PERIÓDICO TCHÊ QUÍMICA
ARTIGO ORIGINAL

Bacillus subtilis COMO CEPA PADRÃO PARA DETECTAR RESÍDUOS DE PENICILINA G E ESTREPTOMICINA EM AMOSTRAS DE LEITE

Bacillus subtilis AS A STANDARD STRAIN TO DETECT PENICILLIN G AND STREPTOMYCIN RESIDUES IN MILK SAMPLES IN KARBALA, IRAQ

Bacillus subtilis كسلالة قياسية للكشف عن متبقيات البنسلين ج والستربتوميسين في عينات الحليب في محافظة كربلاء، العراق

Shamel Aldorri, Sind *
University of Kerbala, College of Science, Razaza, and Western Euphrates Research Unit. Iraq.
University of Kerbala, College of Science, Department of Biology. Iraq.
ORCID: https://orcid.org/0009-0007-2266-0535

Hanan Abdul Kareem Jawad
University of Kerbala, College of Science, Department of Biology. Iraq.
ORCID: https://orcid.org/0009-0001-3217-3753

Abd Oun, Hamid Gehad
University of Kerbala, College of Science, Department of Biology. Iraq.
ORCID: https://orcid.org/0009-0009-1341-9402

Islam AA Almsarrhad
University of Kerbala, College of Science, Razaza, and Western Euphrates Research Unit. Iraq.
ORCID: https://orcid.org/0000-0002-7942-998X

Fatima Ahmed Ghashan
University of Kerbala, College of Science, Razaza, and Western Euphrates Research Unit. Iraq.
ORCID: https://orcid.org/0009-0003-2468-8911

Khalid A. Hussein
University of Kerbala, College of Science, Department of Biology. Iraq.
ORCID: https://orcid.org/0000-0002-9687-8963

Al-Mashkoor Huda Mohammed
University of Kerbala, Environmental and Renewable Energy Research Center, Iraq.
ORCID: https://orcid.org/0009-0006-7641-7680

** Corresponding author*
e-mail: sind.s@uokerbala.edu.iq

Received 02 December 2025; received in revised form 30 February 2026; accepted 14 March 2026

RESUMO

Introdução: O uso inadequado e não regulamentado de medicamentos veterinários na produção de alimentos de origem animal pode ser prejudicial tanto à saúde do consumidor quanto à indústria leiteira. A presença de resíduos de antibióticos no leite contribui para o surgimento de bactérias resistentes a antibióticos, que podem ser transmitidas dos animais aos seres humanos, levando ao desenvolvimento de alergias e ao desequilíbrio da microflora intestinal. **Objetivo**: Este estudo teve como objetivo detectar resíduos dos antibióticos penicilina G e estreptomicina em leite pasteurizado e leite cru na cidade de Karbala, utilizando um isolado local de Bacillus subtilis. **Métodos**: *B. subtilis* HS foi isolado de uma área agrícola e utilizado como micro-organismo teste para a detecção de resíduos de antibióticos em amostras de leite. Um total de 45 amostras de leite, incluindo 32 leites crus provenientes de diferentes fazendas e 13 leites pasteurizados adquiridos em mercados, foi coletado no período de setembro de 2024 a abril de 2025. Os resíduos de antibióticos nas amostras de leite foram detectados por meio do método de difusão em poço em ágar e cromatografia em camada delgada

DOI: 10.52571/PTQ.v23.n52.2026_06_SIND_pgs_61_80.pdf

(CCD). Resultados: O ensaio de difusão em poço em ágar demonstrou que, das 45 amostras analisadas (4/13 de leite pasteurizado e 12/32 de leite cru), 30,76% e 37,5% foram positivas, respectivamente. As amostras de leite apresentaram uma taxa de prevalência total de 35,5% para resíduos de antibióticos. Os resultados da CCD evidenciaram a presença de resíduos de penicilina G em 9 amostras de leite (56,25%), sendo 1 de leite pasteurizado e 8 de leite cru, as quais apresentaram mancha idêntica à do antibiótico padrão ao valor de Rf de 0,85. Quanto à presença de resíduos de estreptomicina, 2 amostras (12,5%) de leite cru apresentaram mancha idêntica à do antibiótico padrão ao valor de Rf de 0,51. Ambos os resíduos de antibióticos foram detectados simultaneamente em 2 amostras (12,5%) de leite cru. **Discussão**: A maior ocorrência de resíduos de antibióticos no leite cru em comparação ao leite pasteurizado pode ser atribuída ao não cumprimento, por parte dos produtores, dos períodos de carência estabelecidos pela Comissão do Codex Alimentarius após a administração de medicamentos aos animais. Contribui também para esse cenário a ausência de controle sobre a comercialização do leite cru, vendido diretamente pelos produtores sem a realização de testes rigorosos. **Conclusões**: A presença de resíduos de antibióticos nas amostras de leite evidencia a necessidade de que as autoridades monitorem a qualidade do leite cru que chega ao mercado consumidor.

Palavras-chave: *Segurança alimentar, cromatografia em camada delgada, ensaio de difusão em poço em ágar, Bacillus subtilis, leite pasteurizado*.

ABSTRACT

Background: The improper and unregulated use of veterinary drugs in animal food production can be harmful to both consumer health and the dairy industry. The presence of antibiotic residues in milk contributes to the emergence of antibiotic-resistant bacteria that could be transmitted from animals to humans, leading to the development of allergies and disturbances in the balance of intestinal microflora. **Aim:** This study aimed to detect penicillin G and streptomycin antibiotic residues in pasteurized and raw milk in Karbala city using a local isolate of *Bacillus subtilis.* **Methods:** *B. subtilis* HS was isolated from an agricultural area and used as a test microorganism for antibiotic residues in milk samples. A total of 45 milk samples, including 32 raw milks from various farms and 13 pasteurized milks from markets, were collected from September 2024 to April 2025. Antibiotic residues in milk samples were detected using Agar Well diffusion method and thin-layer chromatography (TLC). **Results**. Agar well diffusion assay showed that, out of 45 samples (4/13 pasteurized milk and 12/32 raw milk), 30.76% and 37.5% were positive, respectively. The milk samples had a total prevalence rate of 35.5% for antibiotic residues. The results of TLC showed the presence of penicillin G residue was out of 9 milk samples (56.25%), 1 (pasteurized milk), and 8 (raw milk) had an identical spot with the standard antibiotic at Rf value of 0.85. Regarding the presence of streptomycin residue, 2 samples (12.5%) from raw milk showed an identical spot with the standard antibiotic at Rf of 0.51. Both antibiotic residues were detected in 2 samples (12.5%) of raw milk. **Discussion:** The higher occurrence of antibiotic residues in raw milk compared to pasteurized milk samples could be attributed to farmers not following the withdrawal periods set by the Codex Alimentarius Commission after administering medication to their animals. Also, the lack of control over raw milk sales, where farmers are sold directly without undergoing strict testing. **Conclusions:** The presence of antibiotic residues in milk samples necessitates that authorities monitor the quality of raw milk entering the consumer market.

Keywords: *Food safety, thin layer chromatography, Agar well diffusion assay, Bacillus subtills, pasteurized milk.*

الملخص

الخلفية: إنّ الاستخدام غير السليم وغير المنظَّم للأدوية البيطرية في إنتاج الغذاء الحيواني يمكن أن يُلحق أضرارًا بصحة المستهلك وصناعة الألبان على حدٍّ سواء. إذ يُسهم وجود متبقيات المضادات الحيوية في الحليب في ظهور بكتيريا مقاومة للمضادات الحيوية يمكن أن تنتقل من الحيوانات إلى الإنسان، مما يؤدي إلى حدوث حالات تحسّسية واضطرابات في توازن النبيت الجرثومي المعوي. **الهدف:** هدفت هذه الدراسة إلى الكشف عن متبقيات المضادين الحيويين البنسلين G والستربتومايسين في الحليب المبستر والحليب الخام في مدينة كربلاء باستخدام عزلة محلية من بكتيريا *Bacillus subtilis*. **طرائق العمل:** تم عزل السلالة HS *B. subtilis* من منطقة زراعية، واستُخدمت ككائن مجهري اختباري للكشف عن متبقيات المضادات الحيوية في عينات الحليب. جُمعت (45) عينة حليب، منها (32) عينة حليب خام من مزارع مختلفة و(13) عينة حليب مبستر من الأسواق، خلال المدة من أيلول 2024 إلى نيسان 2025. وتم الكشف عن متبقيات المضادات الحيوية باستخدام طريقة Agar Well diffusion وتقنية الكروماتوغرافيا ذات الطبقة الرقيقة (TLC). **النتائج:** أظهر اختبار الانتشار في الآغار بطريقة الحفر أنّه من أصل 45 عينة (13/4 من الحليب المبستر و32/12 من الحليب الخام)، كانت نسب النتائج الإيجابية 30.76% و37.5% على التوالي. وبلغت نسبة الانتشار الكلية لمتبقيات المضادات الحيوية في عينات الحليب 35.5%. وأظهرت نتائج الكروماتوغرافيا ذات الطبقة الرقيقة (TLC) وجود متبقيات البنسلين G في 9 عينات حليب (56.25%)، منها عينة واحدة من الحليب المبستر و8 عينات من الحليب الخام، حيث أظهرت بقعة مماثلة للمضاد القياسي عند قيمة Rf بلغت 0.85. أمّا بالنسبة لمتبقيات الستربتومايسين، فقد أظهرت عينتان (12.5%) من الحليب الخام بقعة مماثلة للمضاد القياسي عند قيمة Rf بلغت 0.51. كما تم الكشف عن كلا متبقيي المضادين الحيويين في عينتين (12.5%) من الحليب الخام. **المناقشة:** يُعزى الارتفاع في نسبة متبقيات المضادات الحيوية في الحليب الخام مقارنةً بالحليب المبستر إلى عدم التزام المزارعين بفترات السحب المحدّدة من قبل هيئة الدستور الغذائي الدولية بعد إعطاء الأدوية للحيوانات. كما يُسهم ضعف الرقابة على بيع الحليب الخام، حيث يُباع مباشرةً دون إخضاعه لاختبارات صارمة، في زيادة

هذه النسبة. **الاستنتاجات:** إنّ وجود متبقيات المضادات الحيوية في عينات الحليب يستدعي ضرورة قيام الجهات المختصّة بمراقبة جودة الحليب الخام الداخل إلى سوق المستهلك.

الكلمات المفتاحية: سلامة الغذاء، الكروموتوغرافيا ذات الطبقة الرقيقة، فحص الانتشار بالحفر، *Bacillus subtilis*، *الحليب المبستر*.

1. INTRODUCTION:

The mitigation of the increasing threat of antimicrobial residues in milk and dairy products is the farmer's responsibility. Antibiotics are widely used in livestock farming for disease prevention, infection treatment, and growth promotion, accounting for more than 70% of global antibiotic consumption (Allen *et al*., 2013; Jechalke *et al*., 2014; Salim *et al*., 2018). Most antibiotics used in animals are the same as or closely related to those used in humans (Marshall and Levy, 2011; Ma *et al*., 2021). It is well known that antibiotic contamination creates selective pressure for resistant bacterial strains (Tello *et al*., 2012; Arsène *et al*., 2022). The most widely used antibiotics for both animal and human are classified within the β-lactam group, which includes: carbapenem, monobactam, cephalosporin, and penicillin (Prescott and Hardefeldt, 2024; Garkavenko *et al*.,2021). Other classes include macrolides such as erythromycin and azithromycin. Aminoglycosides such as streptomycin, gentamycin (Conceição *et al*.,2023). Some antibiotics are incompletely absorbed and metabolized in animals, so the residues are excreted unchanged through urine (Chee-Sanford *et al*., 2009). Consequently, high concentrations of residual antibiotics are released in animal manure (Qian *et al*., 2018).

The presence of antibiotic residues in food products, especially milk, has certain harmful effects on public health. A study conducted by Zhang found that antibiotic residues in the muscles, kidneys, and livers of cattle and sheep, may pose a threat to human health (Zhang *et al*.,2021). Another study found antibiotic residues in milk (Titouche *et al*., 2013; Batah *et al*., 2025). Moreover, poultry may contain antibiotic residues, which can cause numerous adverse health effects in humans. Antibiotic residues have been detected in chicken production (meat and eggs) (Chang *et al*., 2015). The accumulation of penicillin residues in milk led to the development of allergies in some hypersensitive individuals, while the accumulation of sulfamethazine and oxytetracycline could increase the risk of cancer (Hou *et al*., 2015). Also, the presence of gentamicin residues is causing nephropathy and disturbances in the intestinal microbiota (Back *et al*., 2020). Therefore, the detection of veterinary drug residues in food represents an important food safety issue

The bioanalytical techniques used to detect antibiotic residues in food products are mainly categorized into two groups: screening methods and Confirmatory Methods (Cháfer-Pericás *et al*., 2010). Screening methods such as microbial inhibition tests, immunoassays like ELISA, and lateral flow tests are characterized by their quickness, ease of use, and affordability (Ghimpețeanu *et al*., 2022). They detect the presence of antibiotic residue above a certain limit rather than identifying the specific type (Cháfer-Pericás *et al*., 2010). Confirmatory methods, such as chromatography coupled with mass spectrometry (LC-MS/MS) and (GC-MS/MS), require expensive equipment and trained personnel (Wang *et al*.,2022). However, these methods are particularly powerful, capable of identifying and quantifying over 100 different antibiotics at very low levels in a single test (Berendsen *et al*.,2013).

Bacillus subtilis is referred to as a "soil dweller," with its natural niche in soil. It is a spore-forming, Gram-positive, rod-shaped, motile bacterium (Norris *& Wolf,* 1961). *B. subtilis is* considered an effective microorganism that is used to detect antibiotic residues in milk for several reasons: first, the broad sensitivity, where many studies showed that *B. subtilis* is naturally sensitive to a wide range of antibiotics such as penicillin, amoxicillin, cephalosporins, and tetracyclines (Sharma *et al*.,2025). This wide-ranging sensitivity enables a single test of *B. subtilis* to effectively detect contamination of food with a variety of different antibiotics (Lee *et al*., 2007; Navrátilová *et al*., 2024). *B. subtilis* is an ideal organism for commercial test kits because it produces spores, which are a stable, inactive state and highly resistant to both heat and drying. This characteristic gives the test kits a long shelf life and allows them to be stored and transported easily (Gondová *et al*., 2014; Trufanov *et al*., 2015). Also, microbial tests using *B. subtilis* are more practical and quicker for screening than chromatography or mass spectrometry. They are

simpler, faster, and much cheaper (Ferone *et al*.,2020).

1.1. Aims

Due to public health concerns, milk and dairy products containing antibiotic residues above safe levels are not considered safe for people to drink (Plumb, 2018). However, there's been growing concern about how often veterinary drug residues are found in food, especially milk. Despite this, no previous studies have specifically examined antibiotic residues in milk sold in Karbala Province. So, this study aims to detect these residues in pasteurized milk (market) and raw cow milk (dairy farm) in Karbala using the Agar well diffusion method and thin-layer chromatography.

2. MATERIALS AND METHODS:

2.1. Materials

2.1.1 Isolation and Identification of Bacillus subtilis

B. subtilis is isolated selectively, as recommended by Vehapi *et al*. (2023), with a slight modification. Soil samples were collected from an agricultural area in the College of science/, Karbala University. Ten soil samples, taken to a depth of 10 cm, were placed in sterile, sealed bags and transported to the laboratory. From each soil sample, 1 g was weighed and placed in a test tube containing 9 mL of sterile saline solution. To obtain a homogeneous suspension by breaking up aggregated soil particles, the samples were shaken in a vortex machine at the highest speed for 20 minutes. To obtain *B. subtilis* spores, the soil suspension from each sample was heated in a water bath at 80 °C for 20 minutes to eliminate vegetative bacterial cells. Then 10 µL of the soil suspension was transferred and spread on a solid culture surface of sterile nutrient agar. The plates were incubated at 30 °C in an incubator for 24-48 hours.

An initial confirmation test was used to identify the selected isolates, including the Gram stain and catalase tests (Amin *et al*., 2015). All isolates were identified as *B. subtilis*. After that, the Viteck 2 test was performed for more accurate identification. The strain was designated as “HS”.

2.1.2 Preparation of Bacillus subtilis HS spore suspension

The spore suspension of *B. subtilis* HS was prepared according to the method described by El Atabani *et al*. (2014). Under sterile conditions, several colonies of the bacteria were transferred to sterile nutrient agar culture plates and incubated at 30 °C for 10 days to induce bacterial cells to produce spores. At the end of the incubation period, the cells were harvested in 10 mL of sterile saline (0.8% Sodium chloride), then centrifuged at 3000 rpm for 10 minutes (repeated twice). The suspension was heated at 70 °C in a water bath for 30 minutes to kill the vegetative cells. The hot suspension was transferred to a centrifuge and spun at 3000 rpm for 10 minutes; the supernatant was discarded. Another 10 mL of sterile saline was added to wash the remnants of the vegetative cells. The mixture was concentrated at the same speed and duration (repeated twice) to obtain a pure spore’s suspension.

2.1.3 Antibiotic sensitivity test for B. subtilis HS

Antibiotic sensitivity tests for *B. subtilis* HS were performed using the Kirby-Bauer method, following the guidelines for antimicrobial disk susceptibility testing established by the Clinical and Laboratory Standards Institute (CLSI, 2012). Nine types of antibiotics (Oxoid, UK) used have been used. The selected antibiotic belonged to five classes of antimicrobials. These antibiotics include aminoglycosides: Kanamycin (K, 30 µg), Neomycin (N, 30 µg) and Streptomycin (S, 10 µg); β-lactam: Ampicillin (AM, 10 µg), Amoxicillin (AmC, 30 µg) and Penicillin G (P, 10 µg); fluroquinolones: Ciprofloxacin (CIP, 5 µg); phenicols: Chloramphenicol (C, 30 µg); tetracyclines: Tetracycline (Te, 30 µg). An overnight bacterial suspension was made to match the turbidity of a 0.5 McFarland standard. This suspension was then evenly spread across Muller Hinton agar plates using a cotton swab. Four antibiotic disks were impregnated on the plates, with a distance of 10 mm between each. The plates were transferred to a fridge for 20 minutes, then incubated at 35 °C for 24 hours. Zone inhibition around each disk was measured in millimeters (mm), and the results were recorded.

2.1.4 Milk sample collecting

Two types of milk samples were randomly collected from September 2024 to April 2025. Samples of all types were collected from Karbala

province. The first one was different brands of unflavored pasteurized milk samples (13), denoted by (P), purchased at the point of sale. The other one was raw milk samples from cows (32), denoted by (C), that were collected from different farms. The samples were kept under cold conditions until transported to the laboratory.

2.1.5 Preparation of standard antibiotic concentrations

Two antibiotics were used in this study: penicillin G and streptomycin, which were kindly supplied by the State Company for Drug Industries and Medical Appliances, Samarra, Iraq. The stock solution for each antibiotic was prepared at a concentration 0.1 mg/mL by dissolving in methanol. Working stock solutions of Penicillin G and streptomycin were sterilized using a Millipore filter and stored at 4 °C until used.

2.2. Methods

2.2.1 Detection of antibiotic residue using Agar well diffusion method

To perform this test, 100 µL of *B. subtilis* HS spore suspension (diluted with normal saline to achieve a turbidity of McFarland's standard solution (0.5) that equivalent to about $1.5x10^8$ CFU/mL) were transferred to each 100 mL of solid culture medium of Muller- Hinton agar cooled to 45 °C (prior to solidification of the medium) and mix well. The medium was then poured into sterilized Petri dishes and left to solidify at room temperature. Using a sterile cork borer, four holes (8 mm in diameter) spaced 20 mm apart were drilled into the surface of the Muller-Hinton culture medium. Each well was filled with 100 µL of the milk samples, individually. The plates were incubated at 30 °C for 24 hours under aerobic conditions. The presence of antibiotic residues was indicated by the formation of an inhibition zone around the wells (absence of bacterial growth). The inhibition zones around the wells were measured, and the results were recorded (Al-Mashhadany *et al.*,2018). The prevalence percentage of antibiotic residue is determined using Equation 1

$$Prevalence\ (\%) = \left(\frac{Number\ of\ positive\ samples}{Total\ number\ of\ samples\ tested}\right) \times 100 \quad \text{(Eq. 1)}$$

Detection of minimum inhibition zone diameter was performed by preparing a serial dilution of each antibiotic (25, 10, 5, 2, 1 µg/mL). Then add the prepared antibiotic solution to antibiotic-free milk, followed by the extraction of antibiotics from the spiked milk. The steps for the Agar well diffusion method, as mentioned above, were repeated. Then determine the lowest antibiotic concentration that yields the smallest inhibition zone (mm).

2.2.2 Detection of antibiotic residue using the thin-layer chromatography (TLC) technique

2.2.2.1 Mobile phase of TLC

The mobile phase was prepared as described by Kaya and Filazi (2010). This solution was composed of Acetone-chloroform-n-propanol-impregnation liquid (16 + 20 + 27 + 16).

The impregnation liquid was a pre-prepared solution (0.1 N phthalate, pH 3.75, and glycerin, 19+1).

2.2.2.2 Thin-layer chromatography

The samples that showed a positive result (a clear inhibition zone; 4 pasteurized milk, 12 raw milk) were applied to thin-layer chromatography as described by Kaya and Filazi (2010). In the current study, two TLC plates were used, one for the pasteurized milk sample and the other for the raw milk samples. The TLC plate used for pasteurized milk was divided into 6 equal channels (4 pasteurized milk and 2 standard antibiotics), while the TLC plate used for raw milk samples was divided into 14 equal channels (12 raw milk and 2 standard antibiotics). Ten microliters of the extract were spotted onto a glass capillary tube. The plates were then allowed to dry for 5 minutes at room temperature to prevent sample decomposition. Subsequently, each plate was placed in a TLC tank containing the mobile phase, which had been prepared an hour beforehand to achieve an optimal saturated environment, and the tank was covered with a lid. Once the solution front reached to the line drawn below 1 cm from the up edge, the plates were removed, dried, and then transferred to a freshly tank. Two methods were used to visualize the spots: UV detection box at 254 nm (Skorupa and Gierak, 2011; Hayati and Anggraini, 2023) and evaporated Iodine. To calculate the Retardation factor (Rf), sample spots were marked with a pencil. The Rf value is determined using Equation 2 (Kumar *et al.*, 2013).

$$Rf = \frac{distance\ travelld\ by\ component}{distance\ traveled\ by\ solvent} \quad \text{(Eq. 2)}$$

A matching Rf value between a sample and a standard indicates that two compounds are identical (Fink *et al*.,1963; Cieśla *et al*.,2009)

2.2.2.2 Quality control

The limit of detection (LOD) and the limit of quantification (LOQ) to evaluate the sensitivity of TLC for penicillin G and streptomycin, a serial dilution was prepared. Different concentrations (50, 25, 10, 5, 1, and 0.5 μg/mL) were prepared by dissolving the standard antibiotics in methanol to obtain the desired concentrations. These concentrations were used for minimum detectable concentrations, as mentioned by Kaya and Filazi (2010).

The recovery (%) of an antibiotic with a milk sampling was detected by adding a known concentration of (50, 25, 5 μg/mL) of standard antibiotic to a milk-free antibiotic sample. Followed by extracting the antibiotic from milk as described by Tyczkowska *et al*. (1989), one mL of the milk sample was mixed with 1 mL of protein precipitation solution (acetonitrile-methanol-deionized water at a ratio of 40:20:20, respectively) in a centrifuge tube. After gently shaking the tube, it was centrifuged at 3000 rpm for 10 minutes. Then spiking the samples in the TLC plate. Recovery (%) was calculated using Equation 3.

$$Recovery\ (\%) = \frac{\text{Amount detected after extraction}}{\text{Amount of originality added}} \times 100 \quad \text{(Eq. 3)}$$

The detection of Precision (RSD%) of the TLC method was evaluated by performing replicate analyses (n=4) of spiked milk samples with a known concentration of antibiotic (25, 10, and 5 μg/mL. The RF value was measured for each run. The RSD% was calculated using Equation 4. RSD% values below 5% are considered good precision.

$$RSD\ (\%) = \frac{\text{SD}}{\text{Mean}} \times 100 \quad \text{(Eq. 4)}$$

2.3 Statistical Analysis

SPSS version 20 software for descriptive statistical analysis was used for results analysis. To evaluate the prevalence of antibiotic residues, milk samples were categorized as 'positive' or 'negative' based on the presence of an inhibition zone ≥2 mm. Given the unequal group sizes (n=13 vs. n=32) and the presence of small expected frequencies in some categories, **Fisher's Exact Test** was employed to determine if the proportion of positive antibiotic detections differed significantly between raw and pasteurized milk. This approach provides a robust, non-parametric assessment of the association between milk processing type and the presence of veterinary drug residues. Results were considered statistically significant if $P < 0.05$. Also, the Confidence Interval (CI) at 95% was calculated

3. RESULTS AND DISCUSSION:

3.1. Results

3.1.1. Antibiotic sensitivity test for *B. subtilis*

B. subtilis HS was found to be sensitive to all the antibiotics tested. The results, shown in **Table 1**, demonstrated its susceptibility to kanamycin, neomycin, streptomycin, ampicillin, amoxicillin, penicillin G, ciprofloxacin, chloramphenicol, and tetracycline. Because of this broad sensitivity, *B. subtilis* HS is a strong candidate for use as a test microorganism to detect antibiotic residues in milk.

3.1.2. Detection of antibiotic residue in milk using the Agar well diffusion method

The results of the Agar well diffusion method for detecting antibiotic residues showed that, out of 13 pasteurized samples collected from markets, only 4 (30.76%) gave a positive result (formation of an inhibition zone around the holes). Of 32 raw milk samples, only 12 (37.5%) tested positive for antibiotic residues. Altogether, the milk samples showed a total occurrence of antibiotic residues of 35.5%, as presented in **Table 2.** The positive and negative results for the antibiotic residue in milk are shown in **Figure 1.**

The diameter of the inhibition zone was varied between pasteurized and raw milk **Figure 2.** The raw milk showed a larger zone of inhibition than pasteurized milk. Even within the same group, the inhibition zone was varied**.** However, there was no significant difference between

pasteurized and raw milk ($p<0.05=0.52$). Statistically, the 95% confidence interval for both pasteurized and raw milk samples was 14.75%-22.59%.

The minimum inhibition zone diameter for antibiotic residues in milk was detected using Agar well diffusion method at concentrations of 25, 10, 5, 2, and 1 μg/mL). Results in **Table 3** and **Figure 3** showed that the minimum inhibition zone diameters for Penicillin G and Streptomycin were 10 $\mu l/mL$ and 5 $\mu l/mL$, respectively.

3.1.3. Detection of Penicillin G and Streptomycin antibiotic residue in milk using the TLC method

The milk samples that showed a positive result (inhibition zone) were subjected to a TLC test to identify the antibiotic residue present in the milk. Penicillin G and Streptomycin were used as standard antibiotics. As shown in **Table 4**, the Retardation factors (Rf) were varied between and within the milk samples. For the presence of Penicillin G residue, there were 9 milk samples (56.25%), 1 belonging to pasteurized milk; P11 and 8 belonging to raw cow milk; C3, C8, C12, C17, C18, C22, C26, and C30 showed an identical spot with the standard antibiotic penicillin G at Rf value 0.85. Regarding the presence of Streptomycin residue, 2 samples (12.5%) from raw cow milk (C29 and C34) showed an identical spot with the standard antibiotic streptomycin at an Rf of 0.51. On the other hand, the combination of Penicillin G and Streptomycin antibiotic residues was detected in 2 samples (12.5%) from raw cow milk (C1 and C20), which showed 2 spots identical to the standards. However, 3 samples from pasteurized milk (P3, P6, and P7) showed spots that were not identical to the standard antibiotic, with different Rf values.

The precision test (RSD%) values for Penicillin G and Streptomycin are shown in **Table 5**. Where the RSD% for penicillin G was 1.7% and for streptomycin was 2.3%.

3.1.4. Quality control

The sensitivity of the TLC method was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ) for Penicillin G and Streptomycin in milk. The LOD values for Penicillin G were found to be 0.5 μg/mL as shown in **Table 6** and **Figure 4**. For Streptomycin, the LOD values were found to be 1 $\mu g/mL$ as shown in **Table 6** and **Figure 5**. The LOQ values for Penicillin G and Streptomycin were 1.5 $\mu g/mL$ and 3 $\mu g/mL$, respectively (**Table 6**).

The recovery % for Penicillin G was 82-87%, while for Streptomycin was 85-89%, which are considered good values, **Table 6.**

3.2. DISCUSSION

Since Alexander Fleming discovered antibiotics, millions of lives have been saved. Despite that, the future of antibiotics used in medicine is more complicated than anyone expected (Hutchings *et al*., 2019). The overuse of antibiotics contributes to the development of antibiotic-resistant strains. This resistance is based on the concept of Darwinian survival of the fittest (Fleming, 2006). The use of antibiotics is not limited to human pathogenic infections; it also includes animal and plant infections (Arsène *et al*., 2022). Reports of antibiotic residues in milk began appearing in the 1960s, but detections significantly rose after 2000. This issue has drawn considerable attention recently, driven by growing concerns over food safety and public health (Sachi *et al*., 2019). The presence of antimicrobial residues in milk and dairy products is considered a serious threat to public health (Ghimpeţeanu *et al*.,2022).
Therefore, simple, and effective screening tests are important for identifying residues of various classes of antimicrobials in food, particularly milk (Islam *et al*., 2020). In the current study, the occurrence of antibiotic residues in raw milk samples collected from the fields using Agar well diffusion method was higher (37.5%) than in pasteurized milk samples collected from markets in Karbala (30.76%). This could be attributed to several reasons; first, farmers do not follow the withdrawal periods set by the Codex Alimentarius Commission after administering medication to their animals, which can result in contamination of their raw milk (Alimentarius, 2010). As is well known, withdrawal periods refer to the time required for an animal to fully metabolize and eliminate a given antibiotic from its tissues, thereby reducing it to a safe and acceptable level for consumption (Virto *et al*., 2022). Each antibiotic has a specific withdrawal time, such as Penicillin 72 hours, Amoxicillin 60 hours (Burmańczuk *et al*., 2017), and a combination of

Streptomycin with Penicillin 72 hours (Karande *et al*., 2021). Accordingly, milk produced during that time must be disposed of. Secondly, the lack of control over raw milk sales, where farmers sell raw milk directly without undergoing strict testing or meeting quality control standards (Zavala and Revoredo-Giha, 2022). Unlikely, the milk intended for pasteurization is usually tested for antibiotic residues at collection centers; milk exceeding safe limits is rejected, ensuring that the raw milk used for pasteurization has very low or undetectable levels of these residues (Rahman *et al*., 2021).

To sum up, the greater detection of antibiotic residues in raw milk compared to pasteurized milk is largely due to residues originating from farm-level veterinary drug use. However, the pasteurization process, along with the quality control measures in place for milk intended for pasteurization, degrades or inactivates some of these residues, resulting in lower levels in the final product. Despite that, the percentage of total occurrence of antibiotic residue in the current study was 35.5%, which was concordant with other studies with a total occurrence ranging from 28-35%, such as Iran 34% (Olatoye *et al*.,2016), Kenya 30.7% (Orwa *et al*., 2017), and Somalia 30% (Mohamed *et al*., 2020). Although many studies have detected antibiotic residues in milk samples, the overall incidence has varied. This variation was low in some regions (Kumarswamy *et al*., 2018) and high in others (Stella *et al*., 2020). This variation could be attributed to many factors, such as withdrawal time of milk, the method used to detect antibiotic residues, and the stability of the antibiotic in milk (Sachi *et al*., 2019). Also, the experimental season was conducted. A study by Moghadam *et al*. (2016) showed that the winter season had a higher incidence of antibiotic residues in milk than the spring season. Likewise, this study was conducted during the winter season when antibiotic residues are typically more common. This can be attributed to colder temperatures and shorter days, which lead animals to be kept indoors more often, increasing the risk of infections and, in turn, prompting the use of an antibiotic for treatment (Alimohammadi *et al*., 2020).

In the current study, the choice of *B. subtilis* to detect penicillin G and streptomycin residues in milk samples is due to its remarkable sensitivity to these antibiotics (Titouche *et al*., 2022). This led to the next part of the work, which was the use of penicillin G and streptomycin as a standard for performing TLC tests on samples that showed a positive result. Table 2 clearly demonstrated that penicillin G had the highest antibiotic residue in milk samples (56.25%), followed by streptomycin (12.5%) and the combination of penicillin G and streptomycin (12.5%). The reasons for the higher occurrence of penicillin G as an antibiotic residue in milk compared to streptomycin are that penicillin G belongs to the beta-lactam antibiotics, known for its wide spectrum of activity against gram-positive pathogens such as streptococcal and staphylococcal infections (Okonko *et al*., 2009). Also, penicillin G has fewer side effects than streptomycin, which can cause an allergic reaction in cattle (Hirvonen *et al*., 1994). Additionally, the low cost, availability, and effectiveness of treatment for infectious diseases lead farmers in remote areas to use penicillin G without veterinary guidance (Layada *et al*., 2016; Batah *et al*., 2025). In general, this study agrees with other studies that reported a high rate of penicillin G in milk samples compared to other antibiotics (Kaya and Filazi, 2010; Malgwi *et al*., 2023).

Numerous analytical techniques exist for detecting antibiotic residues in milk, including microbiological tests, chromatographic methods, immunochemical assays, and receptor- and enzyme-based tests (Kantiani *et al*., 2009; Kaya and Filazi, 2010). Among these, microbiological tests, particularly the agar well diffusion method, are frequently used in the dairy industry to detect antibiotic residues (Titouche *et al*., 2013; Almashhadany, 2021). The combination of thin-layer chromatography (TLC) with microbiological detection methods, such as the agar well diffusion assay, is an effective approach for detecting antibiotic residues in milk. This method is valued for its simplicity, low cost, sensitivity, and specificity in identifying various antibiotics (Kaya and Filazi, 2010; Piech *et al*., 2016).

At this point, robust disease prevention practices should be implemented to minimize the need for antibiotic overuse in livestock. Three main pillars are considered cornerstones for protecting livestock health: smart husbandry, biosecurity, and hygiene (Paramitadevi *et al*., 2023; Jimenez *et al*., 2023). Smart husbandry, using technology to control and optimize all environmental conditions inside livestock houses, is ideal for controlling disease spread. An example of smart husbandry is the use of sensors to adjust ideal temperature, humidity, and ventilation levels (Ongom, 2023), smart

feeding by balancing the quantity of feed that meets the demands of animals (Makkar, 2016), and utilizing sensors to track animal health, behavior, and productivity (Kistanova *et al*.,2024). The biosecurity sector involves restricting and controlling antibiotic prescribing and vaccinating animals (Renault *et al*., 2021). To achieve biosecurity, many practices are used today, including food safety, limiting and managing invasive species, and controlling the risk of pathogen transfer (zoonosis) (Subasinghe *et al*., 2023). Hygiene is the third crucial sector for protecting livestock health. To prevent disease, it is important to maintain a clean-living environment, provide clean water and food, and practice good personal hygiene. Regular cleaning and disinfection of housing, equipment, and vehicles are also very effective in reducing disease risk (Jimenez *et al*., 2023).

To mitigate the harmful effects of antibiotic residue in milk, it's crucial to educate farmers about these risks. This could be accomplished by using an alternative antibiotic, such as a vaccine or phage therapy. Also, the use of prebiotics and probiotics in animal feed, and the use of traditional medicinal herbs to reduce the need for antibiotics in foodstuffs are considered promising steps towards minimizing the use of antibiotics.

4. CONCLUSIONS:

This study was conducted in Karbala province, where two types of milk samples were collected. Raw milk from cows was collected from the fields, and pasteurized milk from different brands was purchased from the markets. This study clearly demonstrated that the antibiotic residues in raw milk was higher than in pasteurized milk. The presence of antibiotics in milk is a serious public health concern. This issue stems from the improper use of veterinary drugs and the failure to monitor the withdrawal period - the time required between giving antibiotics to the animal and collecting its milk. This could lead to long-lasting illnesses, which would cause medical costs to go up and high mortality rates. Consequently, this points to inefficient and failing public health rules and to farmers using antibiotics carelessly, which is dangerous for consumers.

5. DECLARATIONS

5.1. Study Limitations

The main limitations of this study were the unclear history of antibiotic use among farmers in Karbala city and the lack of a clear schedule and protocols for veterinarians. For these reasons, the results provide an overall picture of antibiotic residues in milk rather than explaining them in detail. However, the cows in the city are distributed in small groups (fewer than 10 per farmer), so the sample size needs to be large to capture the city's geographic distribution, which is very expensive.

Although TLC is a highly accessible and economical screening method for antibiotic residues, its detection threshold often exceeds the strict MRLs mandated by Codex. Advanced analytical platforms (HPLC/LC-MS/MS) offer superior sensitivity but are significantly more expensive. In this study, TLC was utilized as a viable alternative for preliminary contamination assessment despite these sensitivity constraints."

5.2. Acknowledgments

I would like to express my sincere gratitude to the Razaza and Western Euphrates Research Unit at the College of Science, Kerbala University for providing the essential laboratory facilities and tools necessary for this work.

5.3. Funding resources

The authors funded this research. In accordance with the ethical guidelines of the Periódico Tchê Química, which do not allow donations from authors with manuscripts under evaluation (even when research funds are available), or in cases of authors' financial constraints, publication costs were fully absorbed by the journal under our Platinum Open Access policy, through the support of the Araucária Scientific Association (https://acaria.org/). This policy aims to ensure complete independence between the editorial process and any financial aspects, reinforcing our commitment to scientific integrity and equity in knowledge dissemination.

5.4. Conflicts of Interest

The authors declare that they have no competing interests.

5.5. Data Availability

Raw data are available upon request from the corresponding author (sind.s@uokerbala.edu.iq).

5.6. Author Contributions

The contribution of all authors to this manuscript is summarized as follows:
Shamel Aldorri, Sind: Conception and design, manuscript writing, performed analysis and final approval. Hanan Abdul Kareem Jawad: Data collection. Abd Oun, Hamid Gehad: Conception and design , final approval , critical review of statistics. Islam Ahmed Abd Alsahib: Manuscript writing. Fatima Ahmed Ghashan: Manuscript writing. Khalid A. Hussein: Critical review of statistics and performed analysis. Al-Mashkoor Huda Mohammed: Final approval.

5.7. AI and Computational Tools Declaration

The authors declare that no generative artificial intelligence tools or computational language models were used in the conception, design, execution, data collection, data analysis, interpretation, manuscript writing, or any other aspect of this research or manuscript preparation.

No artificial intelligence tools were used for data fabrication, statistical analysis, interpretation of results, or scientific decision-making. Textual polishing and grammatical editing were performed using Grammarly.

5.8. Research Integrity Declaration

The authors certify that this research complies with the standards of research integrity, including no data fabrication, no results falsification, no p-hacking or selective reporting, originality, not previously published, and ethical methods.

5.9. Originality & Plagiarism Statement

This manuscript was screened for plagiarism using Turnitin. The overall similarity index was 13%, which is below the acceptable threshold. The authors declare that this manuscript is original work and has not been previously published or submitted elsewhere for consideration. No unattributed text has been copied from other sources, and all references have been properly cited in accordance with APA guidelines. The authors further confirm that this manuscript is not currently under review by any other journal.

5.10. Open Access License

6. STUDIES INVOLVING HUMAN AND ANIMAL SUBJECTS

This study utilized commercially available bovine milk purchased from a local retail outlet. As the research involved only food-grade animal byproducts and did not involve direct intervention, experimentation, or interaction with live animals, formal ethical approval from an Animal Care and Use Committee was not required.

6.1. Ethics Committee Approval

Not applicable.

6.2. Informed Consent

Not applicable.

7. REFERENCES:

1. Al-Mashhadany, D. A., Nahla, A. A., Zaki, A. M., & Mohammad, V. S. (2018). Detection of antibiotic residues among poultry meat in Erbil city and impact of thermal processing on

remnants. *Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences*, *3*, 237-247. https://doi.org/10.26479/2018.0401.19

2. Alimentarius, C. (2010). Codex alimentarius commission. *Toxicological evaluation of certain veterinary drug residues in food. Disponível em: http://www. codexalimentarius. net/web/jecfa. jsp. Acesso em*, *28*

3. Alimohammadi, M., Askari, S. G., Azghadi, N. M., Taghavimanesh, V., Mohammadimoghadam, T., Bidkhori, M., & Mohammadi, A. A. (2020). Antibiotic residues in raw and pasteurized milk produced in Northeastern Iran were examined using the four-plate test (FPT) method. *International Journal of Food Properties*, *23*(1), 1248-1255. https://doi.org/10.1080/10942912.2020.1800032

4. Allen, H. K., Levine, U. Y., Looft, T., Bandrick, M., & Casey, T. A. (2013). Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends in microbiology*, *21*(3), 114-119 https://doi.org/10.1016/j.tim.2012.11.001

5. Almashhadany, D. A. (2021). Screening of antibiotic residues in raw milk of cows and buffalos by diffusion assays. *Italian journal of food safety*, *10*(3), 9034. https://doi.org/10.4081/ijfs.2021.9034

6. Amin, M., Rakhisi, Z., & Ahmady, A. Z. (2015). Isolation and identification of Bacillus species from soil and evaluation of their antibacterial properties. *Avicenna Journal of Clinical Microbiology and Infection*, *2*(1), 23233. https://DOI: 10.17795/ajcmi-23233

7. Arsène, M. M. J., Davares, A. K. L., Viktorovna, P. I., Andreevna, S. L., Sarra, S., Khelifi, I., & Sergueïevna, D. M. (2022). The public health issue of antibiotic residues in food and feed: Causes, consequences, and potential solutions. *Veterinary world*, *15*(3), 662. https://doi.org/10.14202/vetworld.2022.662-671

8. Back, S.-J.,Park, S.-J.,Moon, J.-S.,Lee, S.-B.,Jo, S.-J.,Nam, T.-J.,Bai, S. C.,Kong, I.-S.,Lee, K.-J.andLee, B.-J. (2020). The effects of dietary heat-killed probiotics bacteria additives in low-fishmeal feed on growth performance, immune responses, and intestinal morphology in juvenile olive flounder paralichthys olivaceus. *Aquaculture Reports*, *18*, 100415. https://doi.org/10.1016/j.aqrep.2020.100415

9. Batah, R., Belbel, Z., Melouah, K., Labied, I., Benaldjia, L., & Berreghis, A. (2025). Detection of antibiotic residues in cow's milk using microbiological screening test in Souk Ahras, Algeria. *Journal of the Hellenic Veterinary Medical Society*, *76*(1), 8865-8870. DOI: 10.12681/jhvms.38195

10. Berendsen, B., Wegh, R., Reinders, T., Lommen, A., & Stolker, A. (2013). Development and validation of a comprehensive LC-MS/MS method for the determination of 46 antibiotics in meat and meat products. *Food Chemistry*, 137(1-4), 160-170.

11. Burmańczuk, A., Tomasz, G., Gbylik-Sikorska, M., Gajda, A., & Kowalski, C. (2017). Withdrawal of amoxicillin and penicillin G procaine from milk after intramammary administration in dairy cows with mastitis. *Journal of Veterinary Research*, *61*(1), 37. https://doi.org/10.1515/jvetres-2017-0005

12. Cháfer-Pericás, C., Maquieira, Á., & Puchades, R. (2010). Fast screening methods to detect antibiotic residues in food samples. *TrAC Trends in Analytical Chemistry*, *29*(9), 1038-1049. https://doi.org/10.1016/j.trac.2010.06.004

13. Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M. and Hanage, W. P. (2015). Antibiotics in agriculture and the risk to human health: how worried should we be? *Evolutionary applications, 8*(3), 240-247. https://doi.org/10.1111/eva.12185

14. Chee-Sanford, J. C., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y. F., Yannarell, A. C., & Aminov, R. I. (2009). Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *Journal of environmental quality*, *38*(3), 1086-1108. https://doi.org/10.2134/jeq2008.0128

15. Cieśla, Ł., Skalicka-Woźniak, K., Hajnos, M., Hawrył, M., & Waksmundzka-Hajnos, M. (2009). Multidimensional TLC procedure for separation of complex natural mixtures spanning a wide polarity range; Application for fingerprint construction and for investigation of systematic relationships

within the Peucedanum genus. *Acta Chromatographica*, *21*(4), 641-657. https://doi.org/10.1556/achrom.21.2009.4.10
16. CLSI. M02-A11: Performance Standards for Antimicrobial Disk Susceptibility Tests, 11th ed.; Clincial and Laboratory Standards Institute: Wayne, PA, USA, 2012; Volume 32, ISBN 1562384856.
17. Conceição, S., Queiroga, M. C., & Laranjo, M. (2023). Antimicrobial resistance in Bacteria from meat and meat products: A one health perspective. *Microorganisms*, *11*(10), 2581. https://doi.org/10.3390/microorganisms11102581
18. El Atabani, A. I., El-Ghareeb, W. R., Elabbasy, M. T., & Ghazaly, E. I. (2014). Oxytetracycline residues in marketed frozen beef livers at Sharkia, Egypt. *Benha Veterinary Medical Journal*, *26*(1), 104-112.
19. Ferone, M., Gowen, A., Fanning, S., & Scannell, A. G. (2020). Microbial detection and identification methods: Bench top assays to omics approaches. Comprehensive Reviews in Food Science and Food Safety, 19(6), 3106-3129. https://doi.org/10.1111/1541-4337.12618
20. Fink, K., Cline, R. E., & Fink, R. M. (1963). Paper chromatography of several classes of compounds: correlated Rf values in a variety of solvent systems. *Analytical Chemistry*, *35*(3), 389-398. https://doi.org/10.1021/ac60196a036
21. Fleming, D. O. (2006). Prudent biosafety practices. *Biological Safety: Principles and Practices*, 361-371.
22. Garkavenko, T. O., Gorbatyuk, O. I., Dybkova, S. M., Kozytska, T. G., Andriiashchuk, V. O., Kukhtyn, M. D., & Horiuk, Y. V. (2021). Screening of Epidemiologically Significant Mechanisms of Antibiotics to β-Lactams in Enterobacteriaceae-Pathogens of Zoonoses. *Journal of Pure & Applied Microbiology*, *15*(3). https://doi.org/10.22207/JPAM.15.3.14
23. Ghimpețeanu, O., Tănase, G., Ștefan, C., Vasile, A., & Cătălina, R. (2022). A review of antibiotic residues in food of animal origin and their health implications. *Journal of Applied Animal Research*, 50(1), 384-394.
24. Gondová, Z., Kožárová, I., Poláková, Z., & Mad'arová, M. (2014). Comparison of four microbiological inhibition tests for the screening of antimicrobial residues in the tissues of food-producing animals. *Italian Journal of Animal Science*, *13*(4), 3521 https://doi.org/10.4081/ijas.2014.3521
25. Hayati, E. K., & Anggraini, M. P. (2023). Chemometric Analysis on Fingerprints of Acalypha Indica L Based on the Different Drying Methods. *Walisongo Journal of Chemistry*, *6*(2), 127-133.
26. Hirvonen, J., Pyörälä, S., Heinäsuo, A., & Jousimies-Somer, H. (1994). Penicillin G and penicillin G-tinidazole treatment of experimentally induced summer mastitis—effect on elimination rates of bacteria and outcome of the disease. *Veterinary microbiology*, *42*(4), 307-315. https://doi.org/10.1016/0378-1135(94)90062-0
27. Hou, J., Wan, W., Mao, D., Wang, C., Mu, Q., Qin, S., & Luo, Y. (2015). Occurrence and distribution of sulfonamides, tetracyclines, quinolones, macrolides, and nitrofurans in livestock manure and amended soils of Northern China. *Environmental Science and Pollution Research*, *22*(6), 4545-4554. https://doi.org/10.1007/s11356-014-3632-y
28. Hutchings, M. I., Truman, A. W., & Wilkinson, B. (2019). Antibiotics: past, present, and future. *Current opinion in microbiology*, *51*, 72-80. https://doi.org/10.1016/j.mib.2019.10.008
29. Islam, M. A., Sharma, A., Ahsan, S., Mazumdar, S., Rudra, K.andPhillips, C. J. (2020). Welfare assessment of dairy cows in small farms in Bangladesh. *Animals, 10*(3), 394. https://doi.org/10.3390/ani10030394
30. Jechalke, S., Heuer, H., Siemens, J., Amelung, W., & Smalla, K. (2014). Fate and effects of veterinary antibiotics in soil. *Trends in microbiology*, *22*(9), 536-545.
31. Jimenez, C. E. P., Keestra, S., Tandon, P., Cumming, O., Pickering, A. J., Moodley, A., & Chandler, C. I. (2023). Biosecurity and water, sanitation, and hygiene (WASH) interventions in animal agricultural settings for reducing infection burden, antibiotic use, and antibiotic resistance: a One Health systematic review. *The Lancet Planetary Health*, *7*(5), e418-e434. https://doi.org/10.1016/S2542-5196(23)00049-9
32. Kantiani, L., Farré, M., & Barcelo, D. (2009). Analytical methodologies for the detection of

β-lactam antibiotics in milk and feed samples. *TrAC Trends in Analytical Chemistry*, *28*(6), 729-744. https://doi.org/10.1016/j.trac.2009.04.005

33. Karande, V. K., Jagtap, R., Kalita, D. J., & Ghumare, B. C. (2021). Determination of streptococcal penicillin residues in cow milk after its intramuscular administration. *J Endocrinol Zool Stud*, *9*(2), 151-156.
34. Kaya, S. E., & Filazi, A. (2010). Determination of antibiotic residues in milk samples. *Kafkas Univ Vet Fak Derg*, *16*(Suppl-A), S31-S35. https://DOI:10.9775/kvfd.2009.1174
35. Kistanova, E., Yotov, S., & Zaimova, D. (2024). Intelligent Animal Husbandry: Present and Future. *Animals*, *14*(11), 1645. https://doi.org/10.3390/ani14111645
36. Kumar, S., Jyotirmayee, K., & Sarangi, M. (2013). Thin-layer chromatography: a tool of biotechnology for isolation of bioactive compounds from medicinal plants. *International Journal of Pharmaceutical Sciences Review and Research*, *18*(1), 126-132.
37. Kumarswamy, N. P., Latha, C., Vrinda, K. M., Sethukekshmi, C., & Mercy, K. A. (2018). Detection of antibiotic residues in raw cow milk in Thrissur, India. *Pharma Innov J*, *7*(8), 452-4.
38. Layada, S., Benouareth, D. E., Coucke, W., & Andjelkovic, M. (2016). Assessment of antibiotic residues in commercial and farm milk collected in the region of Guelma (Algeria). International Journal of Food Contamination, 3(1), 19. https://DOI 10.1186/s40550-016-0042-6
39. Lee, J. B., Chung, H. H., Chung, Y. H., & Lee, K. G. (2007). Development of an analytical protocol for detecting antibiotic residues in various foods. *Food Chemistry*, *105*(4), 1726-1731. https://doi.org/10.1016/j.foodchem.2007.05.030
40. Ma, F., Xu, S., Tang, Z., Li, Z., & Zhang, L. (2021). Use of antimicrobials in food animals and impact of transmission of antimicrobial resistance on humans. *Biosafety and Health*, *3*(1), 32-38. https://doi.org/10.1016/j.bsheal.2020.09.004
41. Makkar, H. P. (2016). Smart livestock feeding strategies for harvesting triple gain–the desired outcomes in planet, people and profit dimensions: a developing country perspective. *Animal Production Science*, *56*(3), 519-534. https://doi.org/10.1071/AN15557
42. Malgwi, K. D., Umaru, B., Chabri, S. A., Daniel, N., Sanya, L., Maina, U. A., & Saka, S. (2023). Assessment of Oxytetracycline and Penicillin G Residues Levels in Raw and Fermented Milk in Maiduguri, Northeastern Nigeria. *Saudi J Med Pharm Sci*, *9*(3), 140-149. https://doi: 10.36348/sjmps.2023.v09i03.001
43. Marshall, B. M., & Levy, S. B. (2011). Food animals and antimicrobials: impacts on human health. *Clinical microbiology reviews*, *24*(4), 718-733. https://doi.org/10.1128/cmr.00002-11
44. Moghadam, M. M., Amiri, M., Riabi, H. R. A., & Riabi, H. R. A. (2016). Evaluation of antibiotic residues in pasteurized and raw milk distributed in the South of Khorasan-e Razavi Province, Iran. *Journal of Clinical and Diagnostic Research: JCDR*, *10*(12), FC31. https://doi.org/10.7860/JCDR/2016/21034.9034
45. Mohamed, M. A., Elmi, A. A. S., Dubad, A. B., Hassan, Y. H. S., Osman, A. M., & Bitrus, A. A. (2020). Antibiotic residue in raw milk collected from dairy farms and markets in Benadir, Somalia. *PAMJ-One Health*, *2*(19).
46. Navrátilová, P., Vorlová, L., Dluhošová, S., Bartáková, K., Hanuš, O., & Samková, E. (2024). Screening Methods for Antimicrobial Residues in the Dairy Chain—The Past and the Present. *Antibiotics*, *13*(11), 1098. https://doi.org/10.3390/antibiotics13111098
47. Norris, J. R., & Wolf, J. (1961). A study of antigens of the aerobic sporeforming bacteria. *Journal of Applied Bacteriology*, *24*(1), 42-56.
48. Okonko, I. O., Fajobi, E. A., Ogunnusi, T. A., Ogunjobi, A. A., & Obiogbolu, C. H. (2009). Antimicrobial chemotherapy and sustainable development: the past, the current trend, and the future.
49. Olatoye, I. O., Daniel, O. F., & Ishola, S. A. (2016). Screening of antibiotics and chemical analysis of penicillin residue in fresh milk and traditional dairy products in Oyo state, Nigeria. *Veterinary world*, *9*(9), 948. https://doi.org/10.14202/vetworld.2016.948-954
50. Ongom, J. (2023). Improving Animal Health and Production through Epidemiological Studies and Technological Integration. *International Journal of Scientific*

and Management Research, *6*(07), 279-293. http://doi.org/10.37502/IJSMR.2023.6718

51. Orwa, J. D., Matofari, J. W., Muliro, P. S., & Lamuka, P. (2017). Assessment of sulphonamides and tetracyclines antibiotic residue contaminants in rural and peri urban dairy value chains in Kenya. *International Journal of Food Contamination*, *4*(1), 5. https://DOI 10.1186/s40550-017-0050-1
52. Paramitadevi, Y. V., Priadi, C. R., Rahmatika, I., Rukmana, A., & Moersidik, S. S. (2023). Integration of water, sanitation, and hygiene program with biosecurity: A one health approach to reduce the prevalence and exposure of antibiotic-resistant bacteria in the livestock community. *Int J One Health*, *9*, 181-193. www.doi.org/10.14202/IJOH.2023.181-193
53. Piech, T., Majer-Dziedzic, B., Kostruba, A., Grzelak, E. M., & Choma, I. M. (2016). Thin-layer chromatography—Direct bioautography as an alternative method for screening of antibiotic residues in milk: A comparative study. *Journal of Liquid Chromatography & Related Technologies*, *39*(5-6), 292-297. https://doi.org/10.1080/10826076.2016.1163468
54. Plumb, D. C. (2018). *Plumb's veterinary drug handbook: Desk*: John Wiley & Sons.
55. Prescott, J. F., & Hardefeldt, L. Y. (2024). Other Beta-lactam Antibiotics: Beta-lactamase Inhibitors, Carbapenems, and Monobactams. *Antimicrobial therapy in veterinary medicine*, 169-186. https://doi.org/10.1002/9781119654629.ch9
56. Qian, X., Gu, J., Sun, W., Wang, X. J., Su, J. Q., & Stedfeld, R. (2018). Diversity, abundance, and persistence of antibiotic resistance genes in various types of animal manure following industrial composting. *Journal of Hazardous materials*, *344*, 716-722. https://doi.org/10.1016/j.jhazmat.2017.11.020
57. Rahman, M. S., Hassan, M. M., & Chowdhury, S. (2021). Determination of antibiotic residues in milk and assessment of human health risk in Bangladesh. *Heliyon*, *7*(8). https://doi.org/10.1016/j.heliyon.2021.e07739
58. Renault, V., Humblet, M. F., & Saegerman, C. (2021). Biosecurity concept: Origins, evolution, and perspectives. *Animals*, *12*(1), 63. https://doi.org/10.3390/ani12010063
59. Sachi, S., Ferdous, J., Sikder, M. H., & Hussani, S. A. K. (2019). Antibiotic residues in milk: Past, present, and future. *Journal of advanced veterinary and animal research*, *6*(3), 315. https://doi.org/10.5455/javar.2019.f350
60. Salim, H. M., Huque, K. S., Kamaruddin, K. M., & Haque Beg, A. (2018). Global restriction of using antibiotic growth promoters and alternative strategies in poultry production. *Science progress*, *101*(1),52-75. https://doi.org/10.3184/003685018X15173975498947
61. Sharma, S., Chauhan, A., Ranjan, A., Srivastav, R., Chauhan, R., Singh, V. N., & Jindal, T. (2025). Antibiotic Resistance Patterns in Cold-Adapted Bacillus subtilis From the Southern Ocean and Comparative Genomic Insights. *Frontiers in Bioscience-Elite*, *17*(2), 38809. http://doi.org/10.31083/FBE38809
62. Skorupa, A., & Gierak, A. (2011). Detection and visualization methods used in thin-layer chromatography. *JPC-Journal of Planar Chromatography-Modern TLC*, *24*(4), 274-280. https://doi.org/10.1556/jpc.24.2011.4.1
63. Stella, O. I. O., Ezenduka, E. V., & Anaelom, N. J. (2020). Screening for tylosin and other antimicrobial residues in fresh and fermented (nono) cow milk in Delta state, South-South, Nigeria. *Veterinary World*, *13*(3), 458. https://doi.org/10.14202/vetworld.2020.458-464
64. Subasinghe, R., Alday-Sanz, V., Bondad-Reantaso, M. G., Jie, H., Shinn, A. P., & Sorgeloos, P. (2023). Biosecurity: Reducing the burden of disease. *Journal of the World Aquaculture Society*, *54*(2), 397-426. https://doi.org/10.1111/jwas.12966
65. Tello, A., Austin, B., & Telfer, T. C. (2012). Selective pressure of antibiotic pollution on bacteria of importance to public health. *Environmental health perspectives*, *120*(8), 1100-1106. https://doi.org/10.1289/ehp.1104650
66. Titouche, Y., Akkou, M., & Houali, K. (2022). Detection of antimicrobial residues in bovine meat marketed at Tizi-Ouzou area (Algeria), by a microbial screening assay. *Journal of the Hellenic Veterinary Medical Society*, *73*(2),

Article 2. https://doi.org/10.12681/jhvms.26396

67. Titouche, Y., Hakem, A., Houali, K., Yabrir, B., Malki, O., Chergui, A., ... & Fiţ, N. I. (2013). Detection of antibiotics residues in raw milk produced in Freha area (Tizi-Ouzou), Algeria.
68. Trufanov, O., Kotyk, A., Trufanova, V., Tereshchenko, O., & Zhukorskiy, O. (2015). Detection of antibiotics, active against Bacillus subtilis, in grain and feed. *Agricultural Science and Practice*, *2*(1), 60-66. https://doi.org/10.15407/agrisp2.01.060
69. Tyczkowska, K., Voyksner, R. D., & Aroson, A. L. (1989). Development of an analytical method for penicilling in bovine milk by liquid chromatography with ultraviolet-visible detection and confirmation by mass spectrometric detection. *Journal of Chromatography B: Biomedical Sciences and Applications*, *490*, 101-113. https://doi.org/10.1016/S0378-4347(00)82765-6
70. Vehapi, M., İnan, B., Kayacan-Cakmakoglu, S., Sagdic, O., & Özçimen, D. (2023). Production of Bacillus subtilis soil isolate as biocontrol agent under bioreactor conditions. *Archives of Microbiology*, *205*(1), 52. https://doi.org/10.1007/s00203-022-03381-z
71. Virto, M., Santamarina-García, G., Amores, G., & Hernández, I. (2022). Antibiotics in dairy production: Where is the problem?. *Dairy*, *3*(3), 541-564. https://doi.org/10.3390/dairy3030039
72. Wang, Y., Lu, R., Wang, W., Wang, Q., Hu, X., & Liu, Q. (2022). A review of detection of antibiotic residues in food by surface-enhanced Raman spectroscopy. *Molecules*, 27(22), 7794. https://doi.org/10.1155/2021/8180154
73. Zavala Nacul, H., & Revoredo-Giha, C. (2022). Food safety and the informal milk supply chain in Kenya. *Agriculture & Food Security*, *11*(1), 8. https://doi.org/10.1186/s40066-021-00349-y
74. Zhang, Y., Lu, J., Yan, Y., Liu, J., & Wang, M. (2021). Antibiotic residues in cattle and sheep meat and human exposure assessment in southern Xinjiang, China. *Food Science & Nutrition*, *9*(11), 6152-6161. https://doi.org/10.1002/fsn3.2568

Table 1. *Antibiotic sensitivity test for B. subtilis HS*

Test group	Disc code	concentration	Inhibition zone (mm)	Zone interpretation
Aminoglycosides				
Kanamycin	K	30 µg	32	S
Neomycin	N	30 µg	26	S
Streptomycin	S	10 µg	24	S
β- lactam				
Ampicillin	AM	10 µg	24	S
Amoxicillin	AmC	30 µg	29	S
Penicillin G	P	10 µg	25	S
Fluroquinolones				
Ciprofloxacin	CIP	5 µg	27	S
Phenicol				
Chloramphenicol	C	30 µg	27	S
Tetracycline				
Tetracycline	Te	30 µg	18	S

Table 2. Confidence Interval (CI 95%) and antibiotic residue ocurance for pasteurized and raw milk samples using the Agar well diffusion method depending on the values of the inhibiotion zone (mm)

Parameters	Pasteurized milk	Inhibition zone (mm) as positive results out of 13	Raw milk	Inhibition zone (mm) as positive results out of 32	Total
	P3	5	C1	14	
	P6	5	C3	10	
	P7	4	C8	16	
	P11	6	C12	24	
			C17	10	
			C18	15	
			C20	13	
			C22	12	
			C26	17	
			C29	13	
			C30	12	
			C34	8	
No. of positive samples		4		12	16
95% CI		3.70%-6.30%		11.05%-16.29%	14.75%-22.59%
Antibiotic residue Occurance		30.7%		37.5%	35.5%

Table 3. *The minimum inhibition zone diameter for antibiotic residues in milk was detected using Agar well diffusion method at concentrations (25, 10, 5, 2 ,1 µg/mL) for Penicillin G and Streptomycin. (+), presence of inhibition zone and (-), no inhibition zone.*

Concentration (µg/mL)	penicillin G	streptomycin
25	+	+
10	+	+
5	-	+
2	-	-
1	-	-

Table 4. *Retardation factor (Rf) of pasteurized milk and raw milk samples compared to standard antibiotics, penicillin G and streptomycin, measured by TLC.*

Milk sample	Rf identical with penicillin G[a]	Rf identical with streptomycin[b]	Not identical
P3	-	-	0.29
P6	-	-	0.71
P7	-	-	0.29
P11	0.85	-	-
C1	0.85	0.51	-
C3	0.85	-	-
C8	0.85	-	-
C12	0.85	-	-
C17	0.85	-	-
C18	0.85	-	-
C20	0.85	0.51	-
C22	0.85	-	-
C26	0.85	-	-
C29	-	0.51	-
C30	0.85	-	-
C34	-	0.51	-

a = Retardation factor of standard penicillin G was 0.85
b = Retardation factor of standard streptomycin was 0.51

Table 5. *The Precision test (RSD%) for (n=4) depending on Retardation Factor (Rf) of spiked milk samples (pasteurized and raw milk) for penicillin G and streptomycin.*

Antibiotic	Mean RF	SD	RSD (%)
Penicillin G	0.85	0.015	1.7%
Streptomycin	0.51	0.012	2.3%

Table 6. *Quality control for the TLC test, including LOD, LOQ, and Recovery (%).*

Antibiotic	LOD	LOQ	Recovery (%)
Penicillin G	0.5 µg/ml	1.5 µg/ml	82-87%
Streptomycin	1 µg/ml	3 µg/ml	85-89%

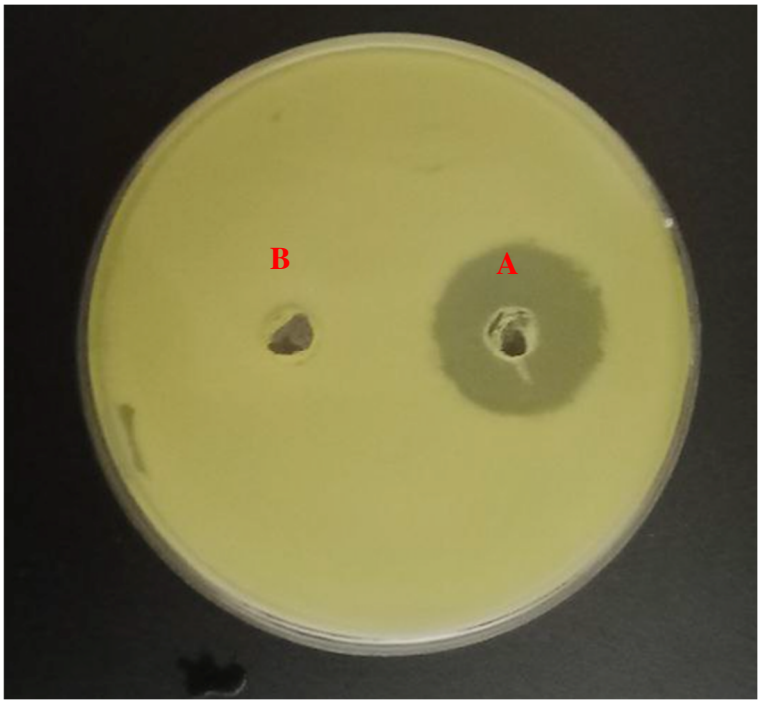

Figure 1. *The inhibition zone (mm) of a milk containing an antibiotic, a positive result (A) and milk without antibiotic, a negative result (B) using the Agar well diffusion method.*

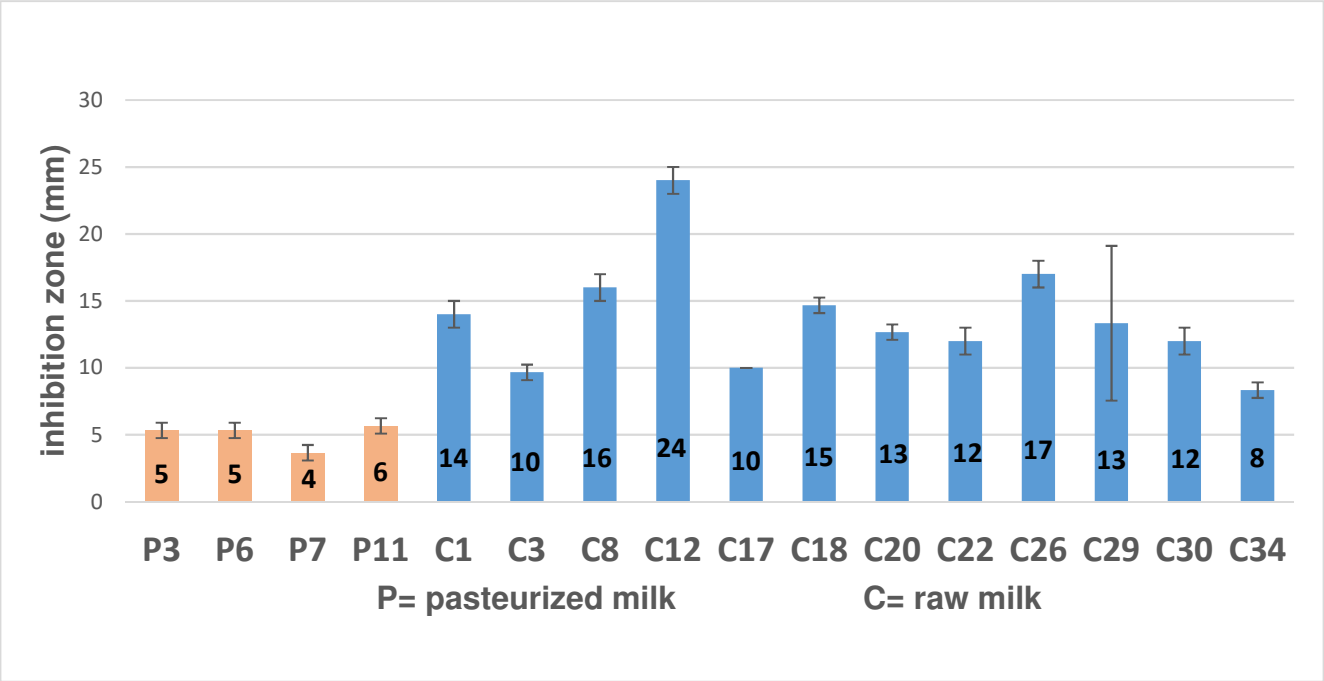

Figure 2. *The dimension of the inhibition zone (mm) of pasteurized milk and raw milk using the Agar well diffusion method. P: Pasteurized milk and C: Cow milk*

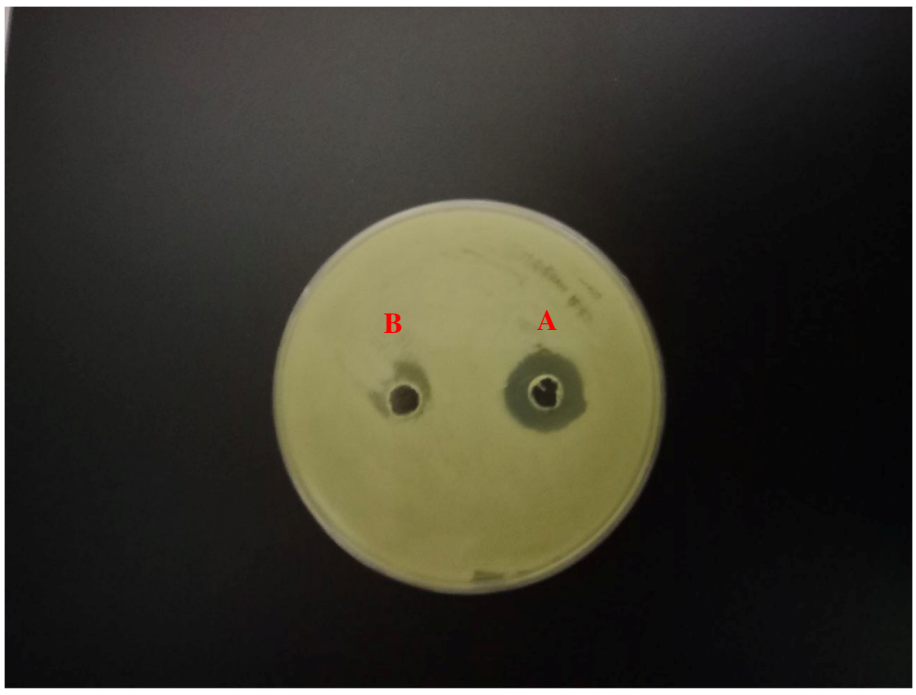

Figure 3. *The minimum inhibition zone diameter for antibiotic residues in milk. (A), penicillin G at concentration 10 µg/mL and streptomycin at 5µg/mL using Agar well diffusion method.*

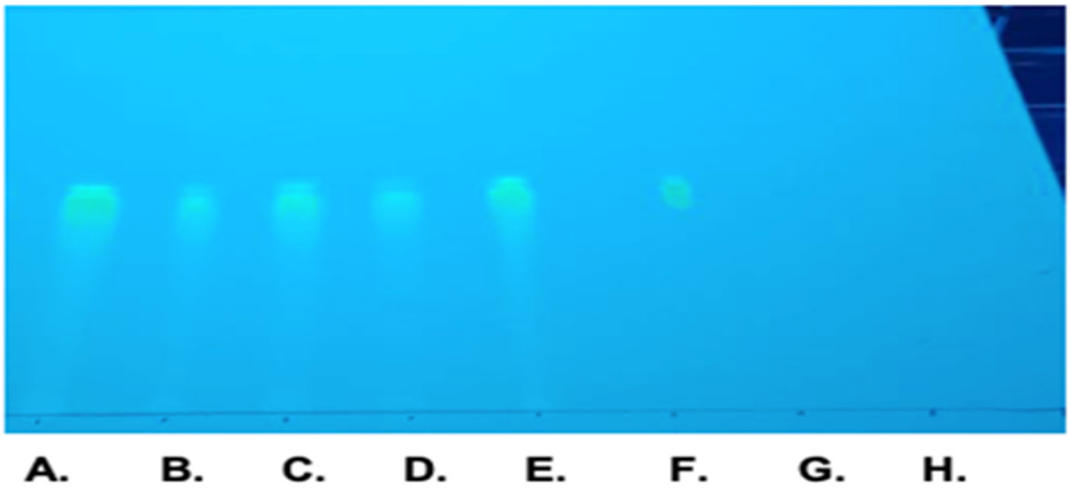

Figure 4. *TLC analysis of penicillin G antibiotic standard at different concentration. Spots corresponding to the following concentration: (A) 100 µg/mL, (B) 50 µg/mL, (C) 25 µg/mL, (D) 10 µg/mL, (E) 5 µg/mL, (F) 1 µg/mL, (G) 0.5 µg/mL and (H) milk-free antibiotic extract used as a negative control.*

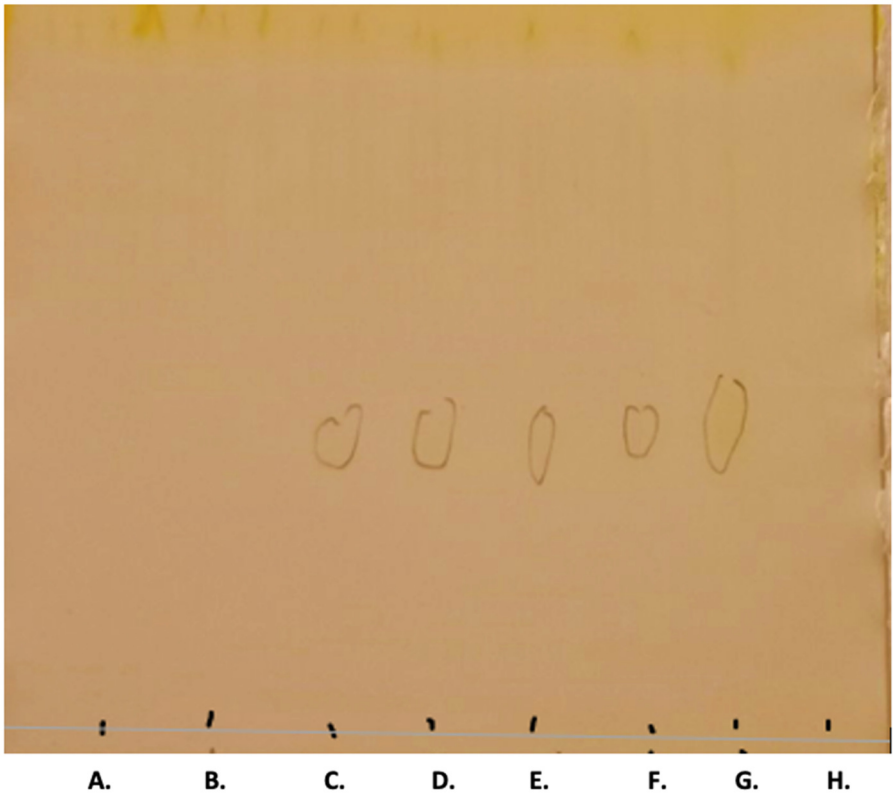

Figure 5. *TLC analysis of streptomycin antibiotic standard at different concentration. Spots corresponding to the following concentration: (A) milk-free antibiotic extract used as a negative control, (B) 100 µg/mL, (C) 50 µg/mL, (D) 25 µg/mL, (E) 10 µg/mL, (F) 5 µg/mL, (G) 1 µg/mL and (H) 0. µg/mL.*