 7405, MP 7801) kullanılarak gerçekleştirildi. Kesitler üreticinin firmanın önerdiği şekilde 1:100 IGF-I primer antikoru (G-17) (sc-1422, Santa Cruz, CA, ABD) ve IGF-IR primer antikoru (C-20) Santa Cruz sc: 713 CA, ABD) ile 4°C'de gece boyunca inkübe edildi. Numuneler PBS ile yeniden yıkandı ve oda sıcaklığında 30 dakika boyunca ImmPRESS reaktifi (IGF-1 için anti-goat MP-7405 ve IGF-IR için anti-rabit MP-7401) ile inkübe edildi. Son olarak görüntüleme için 3.3'-diaminobenzidin (DAB) kullanıldı ve hematoksilen ile karşıt boyama yapıldı. Kesitler ksilol ile temizlendi ve entellan ile kaplandı (19).

Kesitler iki bağımsız gözlemci tarafından aşağıdaki puanlama sistemi kullanılarak değerlendirildi: 0, immün reaksiyon yok; 1, zayıf immün reaksiyon; 2, orta derecede immün reaksiyon; 3, güçlü immün reaksiyon (20).

İstatistiksel Analiz

Elde edilen verilerin istatistiksel analizleri IBM SPSS Statistics version 22 (Statistical Package for Social Science, IBM SPSS, Chicago, U.S.*A.*) programı kullanılarak yapıldı. İki grubun sayısal verileri arasındaki farkın analizinde Mann-Whitney U Testi kullanılarak yapıldı.

Bulgular

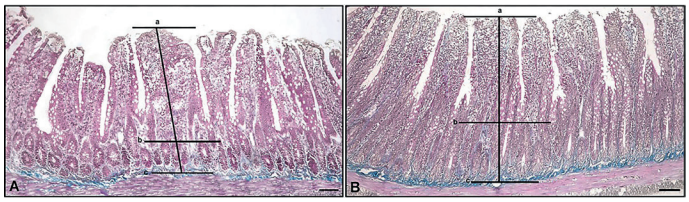
Thirty-Morfometrik bulgular

KAP tedavi grubunda kontrol grubuna göre villus yüksekliği ($p<0,05$), kript derinliği ($p<0,05$), toplam mukozal kalınlık ($p<0,05$), villus/kript oranı ($p<0,05$) ve villus yüzey emilim alanı ($p<0,05$) daha yüksek belirlendi (Şekil 1, Tablo 1).

Tablo 1. Kontrol gruplarının ve deney grubunun villus yüksekliği, kript derinliği, villus/kript oranı, total mukozal kalınlığı ve villus yüzey emilim alanının morfometrik analizi.

Gruplar	N	Villus yüksekliği (Ort±SE)	Kript derinliği (Ort±SE)	Total mukozal kalınlık (Ort ±SE)	Villus /Kript (Ort ±SE)	Yüzey emilim alanı (Ort ±SE)
Kontrol A	10	1009,02±17,1[a]	546,5±28,5[a]	2117,7±48,7[a]	1,84[a]	1,30±0,3[a]
KAP	10	1226,65±35,5[b]	571,5± 23,9 [b]	2267,7 ± 41,5 [b]	2,14[b]	1,47±0.03[b]
P değeri		*0,21*	*0,039*	*0,004*	*0,028*	*0,33*

Aynı sütundaki farklı harfler istatistiksel anlamlılığı gösterir ([a,b])

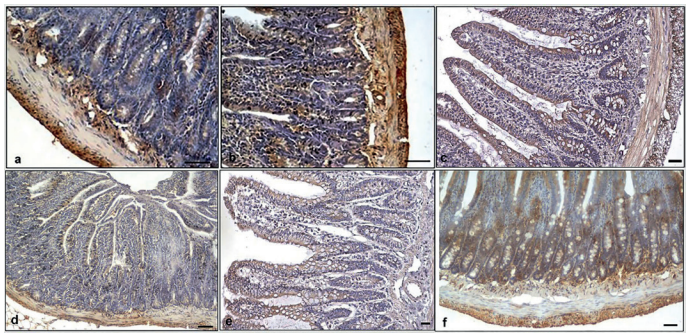


Şekil 1. Ileum mukozasının morfometrik analizi (A) Kontrol grubu (B) KAP tedavi grubu a-b:villus yüksekliği, a-c: total mukozal kalınlık, b-c: kript derinliği. Crossmon's triple staining (Bar: 100 µm)

Immunohistokimyasal Bulgular

IGF-I immunoreaktivitesi

İleumda, tüm gruptaki villus epitel hücrelerinde orta düzeyde immünreaksiyon belirlendi. Kript epitel hücrelerinin kontrol grubunda hafif bir reaksiyon, KAP tedavi grubunda ise orta düzeyde bir reaksiyon belirlendi. IGF-I kontrol gurubu kas tabakası deney grubuna göre daha düşük bir ifade gösterdi. Deney grubunun sirküler kas tabakasında orta düzeyde reaksiyon gösterirken, longitudinal kas tabakasında ifade daha güçlüydü (Şekil 2, Tablo 2) ($p<0,001$).



Şekil 2. İleumda IGF-I ve IGF-IR immunreaksiyonu (a) Kontrol grubu zayıf IGF-I ekspresyonu (b) KAP tedavi grubunda orta düzeyde IGF-I ekspresyonu (c) Deney grubu yüzey epitel hücrelerinde orta, kript hücrelerinde güçlü IGF-I immünoreaksiyonu (d) Kontrol grubu zayıf IGF-IR ekspresyonu (e) Deney grubu yüzey epitel hücrelerinde orta, kript hücrelerinde güçlü IGF-IR immünoreaksiyonu (f) Deney grubu yüzey epitel hücrelerinde orta, kript hücrelerinde güçlü IGF-I immünoreaksiyonu. (Bar: 100 µm -50µm).

Tablo 2. Yüzey epitelinde, kriptlerde, sirküler ve longitudinal kaslarda IGF-I ifadesi.

IGF-I	Kontrol	KAP	*P*
Yüzey epiteli	2,00±0,22[a]	2,12±0,11[a]	-
Kript derinliği	0,81±0,21[a]	2,58±0,17[b]	*0,001*
Sirküler kas	1,21±0,23[a]	2,44±0,15[b]	0,001
Longitudinal Kas	1,48±0,18[a]	2,62±0,23[b]	0,001

Aynı satırdaki farklı harfler istatistiksel anlamlılığı gösterir ([a,b])

IGF-IR immunoreaktivitesi

İleumun villus epitel hücrelerinde orta şiddette bir reaksiyon gözlendi. Kriptlerde ise kontrol gurubundaki reaktivite düşük gözlenirken KAP tedavi grubunda güçlü bir imunorektivite gözlendi. Kas tabakasında ise IGF-I immunreksiyonuna benzer bir ekspresyon belirlendi. IGF-IR ifadesi sirküler kas tabakasında orta düzeyde eksprese olurken,

longitudinal kas tabakasında güçlüydü (Şekil 2, Tablo 3) ($p<0,05$, $p<0,001$).

Tablo 3. Yüzey epitelinde, kriptlerde, sirküler ve longitudinal kaslarda IGF-IR ifadesi.

IGF-IR	Kontrol	KAP	*P*
Yüzey epiteli	2,35±0,23[a]	2,44±0,24[a]	-
Kript derinliği	0,90±0,26[a]	262±0,19[b]	*0,001*
Sirküler kas	1,80 ±0,16[a]	2,25±0,27[b]	0,042
Longitudinal Kas	1,96,±0,15[a]	2,62±0,05[b]	0,001

Aynı satırdaki farklı harfler istatistiksel anlamlılığı gösterir ([a,b])

Tartışma

KAP'nin düşük dozlarda anti-obezite, ağrı kesici, antioksidan ve anti-inflamatuar etkiler gibi çeşitli biyolojik özelliklere sahip olduğu bildirilmiştir. Ancak, KAP sürekli olarak yüksek seviyelerde tüketildiğinde, kişiler mide ekşimesi, ishal, ağrı ve diğer semptomlar gibi GI rahatsızlıkları yaşayabilir. Bu nedenle, KAP alımının GI etkileri giderek daha fazla ilgi görmektedir. Önceki çalışmalarda insanlarda ortalama KAP tüketimini günlük ortalama kişi başına 30-150 mg, farelerde ise 8-37 mg/kg şeklinde ortaya konmuştur (21,22). Sunulan çalışmada düşük doz KAP uygulamasının sıçan ileumundaki etkileri histolojik olarak değerlendirilmiş ve düşük doz KAP uygulamasının sindirimi destekleyici parametrelerinde olumlu etkiler gösterdiği belirlenmiştir. Sindirim sistemi organlarının genel yapısını oluşturan villusların boyunda ve villus boyuyla direk olarak ilişkili olan ve mitotik aktivitelerin yoğunlukta olduğu kript bezlerinin derinliğinde istatistiki olarak bir artış gözlenmiştir.

Uzun süreli ve yüksek konsantrasyonda KAP uygulanması sensorik sinirlerden Substance P'nin salınımının tükenmesine ve sinirlerde desensitizasyon yaratarak ağrının ortadan kalkmasına, dolayısıyla nöyro toksisiteye sebep olur (24). KAP ağrı giderici etkisinin yanında, immun sistem, gastrointestinal, kardiovasküler ve solunum sistemleri olmak üzere pek çok sistem üzerine de etkilidir. KAP'ın etkisi dozuna, uygulama şekline, süresine ve dokuya göre değişmektedir (25,26). Son yıllarda fizyolojik ve farmakolojik etkilerinden dolayı, tıp alanı ve ilaç sanayinde de kullanımı yaygınlaşmıştır. KAP ayrıca, sindirim sistemi fonksiyonlarını arttırarak, sindirime ve toksinlerin atılımına yardımcı olur. Kolonda ve HCT-8 hücreleri üzerinde yapılan çalışmalar KAP'ın barsak epitel hücrelerinde geçirgenliği arttırdığını makromoleküllerin ve iyonların geçişini kolaylaştırdığını bildirmiştir (27,28). Alkol ile deneysel olarak sıçanlarda oluşturulan gastrik lezyonlara karşı kapsaisinin koruyucu etki yaptığı gösterilmiştir (29).

IGF sistemi, gastrointestinal sistemde hücre çoğalması, sağ kalımı ve apoptozis için önde gelen endokrin, parakrin ve otokrin düzenleyici eksen olarak görev yapar (30). Hem IGF-I hem de IGFI-R'in normal hücrelerde mitojenik ve antiapoptotik etkiler gösterdiği bildirilmiştir. Ayrıca Insülin benzeri büyüme faktörü IGF-I'in bağırsak kriptlerinde radyasyon kaynaklı apoptozu engellediğini bildirmiştir (31,32). Bu durum IGF ifadesindeki artışın sindirim sistemini olumlu yönden desteklediğini düşündürmektedir. Çalışmamızda düşük doz KAP uygulamasının kript hücrelerinde IGFI ve IGFI-R'nün ifadesinin arttığını belirledik. İn vivo çalışmalarda IGF'nin bağırsak epiteli üzerindeki proliferatif etkilerini göstermek adına pek çok farklı deneysel model ile çalışmalar sunulmuştur. IGF-I'in yenidoğan domuzlara oral yoldan verilmesi, ince bağırsak ağırlığı, DNA içeriği, protein içeriği ve ince bağırsaktaki villus yüksekliğinin endekslerini artırmıştır (33). Başka bir çalışmada ise farelere intraperitoneal IGF-I uygulamasıyla kısa süreli bir tedaviden sonra bile bağırsak kript derinliğinde artış görülmüştür (34). Yetişkin sıçanlara 14 günlük parenteral IGF-I uygulaması, kript derinliğini ve villus yüksekliğini %30 oranında artırmıştır (29). Dolaysısıyla sindirim sisteminde IGF ailesinin ifadesi sindirim sistemi parametrelerini olumlu yönden desteklemektedir. Çalışmamızda düşük doz KAP uygulamasının yüzey epitelinde IGF-I ve IGF-IR ifadelerini değiştirmediği ancak kript derinliğinde ve iki katmanlı kas tabakası hücrelerinde tedavi sonrası ifadelerde artış olduğunu gösterdi. Epitel hücrelerinde IGF ailesinde bir değişiklik görülmemesinin uygulama süresi ile ilişkili olabileceğini düşünmekteyiz.

Sonuç

Sonuç olarak bu çalışmada düşük doz KAP uygulamasının sindirim sistemi parametrelerini (villus yüksekliği, kript derinliği, total mukozal kalınlık, villus/kript oranı, yüzey emilim alanı) arttığını ve gastrointestinal sistemde hücre çoğalması, sağ kalımı ve apoptozis için önemli bir büyüme faktörü olan IGF-I ve IGF-IR'nün ifadesinin arttığını belirledik. Bu ifadelerdeki artışın ve morfolojik olarak sindirim sistemini destekleyici parametrelerin artışını kullanılan doz ve süreyle ilişkili olduğunu düşünmekteyiz. Dolayısıyla bu durumda Kapsaisinin gösterdiği etkileri IGF-I ve IGF-IR etki mekanizmasını etkileyerek gösterdiği ve tedavinin sindirim sisteminde besin emilimini ve yararlanımını destekleyerek bağırsak morfolojisini olumlu yönde etkilediği sonucuna vardır.

Teşekkür

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Kaynaklar

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RESEARCH ARTICLE

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Probiyotik Sıcak İçecek ve Tüketime Hazır Atıştırmalıkların Spesifik Mikroorganizmalar Açısından İncelenmesi

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Özet

Günümüzde hastalıklar henüz ortaya çıkmadan, yaşam alışkanlıklarını değiştirerek hastalıkları önleyici ya da geciktirici yaklaşımlar gündemdedir. Beslenme alışkanlıklarının değiştirilmesi ve fonksiyonel gıdaların günlük diyete eklenmesi bu yaklaşımların en popülerleridir. Son yıllarda tüketiciler mağazaların kasa çıkışlarında, marketlerde, e-ticaret sitelerinde probiyotik atıştırmalıklar ve sıcak içeceklerle sıklıkla karşılaşmaktadır. Bu çalışma, süt ürünü olmayan probiyotik içeren atıştırmalıklar ve sıcak içeceklerdeki probiyotik mikroorganizma sayısını belirlemek ve ambalaj bilgileri ile karşılaştırmak amacıyla gerçekleştirilmiştir. Bu kapsamda ambalajında probiyotik ifadesi bulunan bar, granola, meyve topu, çikolata, çay ve kahve dahil toplam 28 örnek çeşitli market ve e-ticaret sitelerinden temin edilmiştir. Klasik mikrobiyolojik teknikler kullanılarak, etiket bilgilerinde yer alan *Lactobacillus* spp., *Streptococcus* spp., *Bacillus* spp. ve aerobik koloni sayısı spesifik besiyeri ve üreme ortam koşulları sağlanarak belirlenmiştir. Ambalaj bilgileri incelendiğinde sadece 7 örnekte (5 çay, 1 kahve, 1 bar) probiyotik mikroorganizma türü ve sayısının birlikte belirtildiği gözlenmiştir. 10 örneğin ambalaj bilgisinde probiyotik, probiyotik kültür gibi genel ifadelerin sayı belirtilmeden yer aldığı, 11 örneğin ambalajında ise sayı belirtilmeden spesifik probiyotik mikroorganizma adı kullanıldığı tespit edilmiştir. Mikrobiyolojik analizlerde, 8 örnekte (2 çay, 3 bar, 1 meyve topu, 2 çikolata) probiyotik ve ayrıca bu örneklerden 5'inde (2 çay, 1 bar, 2 çikolata) aerobik koloni sayısı tespit edilebilir seviyenin altında bulunmuştur. Ambalajında mikroorganizma türü ve sayısı belirtilen 2 çay ve 1 kahve örneğinin ambalaj bilgisinden düşük seviyede probiyotik mikroorganizma ihtiva ettiği ortaya konmuştur. Araştırmada incelenen probiyotik içerikli örneklerin %32.1'i (6 atıştırmalık ve 3 sıcak içecek) tek başına tüketildiğinde beklenen olumlu sağlık etkisini meydana getirecek düzeyde (>10^6 kob/g-mL) probiyotik mikroorganizma içermektedir. Bu araştırma sonucunda, tüketicilerin probiyotik içerikli yiyecek ve içecekleri tek başına değil gün içerisinde diğer probiyotik ve prebiyotik nitelikli ürünlerle kombine olarak ve düzenli kullanmalarının beklenen olumlu sağlık etkilerinin ortaya çıkmasına daha fazla yardımcı olacağı sonucuna varılmıştır.

Anahtar Kelimeler: Probiyotik, çay, kahve, granola, bar, çikolata, meyve topu

Investigation of Probiotic Hot Drinks and Ready-to-Eat Snacks in Terms of Specific Microorganisms

Abstract

Nowadays, approaches to prevent or delay diseases by changing life habits before diseases appear are in the spotlight. Changing eating habits and adding functional foods to the daily diet are the most popular approaches. In recent years, consumers have frequently encountered probiotic snacks and hot drinks at checkout counters, in markets, and e-commerce sites. This study was conducted to determine the number of probiotic microorganisms in non-dairy probiotic snacks and hot drinks and to compare them with the packaging information. In this purpose, a total of 28 samples, including bars, granola, fruit balls, chocolate, tea and coffee with the probiotic ingredient on their packaging, were purchased from various markets and e-commerce sites. Using classical microbiological techniques, *Lactobacillus* spp., *Streptococcus* spp., *Bacillus* spp., included in the packaging information, and aerobic colony count was determined by providing specific medium and growth environment conditions. When the packaging information was examined, it was observed that only in 7 samples (5 tea, 1 coffee, 1 bar) the type and number of probiotic microorganisms were stated together. It was determined that general expressions such as probiotic, probiotic culture were included in the packaging information of 10 samples without specifying a number, while the packaging of 11 samples were used the name of a specific probiotic microorganism without specifying a number. In microbiological analyses, probiotics were identified in 8 samples (2 tea, 3 bars, 1 fruit ball, 2 chocolates), and the total number of microorganisms in 5 of these samples (2 tea, 1 bar, 2 chocolates) was found below the detectable level. It was revealed that 2 tea and 1 coffee sample, with the type and number of microorganisms stated on the packaging, contained probiotic microorganisms at a lower level than the packaging information. 32.1% of the probiotic-base samples examined in the study (6 snacks and 3 hot drinks) contained probiotic microorganisms at a level (>10^6 cfu/g-mL) that would produce the expected positive health effect when consumed alone. As a result of this research, it has been concluded that consumers' regular use of probiotic-containing snacks and hot drinks, not just on their own but in combination with other probiotic and prebiotic products throughout the day, will further contribute to the expected positive health effects.

Key words: Probiotic, tea, coffee, granola, bar, chocolate, fruit ball

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Giriş

Günümüzde tüketiciler sürdürülebilir tipte üretilmiş işlenmiş, doğal, sağlıklı, güvenli kabul edilen ve besin değeri yüksek gıdaları talep etmektedir.1 Toplumlarda gıdaların sağlık üzerindeki etkisine dair farkındalığın her geçen gün artması ile birlikte besin değerinin yanı sıra hastalıkları önlemeye veya semptomlarını azaltmaya yardımcı olacak ilave faydalar sağlayan fonksiyonel gıdaların tüketimi de artmaktadır.2 Vejetaryen beslenmenin yaygınlaşması ve süt tüketiminin neden olduğu alerjik reaksiyonlardan korunma isteği, süt ürünü olmayan probiyotik ürünlere olan talebi arttırmıştır.3 Böylece probiyotiklerin süt ürünü haricindeki gıda ürünleriyle birlikte sunulmasına yönelik alternatiflerin geliştirilmesine ilişkin eğilim ortaya çıkmıştır.4 Probiyotik ürün pazarında son yıllarda öne çıkan ürünler arasında yenilikçi yaklaşımlarla üretilen süt ürünü olmayan içecekler, probiyotik tahıl gevreği, kakao ve meyve bazlı ürünler bulunmaktadır.5 Ayrıca, farklı probiyotik mikroorganizma kombinasyonlarını içeren çikolatalar da satışa sunulmuştur.6

Son yıllarda, çay ve kahve gibi sıcak içeceklerde probiyotik katkısıyla fonksiyonel hale getirilmektedir. *Bacillus coagulans* MTCC5856, *Lactobacillus rhamnosus* GG ATCC 53,103, *Lb. acidophilus* LA-5 DSM 13,241, *Bifidobacterium* animalis subsp. *lactis* BB12 DSM 15,954 ve *B. animalis* CNCM-I 2494 suşları bu amaçla başarılı bir şekilde kullanılmaktadır. Probiyotiklerin sıcak içeceklerin hazırlanma sürecinde ve hazırlandıktan 10 dakika sonra da canlı kaldığı gözlenmiştir. Ardından probiyotik bakteri sporları çimlenerek gastrointestinal sistemde canlılıklarını sürdürmekte ve kolonize olmaktadırlar.7 Probiyotik ile zenginleştirilmiş fonksiyonel kahve kavramı yeni bir kavram olmasına karşın sağlık ve kaliteli yaşam eğilimleri arasında kendine revaçta bir yer edinmiştir.8

Probiyotik ürünlerin düzenli kullanımında barsak mikroflorasını modüle etme, büyük moleküllü karbonhidratları parçalayarak insan sağlığına faydalı bileşenleri ortaya çıkarma gibi olumlu etkileri bulunmaktadır. Tüm bu olumlu sağlık etkilerinin görülebilmesi için probiyotik ürünlerde spesifik probiyotik mikroorganizma sayısının en az 6-7 log kob/g-mL ya da toplam mikroorganizma sayısının 8-9 log kob/g-mL olması gerekmektedir.9 Çalışmada, ambalajında probiyotik ifadesi kullanılarak piyasada satışa sunulan süt ürünü olmayan atıştırmalık ve sıcak içeceklerdeki probiyotik ve canlı mikroorganizma sayılarını belirlemek ve böylece bu ürünlerin ambalaj bilgileri ile analiz sonuçlarını karşılaştırarak sağlık açısından yarar sağlama potansiyelini ortaya koymak amaçlanmıştır.

Materyal ve Metod

Araştırmada market ve e-ticaret sitelerinden 9 adet probiyotik çay, 2 adet probiyotik kahve, 3 adet probiyotik granola, 3 adet probiyotik meyve topu, 9 adet probiyotik bar ve 2 adet probiyotik çikolata olmak üzere toplam 28 adet süt ürünü olmayan probiyotik içerikli ürün satın alınmış ve materyal olarak kullanılmıştır. Temin edilen ürünler ambalaj üzerinde belirtilen muhafaza talimatlarına uyularak laboratuvara getirilmiş ve en kısa sürede analizler gerçekleştirilmiştir.

Her bir probiyotik içeren granola, bar, meyve topu ve çikolata örneğinden steril stomacher poşeti içerisine 25 g tartılmış ve üzerine 225 mL Maximum Recovery Diluent (MRD) (Liofilchem, 610077) eklenerek 2 dk süreyle stomacherde homojenize edilmiştir. İlk 10^{-1} dilüsyondan 1 mL alınarak, içerisinde 9 mL MRD bulunan tüplere aktarılarak seri dilüsyonlar hazırlanmış ve mikrobiyolojik ekimler gerçekleştirilmiştir.

Probiyotik içeren çay ve kahve örnekleri ambalaj üzerinde belirtilen hazırlama talimatlarına uyularak hazırlanmış ve ardından her bir örnekten 25 mL alınarak üzerine 225 mL MRD eklenerek 2 dk süreyle stomacherde homojenizasyon işlemine tabi tutulmuştur. İlk 10^{-1} dilüsyondan 1 mL alınarak, içerisinde 9 mL MRD bulunan tüplere aktarılmış ve seri dilüsyonlar hazırlanarak mikrobiyolojik ekimler yapılmıştır.

Lactobacillus spp. izolasyonu için hazırlanan seri dilüsyonlardan 0.1 mL steril pipet kullanılarak de Man Rogosa Sharpe (MRS) (Merck, 110660) agara yayma plak yöntemi ile ekim yapılarak, petriler 37±1°C'de 24-48 saat anaerobik koşullarda inkübe edilmiştir. İnkübasyon sonunda üreme gösteren kolonilere Gram boyama ve katalaz testi uygulanmıştır. Gram pozitif ve katalaz negatif koloniler sayılarak örneklerdeki *Lactobacillus spp*. sayısı belirlenmiştir.10

Streptococcus spp. izolasyonunda örneklere ilişkin seri dilüsyondan 0.1 mL M17 agar (Merck, 115108) bulunan petrilere ekim yapılarak, 37±1°C'de 24 saat aerobik koşullarda inkübasyona bırakılmıştır. 24 saatin sonunda petrilerde gözlenen tipik kolonilere Gram boyama ve katalaz testleri yapılmıştır. Gram pozitif ve katalaz negatif koloniler *Streptococcus spp*. olarak kabul edilmiştir.11

Bacillus spp. sayısı belirlenecek örnekler, vejetatif hücreleri inaktive etmek için 85±1°C'de 2 dk süre ile benmaride bekletilmiş ve hazırlanan seri dilüsyonlardan 0.1 mL alınarak Nutrient agara (Merck, 1.05450) ekim yapılmıştır. Ardından petriler inkübatörde 37±1°C'de 24 saat sürey-

le tutularak inkübasyon sonunda üreyen koloniler sayılmıştır.12

Aerobik koloni sayısının (AKS) belirlenmesinde seri dilüsyonlardan 1 mL alınarak dökme plak tekniği ile steril petrilere ekim yapılmış ve üzerine yaklaşık 50±1°C'ye kadar soğutulmuş steril Plate Count Agar (Merck, 70152) besiyerinden dökülerek iyice homojenize edildikten sonra agarın donmasını takiben 30±1°C'de 48-72 saat inkübasyona bırakılmıştır. Süre sonunda üreme gösteren koloniler sayılarak örneklerdeki AKS belirlenmiştir.13

Bulgular

Mevcut araştırmada, toplam 28 adet örnek mikrobiyolojik yönden incelenmiştir. Bu örneklerden 9'u probiyotik bar, 3'ü probiyotik granola, 3'ü probiyotik meyve topu ve 2'si probiyotik çikolata olmak üzere 17 farklı atıştırmalıktan oluşmaktadır. Atıştırmalık ürünlerin analiz sonuçlarına ilişkin veriler Tablo 1'de sunulmuştur. Sadece bir adet bar örneğinde probiyotik mikroorganizma türü ve sayısı belirtilmiş ve ambalaj bilgisi ile uyumlu sonuçlar elde edilmiştir. Diğer 6 örneğin ambalajında sayı verilmeden probiyotik mikroorganizma türü ya da probiyotik kültür ifadesi kullanılırken, 10 örneğin ambalajında spesifik probiyotik mikroorganizma türü verilmiştir. Bu örneklerden 6'sında (3 bar, 1 meyve topu, 2 çikolata) araştırılan üç farklı türe ilişkin probiyotik ve 3'ünde (1 bar, 2 çikolata) aerobik koloni sayısı tespit edilebilir limitin altında çıkmıştır.

Tablo 1. Bar, granola, meyve topu ve çikolata örneklerinin mikrobiyolojik analiz sonuçları

No	Ürün	Ambalaj bilgisi*	*Bacillus* spp.*	*Lactobacillus* spp.*	*Streptococcus* spp.*	AKS*
1	Bar	Probiyotik kültür	<1 x 10^2	1.4 x 10^7	<1 x 10^2	2.8 x 10^7
2	Bar	Probiyotik kültür	<1 x 10^{2**}	<1 x 10^{2**}	<1 x 10^{2**}	4.2 x 10^7
3	Bar	Probiyotik kültür	<1 x 10^2	4.3 x 10^6	<1 x 10^2	1.8 x 10^7
4	Bar	Probiyotik kültür	<1 x 10^{2**}	<1 x 10^{2**}	<1 x 10^{2**}	1.4 x 10^4
5	Bar	*B. subtilis* (en az 2.7 x 10^6)	3.2 x 10^7	-	-	1.8 x 10^7
6	Bar	*B. coagulans*	2.8 x 10^7	-	-	1.2 x 10^7
7	Bar	*B. coagulans*	<1 x 10^{2**}	-	-	<10
8	Bar	*Lb. coagulans*	1.1 x 10^5	9 x 10^5	-	2 x 10^5
9	Bar	*B. clasui* *Lb. reuteri* *S. thermophilus*	1 x 10^5	9 x 10^4	1 x 10^5	2 x 10^5
1	Granola	*B. coagulans*	8 x 10^4	-	-	3 x 10^5
2	Granola	*B. coagulans*	1 x 10^5	-	-	6 x 10^5
3	Granola	*B. coagulans*	2 x 10^3	-	-	4 x 10^3
1	Meyve topu	Probiyotik kültür	<1 x 10^2	<1 x 10^2	1.5 x 10^7	1 x 10^7
2	Meyve topu	Probiyotik kültür	<1 x 10^2	1 x 10^6	3 x 10^4	3.8 x 10^6
3	Meyve topu	*B. clasui* *Lb. reuteri* *S. thermophilus*	<1 x 10^{2**}	<1 x 10^{2**}	<1 x 10^{2**}	1.5 x 10^6
1	Çikolata	*Lb. plantarum*	-	<1 x 10^{2**}	-	<10
2	Çikolata	*Lb. plantarum*	-	<1 x 10^{2**}	-	<10

*kob/g; **ambalaj bilgisinden düşük tespit edilen değerler

Mikrobiyolojik analizleri gerçekleştirilen 9'u çay ve 2'si kahve olmak üzere toplam 11 adet probiyotik içerikli sıcak içecek örneğine ilişkin bulgular Tablo 2'de özetlenmiştir. Örneklerin 4 adetinin (3 çay, 1 kahve) içerik bilgisinde miktar belirtilmeden probiyotik içerdiği, bir çay örneğinde ise sayı verilerek probiyotik kültür bulundurduğu ifade edilmiştir. 1'i kahve ve 4'ü çay olmak üzere toplam 5 adet sıcak içeceğin ambalajında içerdiği probiyotik mikroorganizma türü ve sayısı belirtilmiştir. Tüm örnekler değerlendirildiğinde 3'ünün ambalajda belirtilen sayının altında mikroorganizma bulundurduğu tespit edilmiştir. 2 farklı çay örneğinde ise varlığı araştırılan probiyotik ve aerobik koloni sayısı tespit edilebilir limitin altında saptanmıştır.

Tablo 2. Sıcak içecek örneklerinin mikrobiyolojik analiz sonuçları

No	İçecek	Ambalaj bilgisi		*Bacillus* spp.*	*Lactobacillus* spp.*	*Streptococcus* spp.*	AKS*
1	Çay	en az 2 x 10^5	*B. coagulans*	2.3 x 10^5	-	-	2.2 x 10^5
2	Çay	en az 2 x 10^5	*B. coagulans*	9 x 10^{4**}	-	-	3.5 x 10^5
3	Çay	-	*B. coagulans*	5 x 10^4			3.4 x 10^6
4	Çay	en az 2 x 10^5	*B. coagulans*	2.1 x 10^5	-	-	4 x 10^5
5	Çay	2 x 10^5	*B. coagulans*	3 x 10^6	-	-	4 x 10^5
6	Çay	-	Probiyotik	3.9 x 10^7	1.4 x 10^5	1.7 x 10^8	1.1 x 10^7
7	Çay	-	Probiyotik	<1 x 10^2	<1 x 10^2	<1 x 10^2	<10
8	Çay	-	Probiyotik	9 x 10^4	<100	7 x 10^5	7 x 10^4
9	Çay	2 x 10^5	Probiyotik kültür	<1 x 10^{2**}	<1 x 10^2	<1 x 10^2	<10
1	Kahve	-	Probiyotik kültür	7 x 10^4	<1 x 10^2	<1 x 10^2	3 x 10^4
2	Kahve	7.2 x 10^8	*B. coagulans*	1.2 x 10^{8**}	-	-	8.2 x 10^6

*kob/mL; **ambalaj bilgisinden düşük tespit edilen değerler

Tartışma ve Sonuç

Geleneksel fermantasyon süreçlerinde ve modern biyoteknolojik işlemlerde geniş bir uygulama alanına sahip *Bacillus* cinsi, çok işlevli sporlu mikroorganizmalar arasındadır ve birçok faydalı özelliğe sahiptir. Ticari probiyotik *Bacillus* suşlarının, anti-mikrobiyal, anti-kanser, anti-oksidan ve vitamin üretme özellikleri bulunmaktadır.14 Sıcak içecek ve atıştırmalıklarda probiyotik mikroorganizma olarak *Bacillus spp*. en yaygın (16 örnek) tespit edilen gruptur. Ambalaj bilgileri incelendiğinde, 5 çay, 1 kahve, 2 bar, 3 granola örneğinde B. *coagulans* ve 1 bar örneğinde B. subtilis tercih edilmiştir. 1 bar ve 1 meyve topu örneğinde B. clasuii iki farklı grup probiyotik ile kombine kullanılmıştır (Tablo1-2). Analizler sonucunda 11 sıcak içecek örneğinin 9'unda *Bacillus spp*. varlığı tespit edilirken diğer 2 örnekte analiz edilen tüm probiyotik bakteri cinsleri tespit edilebilir sınırın altında bulunmuştur. 4 bar ve 3 granola örneğinde yine *Bacillus spp*. farklı düzeylerde tespit edilmiştir. *Bacillus* türlerinin hareketsiz sporları ısıya dirençli olduğundan sıcak içeceklerde kullanılabilecek en uygun probiyotik tercihlerinden biri olmuştur. Ayrıca, sporlarının enzimatik bozulmaya ve midenin asidik koşullarına karşı oldukça

dirençli olması, ince barsaktan başarılı bir şekilde geçmesi15 de bu probiyotik türünün seçiminde önem taşımaktadır.

Doğada yaygın olarak bulunan *Lactobacillus* türleri gıdalarda tercih edilen bir diğer probiyotik türüdür. İnflamatuar barsak hastalığı gibi kronik hastalıklardan korunmada rol oynayan bağırsak mikrobiyotasının önemli bir üyesidir. Ayrıca probiyotik *Lactobacillus* türlerinin çoğu anti-mikrobiyal aktiviteye sahip bakteriyosin üretme, anti-kanser ve bağışıklık baskılayıcı özellikler gösteren enzimler üretirler.16 Ambalaj verilerinde Lb. plantarum 2 çikolata, Lb. *coagulans* 1 bar ve Lb. reuteri 1 bar ve 1 meyve topu örneğinde iki farklı probiyotik ile kombine olarak kullanılmıştır (Tablo1-2). Gerçekleştirilen analizler sonucunda 4 bar, 1 meyve topu ve 1 çay örneğinde *Lactobacillus spp*. farklı düzeylerde tespit edilmiştir (Tablo 1). Bazı *Lactobacillus* türlerinin termofilik karakterde17 olması yanı sıra sporsuz bakterilerin mikrokapsüle edilmesi ile 100ºC'nin üzerindeki sıcaklıklarda canlı kalabilmesi sağlanmaktadır.18 Böylece sıcak içeceklerde laktobasil varlığı açıklanabilmektedir.

Laktik asit bakterileri arasında yer alan *Streptococcus thermophilus* gıdalarda kullanımı güvenli kabul edilen, süt endüstrisinde yaygın kullanım alanı olan ve suş bazında probiyotik özellik gösteren termofilik karakterde endüstriyel bir mikroorganizmadır.19 S. *thermophilus* diğer probiyotiklerle kombine olarak 1 bar ve 1 meyve topu ambalajı içerik bilgisinde bulunmaktadır. Bar örneğinde 1 x 105 kob/g düzeyinde tespit edilirken, meyve topu örneğinde tespit edilebilir limitin altında saptanmıştır. Ambalajında probiyotik ifadesi olan 2 farklı çay örneğinde *S. thermophilus* izole edilmiştir. *S. thermophilus* termofilik karakteri ve mikrokapsülasyon teknolojisi ile sıcak içecek ortamında canlılığını sürdürebilmektedir.

Çalışmada, tüm örnekler AKS içeriği bakımından da değerlendirilmiştir. Analizleri gerçekleştirilen toplam 28 örnek arasında 1 bar, 2 çikolata, 2 çay örneğinde AKS tespit edilebilir limitin altında (<10 kob/g-mL) bulunmuştur. Diğer 23 atıştırmalık ve sıcak içecek örneğinde ise 3 x 10^4 ile 4.2 x10^7 kob/g-mL aralığında saptanmıştır. Probiyotik gıda ürünlerinde istenilen olumlu sağlık etkisini elde etmek için probiyotik mikroorganizma sayıları yanı sıra AKS'ninde 10^8-10^9 kob/g-mL seviyesinde olması gerekmektedir.9 Bu bağlamda, incelenen örneklerin tamamı AKS yönünden düşük düzeyde kalmıştır.

Araştırma sonuçları genel olarak değerlendirildiğinde, 2 çay, 3 bar, 1 meyve topu ve 2 çikolata olmak üzere toplam 8 örnekte spesifik probiyotik mikroorganizma sayısının ve bu örneklerin 5'inde ise AKS'nin tespit edilebilir seviyenin altında olduğu saptanmıştır. Ambalajında mikroorganizma türü ve sayısı belirtilen 2 çay ve 1 kahve örneğinde belirtilenden düşük seviyede probiyotik mikroorganizma bulunduğu ortaya konmuştur.

İçerik bilgisinde probiyotik kültür/probiyotik ifadesi bulunan örneklerde *Lactobacillus spp*. 2 bar, *Streptococcus spp*. 1 meyve topu, *Bacillus spp*. 1 kahve örneğinde üründe tek probiyotik mikroorganizma olarak izole edilmiştir. Ayrıca, 1 meyve topu (*Lactobacillus spp*., *Streptococcus spp*.), 1 çay (*Bacillus spp*., *Streptococcus spp*.) ve diğer 1 çay (*Bacillus spp*., *Lactobacillus spp*., *Streptococcus spp*.) örneğinde karışık probiyotik kültür kullanıldığı tespit edilmiştir (Tablo 1-2). Analiz edilen örneklerden sadece 9 adetinin günlük düzenli tüketimde olumlu sağlık etkisini meydana getirecek düzeyde (>10^6 kob/g-mL) probiyotik mikroorganizma içerdiği belirlenmiştir. 11 örneğin ise başka probiyotik ürünlerle birlikte günlük düzenli tüketilmesinde istenilen sağlık etkilerini tüketiciye sunabileceği sonucuna varılmıştır. Türk Gıda Kodeksi Beslenme Beyanları Yönetmeliği20 ve ekinde son tüketiciye arz edilen gıdalardaki beslenme beyanlarına ilişkin kurallar tanımlanırken, probiyotiklere ilişkin beslenme beyanı ve beyan koşulu tanımlanmamıştır. Bu nedenle probiyotik içeriğine ilişkin ambalaj bilgileri ile araştırma sonucu elde edilen veriler arasında farklılığın tespit edildiği örnekler tağşiş gıda olarak değerlendirilmemiştir. Ancak, gıda mevzuatı beslenme beyanları kapsamının genişletilmesinin belirsizlikleri önleyeceği ve tüketici sağlığı ile haklarını koruma bakımından faydalı olacağı kanaatine varılmıştır.

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Investigation of Mold and Yeast Contaminations in Cheese Samples

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Abstract

Fungi are common contaminants of cheese. Although they are added during the cheesemaking as starter cultures, they might also contaminate the cheese ripening or storage environments. *Candida*, *Penicillium* and *Aspergillus* species can cause serious systemic mycosis in humans and animals. This study aimed to investigate the diversity of the fungal microbiota in 100 samples of various cheese types and screen the azole resistance of *Aspergillus flavus* isolated from these samples. Twenty aged kashar, 20 fresh kashar, 25 white pickled, 25 curd cheese, and 10 cream cheese were collected from different vendors over six months in Bursa Province. Potato Dextrose Agar (PDA) was used to isolate the fungi. Sabouraud Dextrose Agar (SDA) and PDA were used for conventional identification. After microscopic and macroscopic evaluations, isolates were identified species-wise using the Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) system. Seventy-six mold and 81 yeast isolates were isolated and selected from 94 out of 100 cheese samples. As a result of conventional evaluation, 156 isolates (excluding one yeast isolate) could be identified. Based on the MALDI-TOF MS analysis, 55.6% (79/142) of the isolates were yeasts assigned to 31 species across five genera. In comparison, 44.4% were molds assigned to 28 species across five genera. The predominant fungal genus detected was *Candida* (45.1%, 64/142), followed by *Penicillium* (32.4%, 46/142). The most frequently isolated fungal species, *C. famata* (n=37), was found in all cheese types. One strain of *A. flavus* complex was isolated from one curd cheese sample and was susceptible to azole. This study successfully assesses the fungal microbiota of various cheeses from Bursa, consisting of diverse groups of yeasts and molds. While most of the molds consisted of *Penicillium* spp, detecting azole-sensitive *A. flavus* complex underlines the need for regular monitoring of cheese microflora owing to the risk of resistance development.

Keywords: Azole resistance, cheese microbiology, food hygiene, fungal contamination, public health

Introduction

Fungal infections have been on the rise in recent years. These infections include a variety of diseases caused by molds and yeasts that are considered not to be contagious to animals or humans in the past and therefore not recognized as pathogens. This condition seems to be mainly because of changing trends in patient epidemiology; however, this explanation creates a very narrow view, since in theory every fungus could already cause serious systemic mycotic infections especially in immunocompromised patients (1-3). On the other hand, the "One Health" approach brought a new perspective that diseases are multifactorial conditions. In addition to patient factors, variations in the health systems and advanced treatment procedures, environmental changes, geographic locations, increased contact of humans and animals (increased trade, travel, etc.), changes in agricultural procedures (e.g., increased antifungal usage), and nutritional habits, and similar other global factors have serious places (4,5). The One Health approach recently showed itself with a reduce the severe rates of azole-resistant invasive *Aspergillus* infections related to the wide usage of antifungal agents for agricultural purposes (5).

Cheese has a structure appropriate for developing molds and yeasts under suitable temperature and humidity conditions. Molds are saprophytic microorganisms responsible for the spoilage of dairy products, especially of unripened cheese. Different species can grow on various cheese types. *Aspergillus* and *Penicillium* species can grow from 4 °C to 10 °C at low temperatures. Their growth may result

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in a musty off-flavor, and their appearance may be commercially undesirable, resulting in the downgrading of the cheese. There is also significant potential for mycotoxin production (6). Molds may pioneer severe clinical infections, especially in immunocompromised patients such as elders with comorbidities (1). Conidial spread from a microbiologic culture-like area (cheese) will be intense and exposure may lead to infections (1,6). Yeast species are common in soil, hospital environments, and contaminated foodstuffs (7). These species, known as food spoilage microorganisms and opportunistic pathogens, are part of the intestinal microbiota but can cause systemic mycosis in some cases (7,8). Although the most important species causing human disease is *Candida albicans*, other species such as *C. parapsilosis*, *C. famata*, *C. kefir*, and *C. inconspicua* are also among the species frequently isolated in invasive candidiasis cases in recent years (7).

The main goal of this study was to contribute to the "One Health" point of view by investigating the possible fungal exposure originating from cheese, an essential part of everyday nutrition. The study aimed to identify yeast and mold species at genus and species level in yeast-mold populations in cheese samples and also to detect azole resistance in *A. flavus*, the causative agent of mycosis. These fungal species' prevalence and resistance pattern were assessed to provide helpful insights into the food safety risks associated with fungal contamination in cheese. The expected outcome of this work will also contribute to the public health strategy for reducing exposure to fungi and its resultant health effects.

Materials and Methods

Sampling of Cheese Samples

In this study, 100 cheese samples were collected from bazaars in Bursa Province, Türkiye. The samples included aged kashar (n=20), fresh kashar (n=20), white pickled cheese (n=25), curd (n=25), and cream cheese (n=10), with each sample weighing at least 100 grams. All samples were transported under aseptic conditions to the laboratory and stored at 4 °C until analysis.

Isolation and Enumeration of Fungal Species

Samples (10 g) were homogenized in 90 mL of sterile 1% peptone water using a Stomacher 400 (Seward Laboratory Systems, London, UK) for two min at average speed. Samples were serially diluted and spread onto Potato Dextrose Agar (PDA) (Oxoid Ltd., Thermo Fisher Scientific, Massachusetts, USA) (adjusted the pH to 3.5 ± 0.1 with 10% sterile tartaric acid) and incubated for 5-7 days at 25 °C (9). After incubation, mold and yeast colonies were enumerated and selected. The viable colonies were enumerated as log10 CFU g^{-1}. One mold and/or yeast colony was selected from each positive sample and stored at -80 °C for further analysis.

Identification of Fungal Species

The individual colonies were selected according to their color and morphology. Yeast and mold colonies on the plates were examined by direct microscopy for preliminary confirmation and then onto Sabouraud Dextrose Agar (SDA) (RTA Laboratories, Istanbul, Turkey) and PDA, which were then incubated at both 25 °C and 35 °C in ambient atmosphere. After at least 48-h incubation, all new colonies were identified by conventional methods including morphology, germ tube testing, thermotolerance, cornmeal agar, urease testing, pellicle formation in broth, Indian ink capsule observations, cycloheximide resistance, and acid-fast staining for ascospore presence (10-12).

After enough sporulations, all mold colonies were macroscopically examined (growth pattern, speed of growth, pigmentation) (13,14), were tested for thermotolerance, and were microscopically evaluated by both physiologic serum and lactophenol cotton blue staining (Mediko Kimya, Istanbul, Turkey) for special features such as hyphae, septa, vesicles, phialides, conidia, and sexual spores (15). All organisms were stored at -20 °C with 15%-glycerol tryptic soy broth (GBL Laboratories, Istanbul, Turkey) to use in case of further evaluations.

Matrix-assisted laser desorption/Ionization time of flight mass spectrometry (MALDI-TOF-MS) Biotyper instrument equipped with MALDI Biotyper RealTime classification software and FlexControl software (version 3.3) (Bruker Daltonics, Bremen, Germany) was used to identify yeast isolates. A single fresh colony of each yeast isolate was taken from SDA plates, and a thin colony layer was spotted on a 96-spot polished steel MALDI target plate, which was allowed to air dry at room temperature for 30 min. Then, each spot was overlaid by 1 µL of a daily prepared solution of matrix solution (HCCA, α-cyano-4-hydroxycinnamic acid) (16). Afterwards, the sample prepared on the target plate was inserted into the device and analyzed using software associated with the respective system. A score above 2, the threshold value recommended by the manufacturer, was accepted for identification.

To identify mold species by MALDI-TOF MS analysis, subcultures of mold isolates were grown on SDA by incubation at 22-25 °C for 3-5 days. Pre-treatment followed the

procedure with some modifications, as described previously by Becker et al. (2015) (17). A 5 mm diameter mold (including the hyphal bed) was removed from the PDA plates (Oxoid Ltd., Thermo Fisher Scientific) and placed into 5 mL of tubes containing SDB (GBL Laboratories) using a swab before starting sporulation (at an early stage). Tubes were kept in an ultrasonic bath (Elma-Transsonic 460H; Techspan Group, Auckland, New Zealand) for 15 sec and then rotated (Stuart rotator SB3; Novatek Analytical Systems Inc., Istanbul, Turkey) at 18 rpm for 18 h. Then tiny colonies were transferred to the microcentrifuge tube (1.5 mL; Eppendorf AG, Hamburg, Germany) by Pasteur pipettes in the amount of 1.5 mL and centrifuged (Electro-mag M4800M; Elektro-Mag, Istanbul, Turkey) for 2 min at 13,000 rpm. SDB (GBL Laboratories) was removed with a micropipette. The colonies were washed with the water of LC-MS grade (Merck Chromatography-LC-MS grade, 1.15333.2500; Merck KGaA, Darmstadt, Germany), and 1,000 µL was left in each tube. The centrifugation and washing processes were repeated twice and finally the remaining liquid was removed. Then, a volume of 300 µL of deionized water (high-pressure liquid chromatography [HPLC] quality; Merck KGaA) was taken into a new microcentrifuge tube and the visibly prepared colony (5-10 mg) was transferred into this tube. It was mixed with the pipette and vortexed (NUVE NM110; Nüve Laboratory & Sterilization Technology, Ankara, Turkey) for 15 min, followed by centrifugation for 2 min at 13,000 rpm. A volume of 900 µL of ethanol (EtOH; Merck KGaA) was added, and the tubes were again vortexed for 5 min, followed by centrifugation at 13,000 rpm for 2 min. The upper phase (EtOH) was removed without damaging the pellets remaining at the bottom and the centrifugation was repeated. The remaining EtOH was removed with a pipette. Pellets were dried at room temperature for 2-3 min and mixed thoroughly with 1-80 µL of 70% (v/v) formic acid (FA; Merck KGaA) and 1-80 µL of acetonitrile (ACN; Merck KGaA) followed by a centrifugation step for 2 min at 13,000 rpm. A 1 µL clear supernatant was placed onto a ground steel MALDI target plate (Bruker Daltonics) and allowed to dry at room temperature. Subsequently, each sample was overlaid with 1 µL of matrix solution (HCCA, α-cyano-4-hydroxycinnamic acid) and dried at room temperature. Then, placed into the Bruker MicroFlex LT device (Bruker Daltonics), the results were evaluated with MALDI Biotyper RealTime Classification software and FlexControl software (version 3.3) programs.

Screening for Azole Resistance of *Aspergillus flavus* Complex

The test was performed according to Guinea et al. (2018) (18). Mold colony identified as *A. flavus* complex with Biotyper software, version 3.3 was next screened for resistance using the agar-based screening for azole resistance test, which consists of a 4-well plate containing agar supplemented with three azole drugs (itraconazole [ITC] at 4 mg/L, voriconazole [VRC] at 2 mg/L, and posaconazole [POS] at 0.5 mg/L) (Spectrum Chemical MFG Corp., NJ, USA) and a growth control without any antifungal drug. A wet sterile swab was used to collect conidia from an *A. flavus* colony to visually make a 0.5 McFarland suspension in 1 mL of sterile water. A disposable pipette was used to add one drop of the suspension (25 µL) to each of the four wells. The plates were incubated at 35-37 °C for 24-48 h. *A. flavus* ATCC 204304 was used as a control strain.

Results

As seen in Table 1, the yeast count varied between 2.30 and 8.23 log10 CFU g^{-1}, while the mold count varied between 2 and 6.85 log10 CFU g^{-1} in all cheese samples. Aged kashar cheese showed the highest incidence of fungal contamination, with 95% of samples testing positive for yeast at concentrations between 4.78 and 8.23 log10 CFU g^{-1}, with an average of 6.73 log10 CFU g^{-1}. Similarly, mold contamination was common in aged kashar cheese samples, showing the highest prevalence (90%) with an average count of 5.07 log10 CFU g^{-1}. This was followed by curd cheese, which had an 84% prevalence rate, showing a relatively higher risk of yeast contamination. The lowest rate of yeast contamination was found in white pickled cheese, with contamination detected in only 56% of samples. In relation to mold contamination, aged kashar had also the highest prevalence with 90% of the samples. In cream cheese, although the prevalence of yeast was lower, the mold contamination rate was relatively high at 70%.

Table 1: Fungi prevalence and counts (log10 CFU gr^{-1}) in cheese samples

Cheese Type	Samples Count	Positive for Mold			Positive for Yeast		
		No	%	Count	No	%	Count
Aged kashar	20	18	90	Min: 3.0 Max: 6.85 Mean= 5.07 SD=1.23	19	95	Min: 4.78 Max: 8.23 Mean= 6.73 SD=0.97
Fresh kashar	20	11	55	Min: 2.30 Max: 4.90 Mean= 4.07 SD=0.72	14	70	Min: 3.0 Max: 7.18 Mean= 5.22 SD=1.16
White pickled	25	17	68	Min: 2.0 Max: 6.30 Mean= 4.13 SD=1.12	14	56	Min: 3.0 Max: 7.68 Mean= 4.91 SD=1.60
Curd	25	14	56	Min: 2.0 Max: 5.32 Mean= 3.82 SD=0.85	21	84	Min: 2.30 Max: 7.98 Mean= 4.90 SD=1.74
Cream	10	7	70	Min: 3.48 Max: 5.30 Mean= 4.48 SD=0.73	6	60	Min: 3.30 Max: 5.70 Mean= 4.75 SD=0.87

Min: *Minimum;* ***Max:*** *Maximum;* ***Mean:*** *Arithmetic mean ;* ***SD:*** *Standart deviation*

There were 157 isolates (81 yeast and 76 mold isolates) selected after incubation at 25 °C for 5-7 days in PDA of pH 3.5 ± 0.1. As presented in Table 2, fungal contamination

was detected in 94 out of 100 cheese samples. While 156 (80 yeast and 76 mold isolates) of the 157 selected isolates were confirmed conventionally, 142 (79 yeast and 63 mold isolates) were identified at genus and species level by MALDI-TOF MS. The predominant fungal genus detected in 68.1% of the samples was *Candida* (45.1%, 64/142), followed by *Penicillium* genus (32.4%, 46/142). *C. famata* (n=37), *C. zeylanoides* (n=15), *C. lambica* (n=5), *C. kefyr* (n=3), *C. valida* (n=1), *C. parapsilosis* (n=1), *C. intermedia* (n=1) and *C. inconspicua* (n=1) were the identified *Candida* species. *C. famata*, found in all cheese types, was mainly isolated from aged kashar samples (n=13), followed by fresh kashar samples (n=9). Among all selected fungal isolates, only one yeast isolate could not be identified by conventional evaluation, and this isolate was identified as *Geotrichum silvicola* by MALDI-TOF MS.

As a result of MALDI-TOF MS analysis, seven distinct mold species were detected across five types of cheese from a total of 94 out of 100 samples. Various *Penicillium* species, including *P. commune*, *P. digitatum*, *P. camemberti*, *P. italicum*, *P. roqueforti*, and *P. singorense*, were also identified in these samples. *P. commune* was the predominant mold species in 19% (18/94) of the samples. *P. digitatum* was present in 12.8% (12/94) of the samples. The *A. flavus*-oryzae group was isolated and identified from only one (curd cheese), and two or more mold species were isolated from five cheese samples. The azole resistance assay result was negative for the sole *A. flavus* isolate from a curd cheese sample.

Table 2: Identification of fungi isolates from the cheese samples

	Identification of Molds		**Identification of Yeasts**	
Type and number of cheese samples	**Conventional Evaluation**	**MALDI-TOF MS**	**Conventional Identification**	**MALDI-TOF MS**
Aged kashar cheese (n=20/20)	*Penicillium* spp. (n=19)	*Penicillium commune* (n=11) *Penicillium camamberti* (n=3) *Penicillium digitatum* (n=3) [a]NRI (n=2)	*Candida* spp. (n=17)	*Candida famata* (n=13) *Candida zeylanoides* (n=4)
			Yarrowia lipolytica (n=2)	*Yarrowia lipolytica* (n=2)
	Order of *Mucorales* (n=2)	*Rhizopus stolonifera* (n=2)		
Fresh kashar cheese (n=18/20)	*Penicillium* spp. (n=8)	*Penicillium digitatum* (n=3) *Penicillium commune* (n=1) *Penicillium italicum* (n=1) [a]NRI (n=3)	*Candida* spp. (n=11)	*Candida famata* (n=9) *Candida valida (Pichia membranifaciens)* (n=1) *Candida parapsilosis* complex (n=1)
	Order of *Mucorales* (n=3)	*Rhizopus stolonifera* (n=2) *Mucor circinelloides* (n=1)	*Candida kefyr* (n=1)	*Candida kefyr* (n=1)
			Yarrowia lipolytica (n=1)	*Yarrowia lipolytica* (n=1)
	Dematiaceous fungi (*Cladosporium* sp.) (n=1)	*Cladosporium* sp. (n=1)	*Saccharomyces* spp. (n=1)	*Torulaspora delbrueckii* (formerly *Saccharomyces delbrueckii*) (n=1)
			Rhodotorula spp. (n=1)	*Rhodotorula mucilaginosa* (n=1)
White pickled cheese (n=22/25)	*Penicillium* spp. (n=15)	*Penicillium italicum* (n=4) *Penicillium commune* (n=2) *Penicillium roqueforti* (n=2) *Penicillium camamberti* (n=2) *Penicillium singorense* (n=1) [a]NRI (n=4)	*Candida* spp (n=13)	*Candida famata* (n=7) *Candida zeylanoides* (n=4) *Candida lambica* (n= 1) *Candida intermedia* (n=1)
			Saccharomyces spp. (n=2)	*Torulaspora delbrueckii* (formerly *Saccharomyces delbrueckii*) (n=1)
	Order of *Mucorales* (n=2)	*Rhizopus stolonifera* (n=2)		*Kluyveromyces lactis (*formerly *Saccharomyces lactis)* (n= 1)
	Dematiaceous fungi (*Cladosporium* sp.? (n=2)	*Cladosporium* sp. (n=2)		
Curd cheese (n=25/25)	*Penicillium* spp. (n=11)	*Penicillium digitatum* (n=5) *Penicillium commune* (n=2) *Penicillium camamberti* (n=2) [a]NRI (n=2)	*Candida* spp. (n=16)	*Candida famata* (n=6) *Candida zeylanoides* (n=5) *Candida lambica* (n=4) *Candida inconspicua* (n=1)
	Order of *Mucorales* (n=2)	*Rhizopus stolonifer* (n=2)	*Candida kefyr* (n=2)	*Candida kefyr* (n=2)
	Dematiaceous fungi (*Cladosporium* sp.) (n=1)	[a]NRI (n=1)	*Yarrowia lipolytica* (n=2)	*Yarrowia lipolytica* (n=2)
				*Kluyveromyces lactis (*formerly *Saccharomyces lactis)* (n=1) *Torulaspora delbrueckii* (formerly *Saccharomyces delbrueckii*) (n=1)
	Paecilomyces spp. (n=1)	*Byssochlamys spectabilis* (*Paecilomyces variotii*) (n=1)	*Saccharomyces* spp. (n=2)	
	Aspergillus flavus complex (n=1)	*Aspergillus flavus-oryzae* group (n=1)		
				Rhodotorula mucilaginosa (n=1)
			Rhodotorula spp. (n=2)	[a]NRI (n=1)
			[b]NI (n=1)	*Geotrichum silvicola* (n=1)
Cream cheese (n=9/10)	*Penicillium* spp. (n=4)	*Penicillium commune* (n=2) *Penicillium digitatum* (n=1) *Penicillium corylophilum* (n=1)	*Candida* spp. (n=5)	*Candida famata* (n=2) *Candida zeylanoides* (n=2) [a]NRI (n=1)
	Order of *Mucorales* (n=4)	*Rhizopus stolonifera* (n=2) *Mucor circinelloides* (n=1) [a]NRI (n=1)	*Saccharomyces* spp. (n=2)	*Torulaspora delbrueckii* (formerly *Saccharomyces delbrueckii*) (n=1) *Kluyveromyces lactis (*formerly *Saccharomyces lactis)* (n=1)

Discussion

The number of fungal species may increase during ripening, especially in kashar cheeses in direct contact with air. Since one of the dominant flora in aged kashar is *Penicillium spp*, it can be concluded that contamination might have occurred during the ripening stage. *Candida spp*. is the dominant genera in curd cheese; this may be because curd cheese has higher moisture than aged kashar and is a better environment for yeast growth. Lower fungal counts were reported in kashar cheese samples (19-21) and in white cheese samples (22,23) in Türkiye. Godek et al. (2021) (24) found that 74% of the white cheese samples had both yeast and mold, which is relatively higher than what we found. Bouakline et al. (2000) (25) found no fungal contamination

in cream cheese samples. Still, all the soft cheese samples (n=20) had been contaminated with *Geotrichum spp*. and *Candida norvegensis*. While we could identify *Geotrichum silvicola* from one curd cheese sample, *C. norvegensis* was not found in any cheese samples. The samples used in our study were collected from bazaars where they are displayed and sold unpackaged. Exposure to open air, combined with the possible poor hygienic conditions during production, ripening, storage, transportation, and the time spent on market stalls create a potential contamination and breeding environment for fungal species.

While recent studies (26) identified *Mucor* and *Candida* (especially *Issatchenkia orientalis*) as dominant species in cheese, we found *C. famata* as the most prevalent yeast and *P. commune* as the most common mold. Unlike our study, Hameed (2016) (27) found only three yeast species: *C. albicans*, *C. krusei*, and *Debaryomyces hansenii*; *C. albicans* was dominant among all yeasts with 24%, 30%, and 67% ratios from three different cheese samples. Out of all the mold isolates, *Cladosporium cladosporidis* was the dominant species, followed by the only detected *Penicillium* species, which was *P. corylophilum*, and isolated four *Aspergillus* species. *C. albicans* was not detected in our samples, while *P. corylophilum* was only encountered once. Moreover, the dominant mold species was *P. commune* (one of the fungal species known to cause invasive fungal infections) (28), followed by *P. digitatum*, and one *Aspergillus* species was detected in one of our curd cheese samples. Several studies have reported that the dominant mold in cheeses is *Penicillium*, with the most dominant species being *P.* roqueferti (29,30) and *P. communae* (31), respectively. Our data revealed that the predominant fungus was *Candida*, succeeded by *Penicillium*, suggesting significant variations in the fungal species isolated across previous investigations and the prevailing species. The diversity in fungal communities in cheese is thought to be due to regional, environmental, and production-related factors, including differences in sampling, microbial ecology, and climate.

When considering the technologies of these cheeses, the milk used must be heat processed before the starters are added, and these cheeses also do not have fungi as starter cultures. Heat processing inactivates fungi spores due to their thermo-susceptibility (32). After cheese production, there are different ways that the product might get contaminated. The air is one of the common fungal contamination sources. Air quality plays a considerable part during the production, ripening, and storage stages (33). There also is a brined cheese, in which the brine is a potential contamination source. In addition, different areas of the production plant may have different levels of microorganism concentration, although *P. commune* is still the most common contaminant (34).

Azole compounds are used as both antifungal agents in human and veterinary medicine and as fungicides in agriculture (35). Resistance development in fungi seems much more complicated than in bacteria, which can quickly transfer the resistance genes (36). Some studies showed that azole resistance might develop in fungi isolated from infected patients, considering their extensive use (37,38). The prevalence of azole-resistant *Aspergillus spp* is increasing worldwide and is thought to be related to improper use of azole antifungals in agriculture. In a study conducted in France, 208 *Aspergillus* isolates were obtained from clinical and soil samples and eight isolates resisted at least one azole drug (39). In Japan, another study identified two azole-resistant *A. flavus* isolates among 99 *Aspergillus spp*. obtained from 50/692 food samples containing agricultural products (40). Additionally, a separate investigation reported that 18.6% of *Aspergillus* isolates were azole-resistant (41). All these studies indicate that azole is misused in agricultural crop cultivation and that azole resistance is spreading. Proximity to agricultural crop fields, where fungicides are used intensively, and consumption of food contaminated with fungal species may increase the spread of resistant strains into larger populations.

Our study showed that one sample, which constituted 1% of the samples, contained *A. flavus* which was susceptible to azole. The transfers of genes amongst fungi, let alone inter-species, are a very complex process. Application of azoles-both as medicines and also as fungicides in agriculture-needs to be carefully policed. This finding has proved significant in establishing the fact that in our population, *A. flavus* is still susceptible to treatments based on azoles. Azoles are extensively applied in clinical and agricultural use to inhibit fungal growth. Thus, the future research could be on prevalence and resistance profile of *A. flavus* from various types of cheese and different geographical regions showing potential risks and effective management.

Conclusion

In our study, *Candida* was found to be the dominant fungus genera in five different types of cheese. In addition, while the primary source of fungus contamination was determined to be aged kashar cheese, the absence of azole resistance in *A. flavus* isolated from curd cheese was reassuring regarding the prevalence of antifungal resistance. Consumption of cheeses with high levels of fungus genera such as *Candida*, *Penicillium* and *Aspergillus*, which are

known as causative agents of systemic mycosis, can pose significant health risks, particularly for individuals with compromised immune systems. Improving production, ripening, and storage could greatly reduce contamination rates of fungus species in the food production-consumption chain.

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Ultrastructure of the Tongue of Turkeys (*Meleagris gallopavo*-hybrid breed) Reared in Türkiye Using Light and Scanning Electron Microscopy

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Abstract

The tongue is one of the organs involved in the digestive and sensory systems, which plays a vital role in feeding strategies in birds. In our study, macroscopic and microscopic characteristics of the tongue were determined in turkeys raised in Türkiye. Five turkey tongues, cut for consumption, were used. The tongue was divided into apex, corpus, and radix sections and fixed in formaldehyde. After fixation, routine histological tissue procedure was performed. The sections were stained with Crossman's triple staining method and histological features were determined. For scanning electron microscopy, tissue samples fixed by glutaraldehyde solution were coated with gold after the routine procedure and examined under a scanning electron microscope. As a result of the study, the tongue was triangular, and conical papillae were observed between the corpus and radix. Two cornified epithelial structures were identified through histological and electron microscopic findings. Microscopic papillae extending from the connective tissue to the epithelial layer on the dorsal surface of the tongue, extending in different directions were quite prominent. Anterior and posterior lingual glands were present in the corpus and radix of the tongue. Electron microscopic examination showed the opening holes of these glands and conical papillae. Although slight differences were observed, the tongue's structure was generally similar to that of Galliform birds.

Keywords: Morphology, scanning electron microscopy, tongue, turkey.

Introduction

In birds, the upper and lower jaws develop into a beak, while teeth, lips, and cheek formations are absent. The presence of the beak in birds, along with different feeding habits, and living conditions, has led to morphological differentiation of their tongues (1). Anatomically, bird tongues bear a basic resemblance to the tongue segments of mammals (apex, corpus, and radix) and form a triangular organ that fills the entire lower part of the beak. In mammals and aquatic and terrestrial poultry species (geese, ducks, swans, etc.), the tongue functions in solid food intake, grass cutting, drinking, and water filtration (2-6).

The avian tongue is characterized by mechanical papillae (7,8). The formation of a papillar crest consisting of mechanical conical papillae extending caudally and separating the tongue corpus and radix is remarkable (9,10). This crest structure can be observed as single or double. Conical papillae are known to assist in transporting food particles on the tongue, manupilating food, and filtration fluid (11,12).

Studies conducted in different bird species show determined that the histological structure of the tongue form, tongue skeletal apparatus, and tongue epithelium positively correlate with birds' feding habits (11, 12-15). The literature shows that the tongue has been examined macroscopically and microscopically in many bird species such as chicken, quail, parrot, penguin, goose, duck, and domestic

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turkey (6, 16-18).

Turkey (*Meleagris gallopavo*) is a large Galliform bird reported to have been domesticated before Christopher Columbus discovered the Americas, with various subspecies of its wild ancestor spreading over a wide area from southern Canada to southern Mexico (19,20). Turkey meat is widely preferred for its high nutritional value, low fat and cholesterol content, its ability to be processed into various products, and its flavor (21). In Türkiye, turkey meat consumption has increased to meet the increasing population's animal protein needs and with the tendency towards healthy nutrition.

This study aimed to investigate the general morphological structure of the tongue in turkeys raised in Türkiye by using light and scanning electron microscopy. It also aimed to determine the similarities and differences with birds from the same family.

Material and Methods

Supply of Animals

The tongue structures of five healthy adult turkeys slaughtered for consumption were analyzed. Tongues were dissected from each bird and removed from the oral cavities. Macroscopic features were noted.

The procedures used in this investigation were approved by the Siirt University Experimental Animals Application and Research Center under ethics committee report number 2024/05/27.

Light Microscope

The tongue was divided into the apex, corpus, and radix for histological procedures. Tissues were fixed in a 10% neutral buffered formalin solution for 24 hours at room temperature, and routine tissue processing procedures were performed. After dehydration in 70% ethanol, 80% ethanol, 96% ethanol, and absolute ethanol, the tissues were embedded in paraffin and 5 µm sections were taken. Tissue sections were mounted on polylysine-coated slides and incubated in an oven at 37°C for 1 hour. The slides were stained using Masson trichrome staining method modified by Crossman (22). All slides were examined under a light microscope (Nikon Eclipse 80i Microscope, Tokyo, Japan). Photographs were taken with a Nikon Ds Camera Control Unit DS-L1 (Tokyo, Japan).

Scanning Electron Microscope

Tissue samples taken for scanning electron microscopy (SEM) images were kept in 2.5% (pH: 2.7) glutaraldehyde solution for 24 hours. After the first fixation step, they were washed three times at 10-minute intervals in 0.1 M Phosphate buffer (pH 7.4). The second fixation step was completed by rotating osmium tetraoxide at room temperature for 2 hours. The samples were then washed three times for 10 min each in phosphate buffer. Tissues were dehydrated in 25%, 50%, 75%, and 90% ethyl alcohol at +4°C for 15 minutes each. Following dehydration, they were kept in 96% and 100% ethyl alcohol for 30 minutes each. The drying phase was completed in an oven at 60°C for 2 days. Finally, the samples were coated with gold (23). After drying and coating in the incubator, images were taken with SEM (JEOL JSM 5600 LV) at Eskişehir Osmangazi University Central Research Laboratory Application and Research Centre. Nomina Anatomica Avium (24) was referenced for anatomical terminology.

Results

Macroscopic Findings

On macroscopic examination, the tongue was observed to resemble a triangle matching the shape of the beak. The tongue consisted of the apex, corpus, and radix sections. On the dorsal surface of the tongue, a groove structure extending from the apex to the radix and dividing it into two halves was observed. It was observed that the posterior part of the corpus was shaped like the letter "V", with two papillar crest on the right and left sides. Caudally oriented conical papillae were located at the end of the corpus (Figure 1). These papillae were short at the center of the letter "V" and grew larger toward the sides.

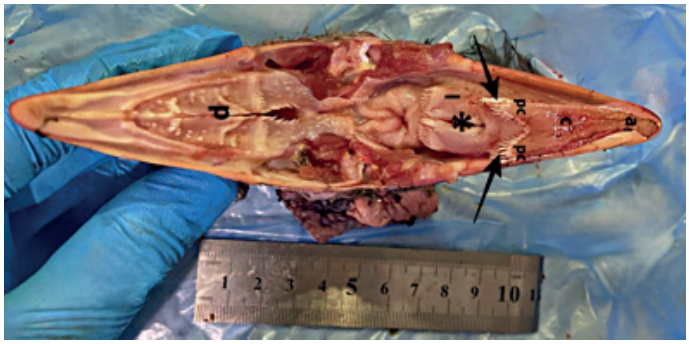

Figure 1. Dorsal view of the beak cavity in a turkey. p: palatinum; l: larynx; pc: papillar crest; c: corpus; a: apex; *: laryngeal cleft; arrow: papillae conicae.

Microscopic Findings

On histological examination, a multilayered epithelium covered the dorsal and ventral surfaces of the tongue (Figure 2a-d). Depending on the tongue region, three different types of epithelium were identified in the tongue mucosa: para-keratinized, ortho-keratinized and non-keratinized epithelium. The dorsal surface of the apex (Figure 2a) and corpus (Figure 2c) were covered with a multilayered par-

akeratinized epithelium, while the ventral surface of the both regions (Figure 2b) was surrounded by a multilayered orthokeratinized epithelium. Multilayered, non-keratinized epithelium covered the radix of the tongue (Figure 2d). The multilayered para-keratinized and ortho-keratinized epithelium consisted of basal, intermediate, and cornified layers (Figure 2a-c, 2f). In contrast, the multilayered non-keratinized epithelium consisted of basal, intermediate, and superficial layers (Figure 2d).

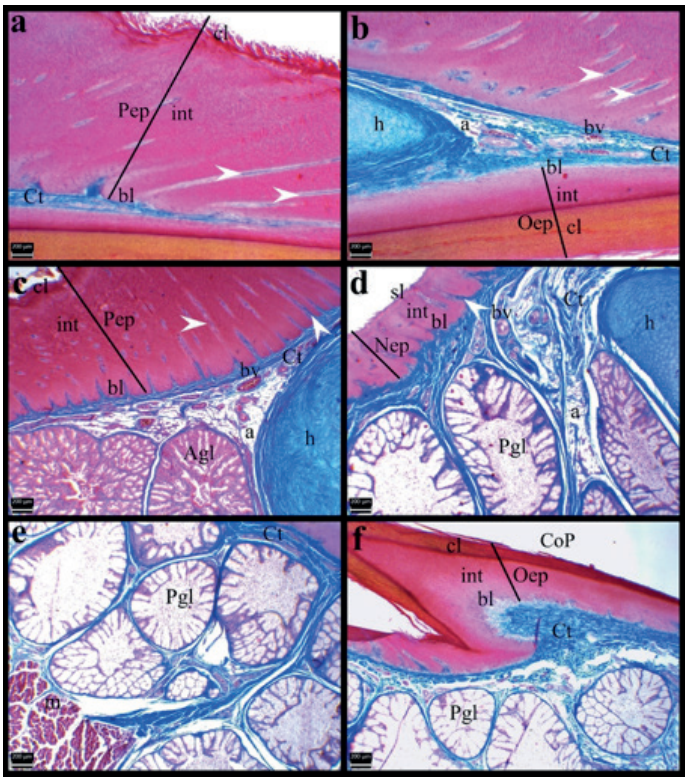

Figure 2. Histological appearance of the apex (a, b), the corpus (c), the radix (d, e) parts of the tongue, and the conical papillae (f). a: adipose tissue, Agl: anterior lingual glands; bl: basal layer, bv: blood vessel, cl: cornified layer, CoP: conical papillae, Ct: connective tissue, h: hyaline cartilage, int: intermediate layer, m: skeletal muscle fibers, Nep: nonkeratinized epithelium, Oep: ortho-keratinized epithelium, Pep: parakeratinized epithelium, Pgl: posterior lingual glands, sl: superficial layer, arrowheads: microscopic papillae. Crossman's triple staining. Bar: 200 µm.

Beneath the epithelial layer, connective tissue containing adipose tissue, nerve plexuses, blood, and lymphatic vessels was identified (Figure 2a, 2d). The connective tissue also contained hyaline cartilage starting at the apex of the tongue and extending towards the radix (Figure 2b-d). No lingual glands were found at the apex of the tongue. However, anterior lingual glands were determined in the connective tissue in the corpus of the tongue (Figure 2c), and posterior lingual glands were found in the connective tissue in the radix of the tongue (Figure 2d, 2e). Skeletal muscle fibers were observed in the connective tissue between the posterior lingual glands at the radix of the tongue (Figure 2e).

On the dorsal surface of the tongue, microscopic papillae extending from the connective tissue to the epithelial layer were prominent (Figure 2a, 2d). Microscopic papillae were denser on the corpus of the tongue (Figure 2c). The microscopic papillae extended at lateral angles at the apex of the tongue (Figure 1a, 1b), whereas they extended at steeper angles at the corpus (Figure 2c) and radix (Figure 2d) parts of the tongue.

Conical papillae were found between the corpus and radix parts of the tongue.
The conical papillae were V-shaped and covered with multilayered orthokeratinized epithelium. Posterior lingual glands were identified in the connective tissue under the conical papillae (Figure 2f).

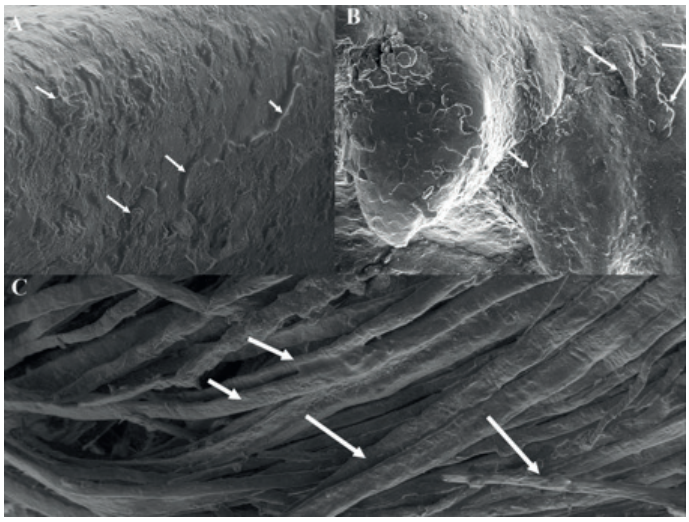

Figure 3. Scanning electron microscopy image of the apex and dorsal parts of the tongue of a turkey. A: Arrow: ortho-keratinized epithelium X100; B: Arrow: ortho-keratinized epithelium X100; C: Arrow: Para-keratinized epithelium X125.

Ultrastructural examination revealed that the apex and corpus of the tongue were covered with ortho-keratinized and para-keratinized epithelium on the dorsal surface (Figure 3). There were diffuse conical papillae between the corpus and radix. Conical papillae and opening holes of the lingual glands were observed around the cohana half (Figure 4).

Figure 4. Scanning electron microscope image of the radix and laryngeal cleft of the turkey.
D: Arrow: Conical papillae X100; E: Arrow: Conical papillae X100.

Discussion

Different feeding habits have caused different tongue forms in birds. Tongue shape shows morphological differences in birds depending on habitat type, food intake, and beak anatomy (25, 26). A good understanding of these differences enables the selection of feed and the development of feeding strategies, especially for breeding species.

Our study found a triangular tongue structure was found in turkeys, similar to birds in the same family (18, 27, 28). The shape of the tongue was reported to be spade-like and oval-tipped in Falconiformes (8, 29), forked and oval-tipped in Strigiformes, and round-tipped in Anseriformes (30). Macroscopically, it was determined to consist of the apex, corpus, and radix as reported in many studies (1, 31). Although the groove dividing the apex and corpus of the tongue into two parts is reported to be absent in chickens (32, 33), we observed this groove structure in our study, similar to studies in domestic turkeys (17), common quail, and Japanese quail (26, 28, 34). The papillar crest consisted of caudally orientated conical papillae arranged in a 'V' shape, similar in structure to chicken (27), quail (26), partridge (35), and turkey (18). It has been reported that the conical papillae transmit food into the esophagus and prevent vomiting (28).

Salivary glands lubricate and protect the mucosa while transferring food to the esophagus (36,37). In a study on chickens (27), microscopic salivary gland opening holes were found on the lingual radix surface. These structures were also observed in our study's SEM examination.

Parakeratinized epithelium was found on the dorsal surface of the apex and corpus of the tongue, while orthokeratinised epithelium was found on the ventral surface of the apex and corpus (18). It has been reported that para-keratinized epithelium is found in the parts where food is collected and food is transported. The ortho-keratinized epithelium is found in the parts of the tongue associated with food intake, grass cutting, and food filtering from water (6, 18). Unlike our study, which was similar to the study conducted on geese, the non-keratinized epithelial structure was determined in the tongue radix, which is considered an exception in the tongue radix. This finding may be attributed to low food contact, consistent with the literature (6).

In our study, as reported in different galliform birds (10, 11, 18, 38, 39), anterior and posterior lingual gland structures were found in the connective tissue in the corpus and radix parts of the tongue. In addition, unlike the literature, more dense microscopic papillae were observed on the corpus in our study. These structures extended laterally at the apex and right angles at the stem and radix.

Although two conical papillae on the posterolateral part of the corpus were reported in domestic turkeys (18), we did not find these structures in our study. Filiform papilla-like structures (or distinct projections of deciduous epithelial cells) have been reported to be found on the anterior parts of the dorsal surface of the tongue in chickens (32) and the posterior parts of the dorsal surface of the tongue in domestic geese (6). In Galliform birds such as *Gallus gallus* (33), *Coturnix coturnix* (26), *Alectorix chukar* (35), and *Meleagris gallopavo* (18), *filiform papillae* structures were not found on the tongue surface. In our study, no *filiform papillae* structure was observed that was similar to that of galliform birds.

Harrison (1964) categorized the tongue in birds into three groups. Domestic turkey tongue was included in the third group because it fills the beak cavity except for the space in the front part of the beak and the mobility in the beak cavity is low (40).

As a result of the study, macroscopic and microscopic descriptions of the tongue were made in turkeys raised in Türkiye. As mentioned in the previous study in turkeys, two cornified epithelial structures were observed. Microscopic papillae extending from the connective tissue to the epithelial layer on the dorsal surface of the tongue with different directions were quite prominent. Our findings indicate that turkey tongues raised in Türkiye are generally similar to those of Galliform birds, with minor differences. The results can be used as a basic data source for the selec-

tion of feed material and veterinary anatomy and surgery in this bird species.

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Partial Characterization of the Mitochondrial DNA (mtDNA) in Gazelles (*Gazella marica*) in Şanlıurfa Province

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Abstract

Gazelles are one of the important species unique to Türkiye, as in most Asian and Middle Eastern countries. Gazelles in Türkiye belong to the Gazella subgutturosa species, which has two subspecies: Şanlıurfa gazelle (*Gazella marica*) and Hatay Mountain gazelle (Gazella gazella). Numbers of Şanlıurfa gazelle (*Gazella marica*) are decreasing in Türkiye as well as all over the world, and today they are found in a limited area on the southern border, mostly around the province of Şanlıurfa. The aim of this study is to examine the 400 bp region of the mitochondrial cytochrome-b (mtDNA *cyt-b*) gene, which is frequently used in phylogenetic studies in gazelles, by sequence analysis. Samples were taken from a total of 40 male and female gazelles from populations in four different holdings in Şanlıurfa province. Sequence analysis results did not reveal any nucleotide diversity in terms of the examined region. the samples examined in after constructed phylogenetic tree which was evaluated together with the sequences covering the same region in the database of the obtained sequence analysis result showed that the samples exanimated were clustered together with *Gazella marica* samples, in line with previous studies. This study implies that the *cyt-b* gene region of mtDNA is not promising to assess genetic variability and phylogenetic relationships among gazelle populations surviving in Şanlıurfa province. Due to its limitations on reveal genetic diversity in *Gazella marica* it should be screened genetic variability and phylogenetic relationships in this grup by using other regions of mtDNA as control region.

Keywords: *Gazella marica*, genetic variability, maternal lineage, mtDNA *cyt-b* gene, sequencing

Introduction

Huge increase in human population and excessive use of natural resources have accelerated climate change and habitat loss. This situation has become a growing threat to sustainable ecology and biodiversity. Türkiye is a geography where the Mediterranean, Iran-Anatolia and the Caucasus biodiversity hotspots intersect. Despite this advantage it has in terms of biodiversity, the risk of losing this advantage is also quite high (1, 2). It has been reported that there are 27 large mammal species in Türkiye and one of these is the gazelle, which is one of the first creatures that comes to mind when Şanlıurfa and the steppe are mentioned. Gazella, the most complex genus in the Antilopinae subfamily among the most diverse hoofed mammals in the Bovidae family, extends from North Africa through the Arabian Peninsula, the Middle East and India to the plains of China and Mongolia (3, 4, 5, 6).

It has been reported that there are 11 species belonging to the Gazella genus, most of which are considered to be at risk today (5, 7). There are two species of gazelle in Türkiye: Şanlıurfa gazelle (*Gazella marica*) and Hatay Mountain gazelle (*Gazella gazella*). Following the report of the IUCN SSC Antelope Specialist Group (2008) on the rapid extinction of gazelle species, breeding and conservation farms

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were established in Türkiye, as in many countries (8, 9, 10). However, since gazelles are one of the most complex and least studied mammal groups, the number of molecular genetic studies, despite their relatively increasing numbers in recent years, is less than the number of studies carried out in most mammal species. Even among the taxonomic classifications made by various research groups, there is still inconsistency. The same situation emerged for these two gazelle species in Türkiye each of them belong to *Gazella subgutturosa* (11, 12). Historical observations regarding the existence of the gazelle genus and its related species in Anatolia were well documented by 13. The Şanlıurfa gazelle (*Gazella marica*) was considered as a subspecies of the Hatay Mountain gazelle (*Gazella gazella*) and they were considered as a single species. In a study conducted with mtDNA sequences in 2011 (12), it was shown that *Gazella marica* is a separate species and immediately afterwards it was shown that there are two species in Türkiye as Şanlıurfa gazelle (*Gazella marica*) and Hatay Mountain gazelle (*Gazella gazella*) by 14 and 15. *Gazella marica*, known as the "Arabian sand gazelle", exits along Yemen, Oman, United Arab Emirates, Saudi Arabia and the southern border of Türkiye. Until the 20th century, gazelles were spreading from the South eastern border of Türkiye to the Northern border of Eastern Anatolia. However, due to reasons such as habitat destruction and illegal hunting, the living spaces of gazelles have narrowed and are limited to Şanlıurfa and surrounding provinces (16, 17).

It has been reported that the risk status of the *Gazella marica* species, whose individual number drops to around 1750-2150, has been included in the "vulnerable" category, like other gazelle species, in the IUCN Red List of Threatened Species within the scope of C2a (i) criteria (7). This means that the number of *Gazella marica* in nature is rapidly decreasing, and if the decrease continues, they will be in danger of extinction. To avoid this extinction, significant efforts are being made to protect the gazelle species. Conservation efforts for the species in Türkiye were initiated with the hunting ban enacted in 1957, the first official studies to collect information about gazelles in the Urfa Region were carried out by Ceylanpınar TİGEM in 1968, and between 1977 and 1982, gazelle breeding and breeding activities were carried out by the Republic of Türkiye Ministry of Agriculture and Forestry General Directorate of Nature Conservation and National Parks has been carried out. Since that date, the gazelle breeding and production station is still run by TİGEM, and breeding sales are also carried out at the 75th Year Gazelle Breeding Station *Gazella marica* was released into the wild in 2021 and 2022 within the scope of reintroduction, allowing them to reproduce there (8).

Under the leadership of the institution, they strive at national and international levels to protect ecological systems and biodiversity in Türkiye. On the other hand, it is seen that research examining gazelles in both Şanlıurfa and Hatay regions in Türkiye, in terms of molecular markers 13, 14, 18, 19 as well as morphometric and reproductive characteristics and habitat needs, has gained momentum after the first decade of the twenty-first century (8, 18, 20, 21). "Conservation and Sustainable Management of Türkiye'sSteppe Ecosystems" project which was also carried out between 2019-2020 with the cooperation of the United Nations Food and Agriculture Organization (FAO) and the Ministry of Agriculture and Forestry, General Directorate of Nature Conservation and National Parks (DKMPGM), General Directorate of Plant Production (BÜGEM) and General Directorate of Forestry (OGM) and with the support of the Global Environment Facility (GEF). In Şanlıurfa Steppe Conservatıon Strategy and Action Plan 2021-2030 prepared within the scope of the project (22), advisory targets for measuring genetic diversity were put forward in order to protect the Şanlıurfa steppe biological diversity at the levels of ecosystem, species, genetic and ecological process diversity. It is already known that estimating genetic diversity at regular intervals with reliable markers is of great importance in determining the risk status of the species, monitoring it, determining the conservation strategy and observing the results of conservation activities.

Although molecular studies have been carried out by various genetic markers to understand the genetic background of the species and to elucidate taxonomic confusion, these are very few in number when compared to domestic farm animals (13, 14, 19, 23, 24 25).

Our aim in this study is to examine the 400 bp long region of the mtDNA cyt b gene by sequence analysis in the blood sample obtained from forty Gazelle *marica* individuals located within the borders of Şanlıurfa province, and to compare it with the sequences belonging to the same region in the database to both review its phylogenetic location and to determine the current genetic diversity through nucleotide diversity.

Material and Method

The research permissions were obtained with the letter dated 29.09.2023 and numbered E-21264211-288.04-11423456 from the Ministry of Agriculture and Forestry, General Directorate of Nature Conservation and National Parks. Ethical approval was also obtained by Harran

University Animal Experiments Local Ethics Committee Presidency with decision number 2023/07/20 and ethics committee permission dated 12/09/2023 and numbered 244460.

All blood samples were taken from the Vena jugularis of male (n=16) and female (n=24) gazelles belonging to the *Gazella marica* species, found in four different locations within the borders of Şanlıurfa Province. DNA isolations from these 40 blood samples were performed using commercial kits, according to the manufacturing instructions. Isolated DNA samples were used for PCR amplifications. The 400 bp located on 5′ -region of the mtDNA *cyt-b* gene belonging to gazelle mtDNA genome was amplified by using primers L14724: 5'- TGACTAATGATAGAAAAAC-CATCGTTG and H15149: 5'- TAACTGTTGCTCCT-CAAAAAGATATTTGTCCTCA (26, 27, 28). Following to amplifications PCR products were purified and sequenced. The sequencing results were aligned and trimmed by using BioEdit and CLUSTALW (http://www.ebi.ac.uk/clustalw/). Using the sequence obtained from sequencer and 105 sequences (KM978962, GU384826, GU384835, GU384836, GU384840, GU384844, GU384856,GU384864, GU384866, GU384867, GU384869, AF187715, AF187716, DQ269164, AF187696, AF187718, AF187719, AF187699, AF187698, MH360717, KU560654, JN632643, MH360718, MH360720, MF180128,MF179979, KU560636, KM582095, NC_020704, MF179985, MF179984, MF179983, MF179982, MF179981, MF179980, MF179978, MF179977, MF179976, MF179975, MF179974, MF179973, MF179972, MF179971, MF179970, KU560635, KM582114, KM582113, KM582112, KM582102, KM582101, KM582100, KM582098, KM582097, KM582096, JN410259.1:11-424, KM582111, NC_020708, KU560634, MT811637, AF187702, MT811636, MT811635, MT811634, MT811633, MT811632, MT811631, MT811626, MT811625, MT811623, AF187696, KP729619, KP729618, KM387302, KM387301, KM387300, KM387299, KM387298, KM387297, KM387296, KM387295,KU560633, MT811638, MT811630, MT811628, MT811627, NC_020703,MT811629, KU560631, NC_020709, KM582103,AF187699, KU560632, KM978968, KU560653, OQ595233, KM978991, KM978990, KM978989, KM978984, KM978980, KM978977, KM978972, KM978971, KM978967, KM978964, KM978962) from public database (https://www.ncbi.nlm.nih.gov/nucleotide/) phylogenetic tree was constructed by MEGA version 10.2.6 according to the neighbour-joining method using Tamura-Nei model (29, 30, 31).

Results and Discussion

All of the DNA samples obtained were amplified by PCR (Figure 1), but the sequence analysis results of 37 of them were found suitable for evaluation.

Figure 1. Electropherogram of 400 bp PCR products

Figure 1. Electropherogram of 400 bp PCR products

However, unfortunately, all the sequences of the 37 samples obtained were identical to each other. For this reason, the diversity parameters of the population could not be estimated. Those who investigated before (13, 14) did not find any nucleotide diversity in Şanlıurfa gazelle population either. However, examination of the mtDNA cyt b region in Iranian *Gazella subgutturosa* in 2019 revealed diversity although it was low (24). According to phylogenetic tree constructed the sample was grouped with sequences obtained from *Gazella subgutturosa* and *Gazella subgutturosa marica* species, Gazella *dorcas* x *leptoceros*, aswell (AF187702, AF187718, AF187696, MH360720, KM582098, KM58210) (Figure 2). This clustering was consistent with the findings of researchers who previously clarified the taxonomic status of gazelles in the city of Şanlıurfa (13, 14).

Figure 2. Neighbour-joining phylogenetic tree constructed with the previous reported sequences from database

Sequence analysis of the 400 bp region in the mtDNA *cyt-b* gene, which is most used in researching maternal diversity and lineages in the Gazelle breed, did not reveal any nucleotide diversity as in previous research results (13, 14). Examination of the Turkish gazelle population with autosomal markers also shows that diversity is significantly restricted. A more recent study examined an exonic region of the MHC class II DRB gene and found the estimated diversity parameters to be lower than the diversity estimated from the same region of the bovine species (19). This situation strengthened the impression that genetic diversity is low in the gazelle population in Türkiye (19). By examining the mtDNA cyt b in studies conducted in China (25, 32) and Iran (24), low genetic diversity was determined, although the region examined was wider in some of them (25, 32). Additionally, a low level of genetic diversity was observed in the examination of Mitochondrial Cytochrome-b, Cytochrome-c and d-loop in *Antilope cervicapra* (33). Previous studies carried out by using first generation markers based on biochemical and karyotypic variations also revealed low diversity in the specie (34, 35). This may be due to the underlying homogeneity of the species, or a recent bottleneck may have led to this reduced diversity, considering that their numbers have dropped to around 300 in the recent past in Türkiye (16). However, it also should be kept in mind that different results may be obtained by examining the control region of mtDN*A*. As a matter of fact, 36 in their study on *Gazella marica* in the wild, determined a low nucleotide diversity and a medium level of haplotype diversity with seven different haplotype groups. Although more than 70% of the samples in the study carried the predominant haplotype, it was greater than the diversity revealed by the mtDNA cyb gene.

As a result, it was shown by 14, that the gazelles in Şanlıurfa belong to the *Gazella marica* specie. Around the same time 15, analysed the samples obtained from Ceylanpınar in terms of the same mitochondrial region and reported that these samples were grouped together with *Gazella marica*. Following these, 13's study also gave this result. Our study confirms these previous studies. In these previous studies it was shown that the mtDNA *cyt-b* region of *Gazella marica* and *Gazella gazella* are clearly separated by 23 variable regions. However, its success in reflecting the genetic diversity within the species should be evaluated together with the results from studies with the control region, which is a more protected region of mtDNA in the same populations. To determine appropriate conservation strategies, genetic diversity both within and between populations must be evaluated with appropriate molecular and phenotypic markers. Both 14's showing that gazelles in Türkiye are diphylogenic by using the mtDNA *cyt-b* region and Saatoglu's study (13) show that the C′ T substitution in the INRA126 microsatellite on the Y chromosome can be used to distinguish species have paved the way for the pure conservation of these species. However, to create successful conservation strategies, genetic diversity in both wildlife and the captive breeding gazelle population at the institute must be monitored with autosomal neutral markers. As it was underlined by 24, microsatellite markers may provide a more objective estimate of genetic diversity and have the potential to make a significant contribution to conservation studies due to their power to show the possible structuring in populations.

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