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Full Length Article

Screening of Beta-Lactam and Oxytetracycline Residues in Tetra Pack Milk and their Impact on Fermentation

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Abstract

The aim of this study was to detect the antibiotic residues in tetra milk packs (MP) samples MP1, MP2, MP3, MP4, MP5, MP6 and MP7 marketed in Hyderabad and their influence was noted on the fermentability trend of yogurt. The results revealed the presence of antibiotic residues in the following dairy products: MP1 (50%), MP5 (30%), MP6 and MP4 (10%). These residues were identified as beta-lactam and oxytetracycline antibiotics. In the evaluation of their impact on the fermentability trend, positive milk samples were further processed for the yogurt preparation parallel to control (pure) milk. For monitoring the fermentability trend of yogurt, pH values and titratable acidity were observed at intervals of an hour during fermentation of milk. The observed values of pH and acidity were compared with the values of milk base of control sample. Among all the milk samples used for yogurt preparation, the fermentability trend was delayed due to the impact of antibiotic residues on the milk fermentation and the mean pH and acidity values were inversely but slowly dropped as compared to the control samples. Based on the findings of the current study, it is concluded that the presence of antibiotic residues in raw market milk is quite common. Furthermore, the detection of antibiotic residues in Tetra pack milk is a significant health concern for society.

Keywords: Beta-lactam; Fermentability; Oxytetracycline; Residues; Tetra pack milk

Introduction

Milk is one of the most widely consumed foods, according to the documentation of the United Nations' Food and Agriculture Organization. It is not only vital for nutrition but also has a significant economic impact. Milk and its derivatives are consumed by approximately 6 billion people worldwide (Henchion *et al.* 2021).

Pakistan is the 4th largest milk producer in the world, but its milk marketing system is still underdeveloped and informal. Only 5–7% of the total milk production is converted into tetra-pack milk, while approximately 93–95% of the milk is sold through informal milk marketing sources, which poses a risk of contamination with hazardous substances such as antibiotic residues (Yunus *et al.* 2022).

Tetra Pack has a hygienic wrapping technology compared to other preserving methods, which have the stuff and their wrapping combined initially and then sterilized. In the Tetra Pak technique, the contents and their packaging are sterilized separately and sealed in a hygienic environment. They offer a wide range of food products, including milk, juices, vegetables and fruits which are processed and sealed using the ultra-heat treatment (UHT) method. These hygienic packages have the ability to preserve food for up to a year without the need for refrigeration, reducing transportation and storage costs while increasing the shelf life of the products (Guardian 2011). Tetra Pak, with a workforce of over 24,800 employees and operations in more than 160 countries, holds the title of the world's largest food packaging producer by revenue. Pakistan caters to expanding markets in Asia and the Middle East, where dairy consumption, particularly of Ultra-High-Temperature processed milk, is on the rise. At that time, Tetra Pak's global sales constituted two-thirds of the dairy packaging industry (Vakh and Tobiszewski 2023).

Due to certain benefits, antibiotics have recently been widely used worldwide for both promoting animal growth and treating them. Approximately 6,315 tons of antibiotics are used in animals annually across the globe (Boeckel *et al.* 2015). Many antibiotics remain biologically active and long-lasting once they are released into the environment, often exhibiting broad-spectrum activity (Sarmah *et al.* 2006;

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Sardar *et al.* 2021). Research findings have shown that extended coagulation time in yogurt production, which was associated with high antibiotic residues, has a significant negative impact on the performance of the yogurt starter culture. This delay affects the coagulation period of the fermentation process (Upadhyay *et al.* 2014). Milk is highly consumable and lactic acid bacteria are used to extend the shelf life of milk through fermentation. The presence of lactic acid bacteria in milk fermentation enhances the activity of cultured bacteria. Milk is also recognized as an ecosystem for lactic acid bacteria. Milk fermentation is a cost-effective and relatively straightforward process. The use of lactic acid bacteria in milk fermentation and enhancement improves the quality and reduces the bitterness of the milk (Hassan *et al*. 2014).

In Sindh province, no reliable program for testing drug residues, especially in tetra pack milk, has been found. Controlling antimicrobial drug residues in animal origin food products is based on current Agricultural and Food guidelines to provide customers confidence in the safety and wholesomeness of their food. Considering the significance of the issue, this research study was undertaken to identify antibiotic residues in Tetra pack milk available in the study area (Hyderabad). Additionally, the study explored the impact of these antibiotic residues on the milk's fermentability trend.

Materials and Methods

Collection of tetra pack milk samples

Seven different processed batches of the most commonly used tetra pack milk samples tagged with MP1, MP2, MP3, MP4, MP5, MP6 and MP7 from various brands and companies were collected from supermarkets and local markets in Hyderabad for screening antibiotic drug residues. All the collected milk samples were brought to the laboratory of the Department of Animal Products Technology at Sindh Agriculture University, Tandojam, Pakistan and stored at refrigeration temperature until the analysis was completed.

Screening of antibiotic residues from tetra pack milk

All the collected tetra pack milk samples were analyzed using the Beta Star Combo commercial testing kit, following the protocol as reported by Wali and Deri (2022). Milk samples (0.4 mL) were drawn using an automatic pipette and then transferred into a reagent micro-well. The dipstick was immersed into the micro-well containing the milk sample and incubated for 5 min in a water bath at 47°C. For result observation, the color intensity markers were compared with the color intensity of the central line (Control line) – the upper line for oxytetracycline and the bottom line for *β*-lactam on the dipstick. Additionally, the color intensity of the test lines was expected to be darker than that of the control lines. Negative results indicated the absence of antibiotic residues, while positive results were identified by a lighter color intensity of the test line compared to the control line.

Preparation of yogurt

All the tetra pack milk samples containing positive antibiotic residues were processed alongside a control milk sample for yogurt preparation. To prepare the yogurt, the milk was pasteurized at 90ºC for 10 min and then cooled to 45ºC using running tap water. Afterward, it was inoculated with a 3.0% artisan yogurt culture and incubated at 42ºC until the pH decreased within the range of 4.5 to 4.8 and the acidity increased to a range of 0.78.

Impact of antibiotic residues on the fermentability trend of milk

All the tetra pack milk samples with antibiotic residues were utilized in the preparation of yogurt. Initially, an artisanal starter culture was created by fermenting milk with natural yogurt culture and purifying it through seven re-culturing steps. The purified starter culture was subsequently employed in an experimental trial during the production of various batches of yogurt. Yogurt was prepared using tetra pack milk samples containing antibiotic residues (positive) in addition to pure (control) milk. To assess the fermentability trend, all batches of yogurt were evaluated at hourly intervals until complete fermentation (reaching a pH of 4.6).

Fermentability trend

The fermentability trend of yogurt was assessed by monitoring pH values and titratable acidity at one hour intervals throughout the milk fermentation period. The observed pH and acidity values were compared with those of the control (pure) milk sample.

pH value

The pH value was determined using a digital pH meter (Hanna Instruments, Model No. HI 8424). Prior to analyzing the yogurt samples, the pH meter was calibrated using a standard solution. Yogurt samples were placed in a beaker and the electrode and temperature probe of the pH meter were inserted into the sample and the readings were recorded.

Titratable acidity

The titratable acidity of all yogurt samples was determined following the method provided by the Association of Official Analytical Chemists (Baur and Ensminger 1977). For this, 9 mL of yogurt samples were titrated using a titration kit with N/10 NaOH solution, while phenolphthalein (3 to 5 drops) served as the indicator. The volume of the alkali used was recorded and calculated using the following formula:

$$Titratable\ acidity\ \% = \frac{Volume\ of\ 0.1\ NaOH\ used\ x\ 0.009}{Volume\ of\ sample\ taken}\ x\ 100$$

Statistical analysis

The data obtained was tabulated and analyzed by using statistical tools; descriptive statistics, Analysis of variance and Tukey's test with the implementation of a computerized package *i.e.*, Student Edition of Statistics (SXW), version 8.1 (Copyright 2005, Analytical Software, USA).

Results

Occurrence of antibiotic residues in tetra pack milk

Among all the tetra pack milk brands, the highest number of positive antibiotic residue findings was in the MP1 brand (50%) followed by MP2 brand (30%) and MP5 brand (30%). Only a single sample from MP6 and MP4 brands (10%) tested positive while not a single sample of MP7 and MP3 milk brands were found positive for antibiotic residues (Fig. 1).

Occurrence of β-lactam and oxytetracycline residues in tetra-pack milk

Among the Tetra pack milk samples, antibiotic residues were detected in the MP1 milk brand at 30%, with positive findings for oxytetracycline. Additionally, 20% of the samples showed the presence of β-lactam residues. In the case of MP5 milk brand, 20% of the samples tested positive for β-lactam, and 10% had oxytetracycline residues. Conversely, in MP2 milk brand, 10% of the samples contained β-lactam residues, while 20% had oxytetracycline residues. Moreover, only 10% of samples from MP6 and MP4 milk brands tested positive for β-lactam residues and they were free from Oxytetracycline residues. Furthermore, the milk samples from MP7 and MP3 brands tested negative for both β-lactam and Oxytetracycline antibiotic residues (Fig. 2).

Impact of antibiotic residues on the fermentability trend

In the second phase of the study, following the detection of antibiotic residues in the tetra-pack milk samples, the positive milk samples were subjected to further processing to evaluate their impact on the fermentation trend. This involved preparing yogurt from these positive milk samples, conducted in parallel with a control group using pure milk. To monitor the fermentation trend of the yogurt, pH values and titratable acidity were observed at one-hour intervals throughout the milk fermentation process.

pH value

To assess the impact of antibiotic residues on yogurt fermentation, the pH trends of these residues are illustrated in (Fig. 3). Among all the milk samples utilized for yogurt preparation, the fermentability trend was notably delayed due to the presence of antibiotic residues. The pH values gradually decreased from 6.70 to 5.10 over an 8 h period in MP5 from 6.60 to 5.20 in MP2 milk brand over 7 h and from 6.80 to 4.90 in MP4 milk brand over 7 h. Similarly, in MP1 milk brand, the pH dropped from 6.60 to 5.00 within 6 h and in MP6 milk brand, it went from 6.60 to 4.90 over 5 h. In contrast, the control milk achieved the required pH values, transitioning from 6.70 to 4.70 within just 3 h, in comparison to all antibiotic residue-positive tetra pack milk samples.

Titratable acidity

To examine the influence of antibiotic residues on yogurt fermentation, the trend in titratable acidity of antibiotic residue results is depicted in (Fig. 4). Among all the milk samples used for yogurt preparation, the fermentation trend was notably delayed due to the presence of antibiotic residues. The desired level of acidity increased gradually, reaching 0.61% after 8 h in MP5 milk brand, 0.64% after 7 h in MP2 and 0.69% after 7 h in MP4 milk brand. Similarly, in MP1, the acidity increased from 0.16 to 0.68% over 6 h and in MP6 milk brand, it rose from 0.15 to 0.7% over 5 h. In contrast, the control milk achieved the required acidity, reaching 0.80%, within just 3 h, compared to all antibiotic residue-positive tetra pack milk samples.

Discussion

In the present study, among all the tetra pack milk brands, the highest number of positive antibiotic residue samples was found in MP1 milk brand sample (55%), followed by MP2 and MP5, both at 30%. In contrast, only one sample from MP6 and MP4 milk brands (10%) tested positive for antibiotic residues, while not a single sample from MP7 and MP3 milk brands showed any such contamination. In a previous study conducted by Shirin *et al.* (2014), it was reported that 30.14% of pasteurized milk samples in the West Azerbaijan state of Iran were tainted with various antibiotics. Another study in Iran by Aalipour *et al.* (2015) found an occurrence of 19.78% of tetracycline residues in the milk samples available in the market. On the other hand, in Pakistan, different researchers like Mangsi *et al.* (2021) detected β-lactam antibiotic residues in 36.50% of the positive milk samples. Similarly, Zhang *et al.* (2022) detected penicillin antibiotic residues in milk samples supplied to households in Italy. The results of this study revealed a higher occurrence of antibiotic residues in milk compared to the total prevalence (18 and 18.6%) reported in Chattogram by Bari *et al.* (2020).

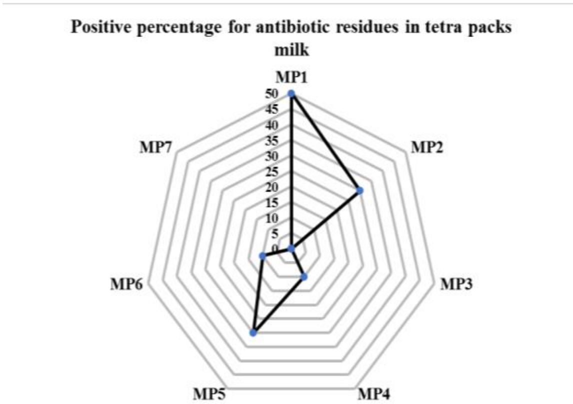

Fig. 1: Positive percent of antibiotics residues in tetra pack milk samples (MP1, MP2, MP3, MP4 MP5 and MP6)

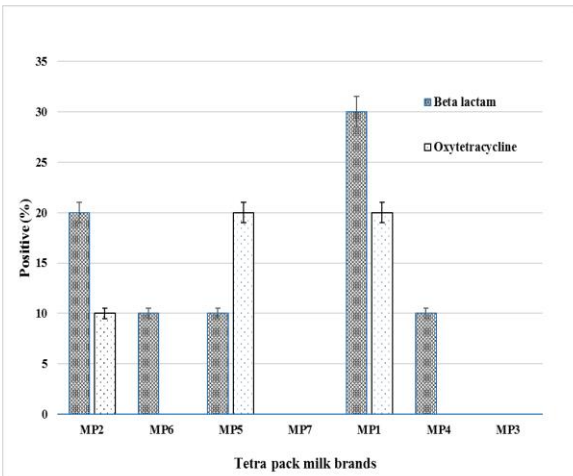

Fig. 2: Positive percent (%) of antibiotics in Tetra pack milk samples (MP1, MP2, MP3, MP4 MP5 and MP6). Each sample represents different Tetra pack milk

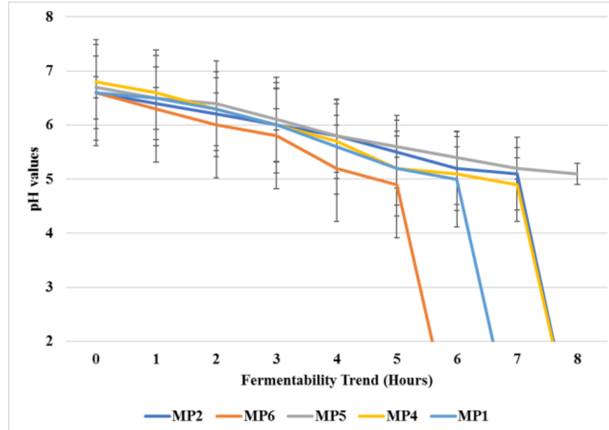

Fig. 3: Fermentability trend (pH value) of antibiotics residues positive tetra pack milk samples (MP1, MP2, MP4, MP5 and MP6). Each sample represents different Tetra pack milk

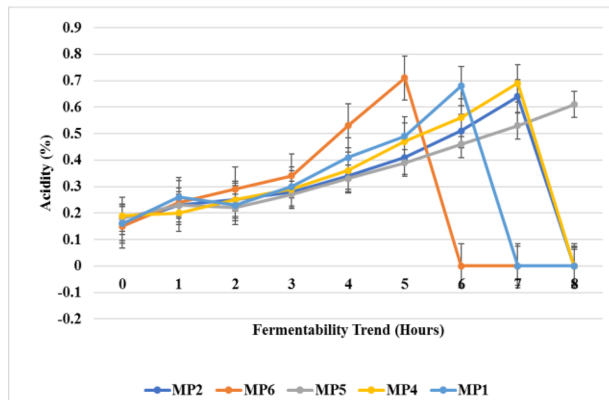

Fig. 4: Fermentability trend (acidity) of antibiotics residues positive tetra pack milk samples (MP1, MP2, MP3, MP4, MP5 and MP6). Each sample represents different Tetra pack milk

Generally, incidents of antibiotic contamination in milk are quite common in advanced countries, primarily due to the higher doses of antibiotics administered to treat sick animals. In contrast, in underdeveloped and third-world countries, antibiotic contamination in milk may arise from the lack of adequate withdrawal periods after the application of antibiotics in dairy and meat production, as well as intentional adulteration of milk with antimicrobial agents to inhibit the growth of spoilage-causing microorganisms, thereby extending the shelf life of milk and milk products (McEwen *et al.* 1992).

In the current study, the number of positive samples for antibiotic residues in various tetra pack milk brands exhibited variations, and these findings were compared with the results of previous research conducted on both raw and processed milk in Iran, Bangladesh and Ghana as reported by Mohsenzadeh and Bahranipour (2008). This variability in the presence of antibiotic residues in milk can be attributed to the differing processing temperatures employed during pasteurization and ultra-heat treatments in various dairy processing facilities. Additionally, regional differences in terms of prevalent animal diseases and the utilization of different antibiotics for treating various infectious issues may also contribute to this variation as published by Mahmoudi *et al.* (2014).

In the current study, it was found that 20% of the MP1 and MP5 milk brands and 10% each of MP2, MP6 and MP4 pack milk samples, tested positive for β-lactam antibiotic residues. In contrast, 30% of MP1, 20% of MP2 and 10% of MP5 tetra pack milk samples were found to be positive for oxytetracycline, while MP6 and MP4 milk samples were free from both types of antibiotics (β-lactam and oxytetracycline). Worldwide, most of the data on the prevalence of antibiotic residues in milk and dairy products (21.88%) has been conducted in China, followed by Spain (13.39%), Germany (4.91%), the United States of America (4.46%) and Italy (4.01%). It's noteworthy that a significant amount of research on antibiotics has been conducted in health-conscious developed countries rather than in developing countries as reported by Sachi *et al.* (2019).

Furthermore, various research approaches have been undertaken for different types of antibiotics. The highest proportion of research has focused on the β-lactam group (comprising penicillin at 56.39% and cephalosporin at 43.61%), accounting for 36.54% of the total, whereas the least attention has been given to aminoglycosides at 10.44%.

Antibiotics can gain direct or indirect access to milk, leading to contamination whether they are used for treatment or during the processing and preservation of milk and dairy products (Nisha 2008). In a study conducted by Bari *et al.* (2020), it was reported that a significant number of commercial milk samples were found positive for oxytetracycline (6%), with 4% of samples testing positive for amoxicillin, both in individual commercial samples and household samples. In Kenya, milk production with β-lactam residues is a common occurrence due to the frequent use of antibiotics for animal treatment by small dairy farmers, often exceeding recognized maximum limits (Shitandi and Sternesjo 2004). Similarly, Abebew *et al.* (2014) reported findings from Ethiopia, where a higher number of milk samples tested positive for oxytetracycline (83.33%) and β-lactam, specifically penicillin (16.66%). These levels exceeded critical values and maximum residual limits due to their imprudent use in dairy farms. In line with the results of the current study, Layada *et al.* (2016) found that 10% of processed milk samples and 20% of untreated raw milk samples in Algeria tested positive for various antibiotic residues, including oxytetracycline, penicillin, gentamicin, and Neosporin. These figures were notably higher than the occurrence of antibiotic residues in milk. In line with these results, Mangsi *et al.* (2021) reported a similar trend in the fermentation of antibiotic residue-positive milk samples. They found that the decrease in pH, the increase in acidity percent, and the fermentability trend during yogurt preparation were delayed compared to the control milk without antibiotic residues, which had a pH of 4.70 and acidity of 0.75%. Numerous studies have confirmed that the presence and extent of antibiotics in milk can hinder the activity of lactic acid bacteria (Vintila *et al.* 2013), leading to a delay in lactic acid production and extending the fermentation time of milk compared to antibiotic-free milk (Berruga *et al.* 2007). Sarmah *et al.* (2006) reported similar findings, where the profound antimicrobial effect of antibiotic residues inhibited the growth of yogurt starter culture microorganisms, resulting in a delay in coagulation time by altering the pH and acidity values of the milk during the fermentation process. These findings were corroborated in the current study, where pure milk samples were converted into yogurt within 3 h and achieved desirable pH and acidity values compared to all antibiotic residue-positive tetra pack milk samples. Erdogan *et al.* (2001) documented a similar trend in yogurt fermentability, reporting that the pH (4.10) and acidity (0.75%) in control milk allowed the fermentation process to be completed within 3 h. In contrast, milk samples with Penicillin residues achieved a pH of 5.53 and acidity of 0.35% beyond the stipulated time (7–8 h) for fermentation. This variation in pH and acidity values in yogurt may be attributed to the concentration of Penicillin and other antibiotics influencing the activity of yogurt starter cultures, resulting in inadequate acid development (Mishra *et al.* 2011).

Conclusion

The presence of antibiotic residues and the adulteration of raw market milk are widespread issues, and the occurrence of antibiotic residues in Tetra pack milk poses a significant health concern for urban society. Yogurt produced using milk samples that had antibiotic residues did not meet the expected quality criteria because the lower acid production was linked to the vulnerability of the starter culture to antibiotics. Conversely, antibiotics had a negative impact on the yogurt's fermentability trend and its overall quality, mainly because of their increased effectiveness against a wide spectrum of microorganisms.

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Not applicable.

Author Contributions

GSB and GBK planned the experiments; NM and QD carried out the experiments; AHS and MAJ analyzed the data; QR conceived the experiment; MBA wrote the manuscript.

Conflicts of Interest

The authors declare that there is no conflict of interest for this publication.

Data Availability

Available data can be shared on demand.

Ethics Approval

Not applicable.

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