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Transforming growth factor-beta levels in healthy cow's and buffalo's milk*

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Summary

Aim: Due to the numerous bioactive molecules and components it contains, milk significantly contributes to the proper growth and physiological development of both humans and animals during the neonatal period, strengthening the body's defenses and providing protection against infections and diseases. In addition to its nutritional properties, milk contains abundant growth factors, such as transforming growth factor-beta (TGF-β). This study aimed to investigate the TGF-β level in milk from healthy buffaloes and cows.

Material and methods: The study material comprised milk samples from Jersey breed cows aged between 4 and 9 years, and Anatolian water buffaloes aged between 3 and 10 years, in lactation. Samples of milk were collected from the four mammary lobes of cows and buffaloes without mastitis. The TGF-β level was measured with a bovine-specific enzyme-linked immunosorbent assay kit.

Results: The TGF-β level in cow's milk ranged from 3.07 to 6.90 ng/ml, with a mean value of 4.67 ± 1.08 ng/ml. In cow's milk, the TGF-β level showed a positive correlation with both the age of the cow ($r = 0.914$, $p < 0.01$) and daily milk production ($r = 0.961$, $p < 0.01$). The TGF-β level in buffalo's milk ranged from 5.20 ng/ml to 8.32 ng/ml, with a mean value of 7.10 ± 1.21 ng/ml. In buffalo's milk, the TGF-β level showed a positive correlation with both the age of the buffaloes ($r = 0.859$, $p < 0.01$) and daily milk production ($r = 0.940$, $p < 0.01$). A comparison of TGF-β levels in cow's and buffalo's milk revealed a statistically significant difference ($p < 0.001$).

Conclusions: In conclusion, the findings of this study show that milk from Anatolian water buffaloes contains higher levels of TGF-β than milk from Jersey breed cows. The present study contributes to the scientific understanding of bioactive compounds in cow's and buffalo's milk and their significance in human nutrition. Further research is needed to evaluate TGF-β levels in a larger number of milk samples from other milk-producing species.

Keywords: buffalo, cow, milk, transforming growth factor-beta

The presence of multiple bioactive components in milk facilitates neonatal growth and development in both humans and animals while also enhancing immune competence and resistance to infectious diseases (1, 3, 6, 10, 21, 24, 29). Due to their strong bioactivity, cow's colostrum and mature milk are used as important protein components in the manufacturing of baby formula. Compared with cow's milk, buffalo's milk contains higher levels of bioactive components (1, 4). Buffalo converts dietary carotenoids into vitamin A, which is secreted into milk, providing this essential micronutrient to neonates and humans (29). Buffalo's milk contains approximately twice the lipid content of cow's milk, has a higher proportion of saturated fatty acids, and provides more energy (17).

Breastfeeding in newborns has been shown to reduce the prevalence of many pathological disorders, such as allergic diseases, diabetes, Crohn's syndrome, and mucosal ulceration of the colon (23, 31). Scientific studies suggest that bioactive growth-promoting components in human and animal colostrum and milk confer substantial benefits for neonatal health and may also play a role in the prevention or modulation of adult diseases (9, 24). Among the most important growth factors in that group is transforming growth factor-beta (TGF-β). TGF-β, which is synthesized by many cell types, has an important function in the cellular regulation mechanisms, including cellular divi-

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sion, cell specialization, adhesion, and apoptosis (7). TGF-β has a function in both embryonic maturation and mammary tissue morphogenesis (22). Through its biphasic regulatory properties, TGF-β modulates immune responses by maintaining cellular homeostasis and controlling inflammation (16).

The biosynthesis of TGF-β involves the production of an inactive precursor, which is activated through several mechanisms, including changes in pH and the action of enzymes, such as neuraminidase (19). Newborn tissues rapidly absorb TGF-β, suppress proinflammatory secretion of cytokines, suppress Th1 activity, enhance non-inflammatory protective responses at mucosal barriers, and stimulate the synthesis of immunoglobulin A (IgA) (5). TGF-β has antagonistic effects on IL-1β, TNF-α, and interferon-gamma (IFN-γ), which are cytokines that regulate inflammatory responses (30). In developing human intestinal epithelial cells, TGF-β1 suppresses TNF-triggered IL-8 synthesis, while TGF-β2 inhibits IL-8 secretion stimulated by IL-1β (15). TGF-β2-mediated inhibition of IL-1β has been shown to suppress the extracellular signal-regulated kinase (ERK) pathway, thereby reducing production both *in vivo* and *in vitro* (15, 25). TGF-β derived from breast milk is recognized for its protective role, which consists primarily in inhibiting intestinal epithelial cell apoptosis and downregulating cytokine expression by macrophages (15). Follicular cells synthesize both ligands and receptors of the TGF-β superfamily, which are essential for regulating follicular development, cell proliferation, steroidogenesis, and ovulation (11). TGF-β1 promotes vascular instability, apoptosis, and remodeling of the extracellular matrix during luteolysis in cattle (8).

In light of scientific studies, TGF-β found in milk appears to have significant positive effects on the local functions of the mammary gland in both humans and animals, as well as on the gastrointestinal system (15, 22). In a literature review, no scientific report was found in which TGF-β levels in milk from buffaloes and cows were measured comparatively. This study aimed to evaluate the levels of TGF-β in the milk of healthy cows and buffaloes in lactation and to determine which milk contains higher levels of TGF-β.

Material and methods

Milk samples. The present study used milk samples from lactating Jersey cows aged 4-9 years and Anatolian water buffalo aged 3-10 years in the Giresun province. The body condition score (BCS) of the animals was not recorded. All animals were clinically healthy, and milk was collected from animals managed under standard feeding and care conditions to minimize potential variability in milk composition. Milk samples were collected from cows and buffaloes during the morning milking session and screened for subclinical mastitis with the California Mastitis Test (CMT). No bacteriological examination of milk samples was performed. A total of 240 milk samples were collected and stored at –20°C for up to two months before analysis. The somatic cell count was subsequently determined by microscopic evaluation of leukocyte content. Milk samples obtained from 30 healthy cows with negative CMT results and SCC < 200.000 cells/ml and from 30 buffaloes constituted the study material. Somatic cell counts were determined using the MILKANA® Somatic Scan (Mayasan A.Ş., İstanbul, Turkey).

California mastitis test. Milk from each udder quarter of cows and buffaloes was milked directly into the four compartments of a California Mastitis Test (CMT) plate. CMT solution was added to the milk in the compartments at a ratio of 1 : 1. The consistency scores in the four separate compartments of the CMT test container, which was rotated with circular movements, were recorded as (–), (+1), (+2), and (+3) (28).

Somatic cell count. Milk samples from each mammary lobe were collected into glass centrifuge tubes and centrifuged at 1550 × g for 10 minutes. After centrifugation, the cream layer was gently removed from the surface, and the tubes were placed upside down on a rack for 2 minutes to allow residual liquid to drain. Subsequently, 1 ml of the sediment settled at the bottom of the tubes was taken using a sterile loop and evenly distributed onto clean microscope slides. The slides were fixed in methanol for 3 minutes, stained with 0.2% toluidine blue, and subsequently air-dried. Following the application of immersion oil to each slide, somatic cells were counted in 20 randomly selected microscopic fields under a 100 × oil-immersion objective. The mean number of somatic cells per field was determined by dividing the total cell count by the number of fields examined. This value was used to estimate the somatic cell concentration per milliliter of milk in samples from both cows and buffaloes (10, 14). The evaluation of somatic cell count is presented in Table 1.

Tab. 1. Evaluation of somatic cell count

Mean cell number	Score	Number of cells per ml of milk
1-5	+	< 200.000
6-20	++	> 200.000
> 20	+++	> 1.000.000

Preparation of milk serum. Milk serum was obtained from cows and buffaloes following a protocol described by Alais (2). The process was initiated by adding 1 ml of 0.3% chymosin to each glass tube containing milk. To facilitate coagulation, the tubes were incubated in a water bath at 37°C for 20 minutes. The tubes were kept at room temperature for 80 minutes to allow milk serum to separate. At the end of this period, the milk clot formed in the tube was carefully removed. The milk serum was then filtered through a membrane filter into clean tubes. The samples were centrifuged at 1550 × g for 5 minutes, after which the cream layer was removed. The resulting milk serum was aliquoted into three microcentrifuge tubes and stored at –20°C until TGF-β analysis.

Measurement of TGF-β levels. Milk TGF-β concentrations were measured using a commercially available bovine-specific Enzyme-linked immunosorbent assay kit (E2049Bo, Bioassay Technology Laboratory, Shanghai,

China). All procedures were conducted in accordance with the manufacturer's instructions, and all standards and samples were analyzed in duplicate. The kit had a detection range of 0.5-150 ng/ml and a sensitivity of 0.32 ng/ml. Before analysis, all reagents and samples were allowed to thaw at room temperature (21°C) for 30 minutes. A 160 ng/ml stock solution was serially diluted to obtain standard concentrations of 5 ng/ml, 10 ng/ml, 20 ng/ml, 40 ng/ml, and 80 ng/ml in accordance with the manufacturer's protocol. The microplate wells were labeled for standards, blanks, and samples. Each standard well received 50 µl of the corresponding standard solution in duplicate. For the sample wells, 40 µl of milk serum and 10 µl of TGF-β antibody were added. Subsequently, 50 µl of streptavidin-HRP was dispensed into all wells. After sealing with adhesive film, the plate was incubated at 37°C for 60 minutes. Following incubation, the contents of each well were removed, and the wells were rinsed with 350 µl of wash buffer using a multichannel pipette. The plate was then inverted onto absorbent paper and gently tapped to remove any residual liquid. The washing step was repeated twice more. Next, all wells received 50 µl each of Chromogen A and Chromogen B, and the contents were gently mixed. The plate was covered again and incubated in the dark at 37°C for ten minutes. The reaction was terminated by adding 50 µl of stop solution to each well. Absorbance was measured with a microplate reader (Infinite F50, Tecan Austria GmbH, Grödig, Austria) at 450 nm within 15 minutes. The TGF-β level in each milk sample was calculated from the standard curve generated using known concentrations and was expressed in ng/ml.

Statistical analysis. The data obtained in this study were tested for normality using the Shapiro-Wilk test before significance testing. Since the data met the assumption of normal distribution, an Independent Samples t-test was employed to determine differences in TGF-β levels between cow's and buffalo's milk. The relationships between TGF-β levels in cow's and buffalo's milk, age, and daily milk production were assessed using Pearson's correlation test. Data from repeated experiments are presented as mean ± SD, with a 95% confidence interval. A p-value of less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS Statistics 22.0 software (IBM Corp., Armonk, NY, USA).

Results and discussion

TGF-β level in cow's and buffalo's milk. Milk from Anatolian water buffaloes contained significantly higher levels of TGF-β than milk from Jersey cows. Comparison between the two species revealed that buffalo's milk contained significantly higher TGF-β levels than cow's milk ($p < 0.001$).

TGF-β level in cow's milk. The distribution of TGF-β levels in cow's milk is shown in Figures 1 and 2. The TGF-β concentration ranged from 3.07 to 6.90 ng/ml, with a mean value of 4.67 ± 1.08 ng/ml.

Correlation between age and TGF-β levels in cow's milk. A significant positive correlation was observed between TGF-β level in cow's milk and age ($r = 0.914$; $p < 0.01$). The relationship between cow's milk TGF-β levels and age is illustrated in Figure 3.

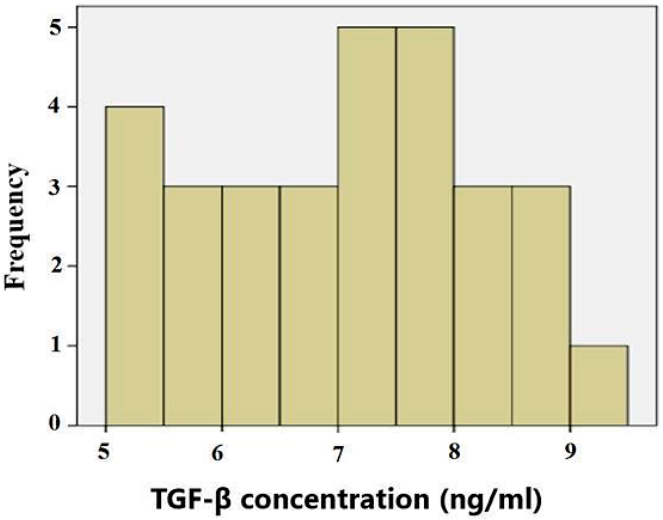

Fig. 1. Histogram of TGF-β level in cow's milk

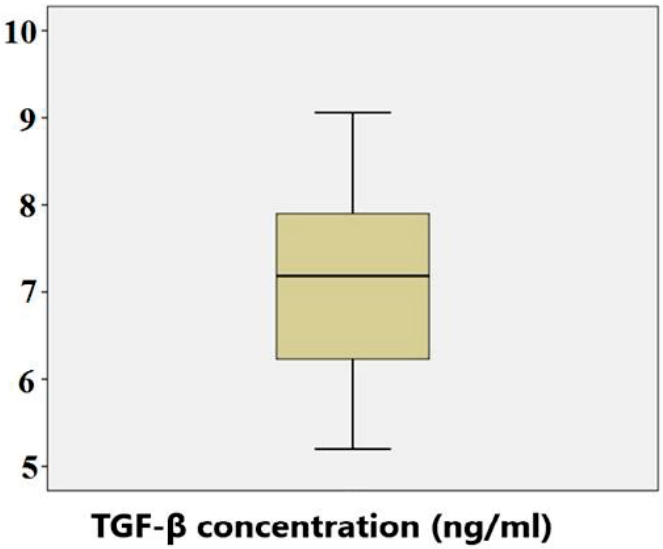

Fig. 2. Distribution of TGF-β levels in cow's milk: The horizontal line represents the arithmetic mean, the box indicates the standard deviation of the means, and the bars show the range of TGF-β levels

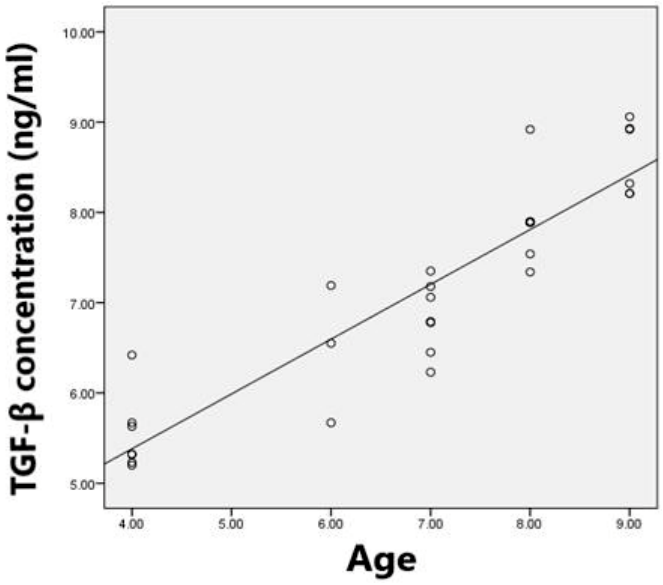

Fig. 3. Correlation between age and milk TGF-β level (ng/ml) in cows

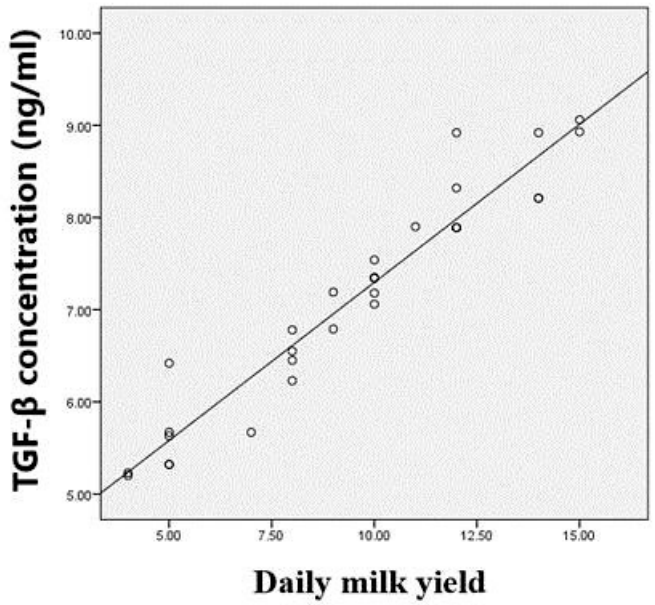

Fig. 4. Correlation between TGF-β levels (ng/ml) and daily milk production in cows. Pearson's correlation (r = 0.961, p < 0.01)

Correlation between TGF-β levels in cow's milk and daily milk production. The correlation between TGF-β levels in cow's milk and daily milk production was significantly positive ($r = 0.961$; $p < 0.01$). The correlation between TGF-β levels in cow's milk and daily milk production is shown in Figure 4.

TGF-β level in buffalo's milk. The distribution of TGF-β levels in buffalo's milk is shown in Figures 5 and 6. The TGF-β concentration in buffalo's milk ranged from 5.20 ng/ml to 8.32 ng/ml, with a mean value of 7.10 ± 1.21 ng/ml.

Correlation between TGF-β levels and age in buffalo's milk. A significant positive correlation was found between TGF-β levels and age in buffalo's milk

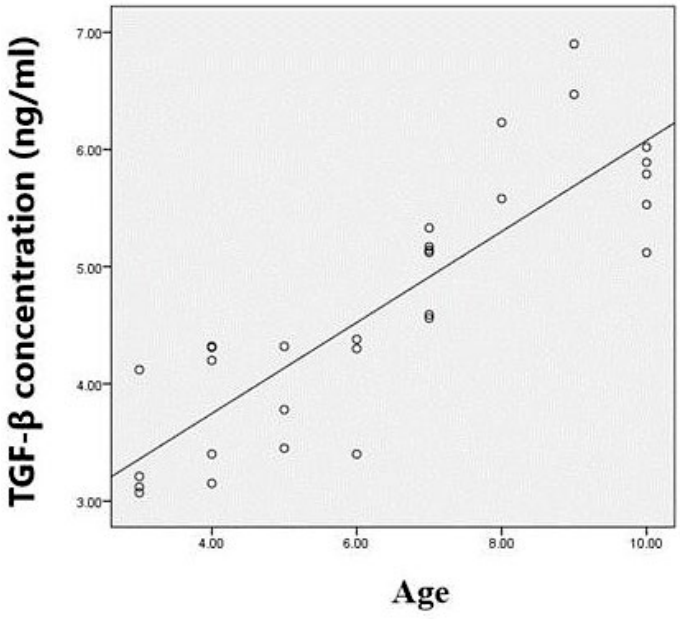

Fig. 7. Correlation of TGF-β levels (ng/ml) with age in buffalo's milk. Pearson's correlation (r = 0.859, p < 0.01)

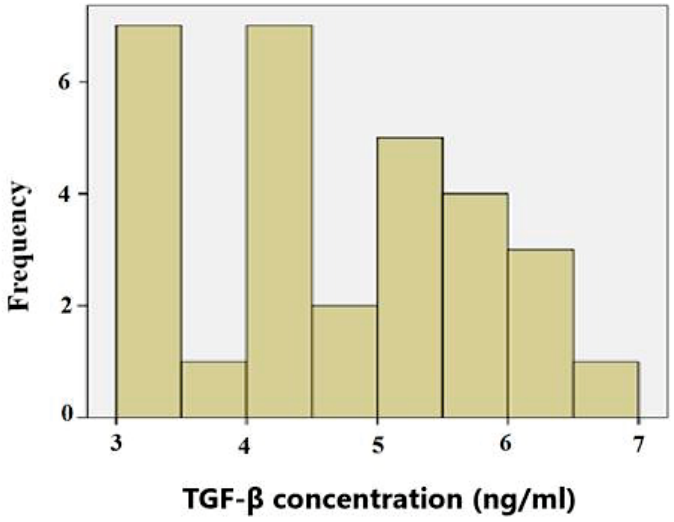

Fig. 5. Histogram of TGF-β levels in buffalo's milk

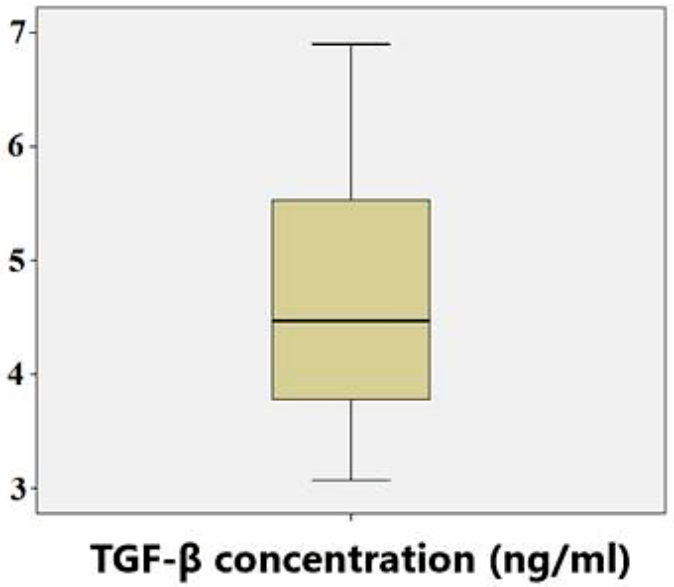

Fig. 6. Distribution of TGF-β levels in buffalo's milk samples: The horizontal line represents the arithmetic mean, the box indicates the standard deviation of the means, and the bars show the range of TGF-β levels

($r = 0.859$; $p < 0.01$). The relationship between TGF-β levels in buffalo's milk and age is shown in Figure 7.

Correlation between TGF-β levels in buffalo's milk and daily milk production. TGF-β level showed a strong positive association with the daily milk production in buffaloes ($r = 0.940$; $p < 0.01$). This relationship is illustrated graphically in Figure 8.

Human breast milk provides the neonate with a variety of bioactive components that protect him against infection and inflammation, support immune system maturation, organ development, and the establishment of a healthy microbiota (3). Peptides found in the composition of milk, including TGF-β, interleukins, interferons, lymphokines, chemokines, and tumor necrosis factors, have critical roles in immune responses. These peptides are produced by mammary epithelial cells or milk leukocytes, primarily T cells and macrophages (9, 13). The predominant growth factor pres-

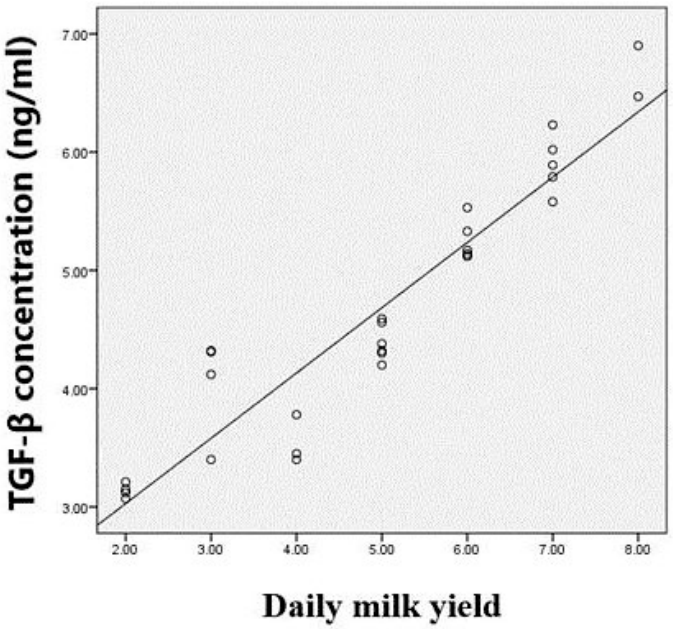

Fig. 8. Correlation between milk TGF-β levels (ng/ml) and daily milk production in buffaloes. Pearson's correlation ($r = 0.940$, $p < 0.01$)

ent in maternal colostrum is TGF-β2, with TGF-β3 and TGF-β1 occurring at lower concentrations (18). Studies have demonstrated that mRNAs for TGF-β1 and TGF-β2 are expressed in cells found in breast milk, with the concentration of TGF-β in human colostrum amounting to 1365.7 ± 242.9 ng/ml (27). Hirata et al. (12) documented that the concentrations of TGF-β1 and TGF-β2 in maternal colostrum were 954.7 pg/ml and 5173.2 pg/ml, respectively, while in mature milk, TGF-β1 and TGF-β2 levels were 498 pg/ml and 1263.7 pg/ml, respectively.

TGF-β plays a key role in regulating cellular homeostasis and inflammatory responses, and it modulates immune responses through biphasic effects, either inducing or suppressing them (16, 19). TGF-β, abundantly present in the neonatal microenvironment, suppresses pro-inflammatory cytokine production, downregulates Th1-mediated immune responses, and enhances non-inflammatory mucosal defense mechanisms (5). TGF-β belongs to the family of polypeptide growth factors that regulate cellular growth and differentiation. TGF-β, synthesized by various cell types, plays a critical role in regulating cellular functions, such as proliferation, differentiation, adhesion, and programmed cell death (7).

TGF-β is involved in the regulation of both embryogenesis and the morphogenetic development of the mammary gland (22). *In vivo* studies have demonstrated that commercially available bovine milk retains TGF-β bioactivity and provides protection against lipopolysaccharide-induced tissue injury and mortality in mice following oral administration. In humans, oral consumption of bovine milk has been associated with increased plasma TGF-β2 levels, observed approximately four hours after intake (21). Additionally, evidence suggests that daily synbiotic supplementation in lactating mothers increases IgA and TGF-β2 concentrations in breast milk, aiding to prevent diarrhoea in breastfed infants (20).

Considering the biological effects of TGF-β in the body, knowing its levels in milk is important for human health. In this study, the TGF-β level in cow's milk, the most commonly consumed mammalian milk by humans, was found to range between 3.07 ng/ml and 6.90 ng/ml, with a mean of 4.67 ± 1.08 ng/ml. A positive correlation was observed between TGF-β levels in cow's milk and both the age of the cow ($p < 0.01$) and daily milk production ($p < 0.01$). The TGF-β level in buffalo's milk ranged from 5.20 ng/ml to 8.32 ng/ml, with a mean of 7.10 ± 1.21 ng/ml. Similarly, TGF-β levels in buffalo's milk showed a positive correlation with the age of the buffalo ($p < 0.01$) and daily milk production ($p < 0.01$). In this study, TGF-β levels were significantly higher in Anatolian water buffalo's milk than in Jersey cow's milk ($p < 0.001$). Nonetheless, these findings should be confirmed by data from larger populations of animals.

The level of TGF-β in milk is influenced by species, breed, genetic makeup, nutrition, climate, geographical location, and pasteurization, as well as heat treatment, which can affect the bioactivity of TGF-β and other cytokines. Research indicates that TGF-β remains well-preserved in breast milk following pasteurization (26). Studies have shown that orally administered commercially available bovine milk preserves TGF-β bioactivity and protects mice from lipopolysaccharide-induced tissue damage and death (21). The TGF-β2 isoform, in particular, has been shown to attenuate inflammatory responses mediated by macrophages in the immature intestine, thereby offering protection against inflammation-associated mucosal injury (15). Beyond its immunomodulatory effects, TGF-β is involved in mammary gland development, embryogenesis, and the regulation of cell lineage commitment (22).

The bioactive components within the biochemical profile of colostrum and mature milk make these fluids vital for the survival of every newborn and the progression of immunological development. In humans, nearly all age groups require the consumption of milk and dairy products as part of a healthy diet. The TGF-β superfamily comprises signaling molecules that influence cell proliferation, differentiation, apoptosis, and ageing. Expressed and synthesized in almost all cell types, TGF-β regulates various cellular functions. Although it is well established that TGF-β, produced by mammary epithelial cells, T cells, leukocytes, and macrophages and subsequently secreted into milk, is present in the milk of various mammalian species, studies on this topic remain limited. TGF-β, which exerts biphasic effects, modulates inflammatory processes and influences embryonic and mammary gland development, as well as follicular maturation, ovulation, and steroidogenesis. It also promotes collagen

synthesis and angiogenesis to accelerate wound healing and contributes to tumor suppression in early stages, while supporting tumor progression in later stages. Additionally, TGF-β has been reported to protect the developing intestine against inflammatory mucosal damage. Determining the concentration of TGF-β in milk is important for public health because of its beneficial effects on the body. In conclusion, our findings indicate that TGF-β levels are higher in buffalo's milk than in cow's milk. Significant correlations were observed between TGF-β concentrations and both age and daily milk yield in cows and buffaloes. Therefore, it is essential to measure TGF-β levels in milk from a larger number of cows and buffaloes of various breeds and to establish reference values for TGF-β content in cow and buffalo's milk. Furthermore, these findings should be supported by TGF-β measurements in milk from other species consumed by humans, such as goats and sheep, across different regions and lactation periods.

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