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RESEARCH ARTICLE

Enhancing Dairy Safety: A User-Friendly Rapid Kit for Sensitive Detection of Antibiotic Residues in Milk

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Abstract

Milk is a nutrient-dense food that provides essential nutrients to support growth, development, and overall health. Currently, over 80% of food animals receive antibiotics as therapeutics, feed additives, and growth promoters. Significant amounts of antibiotics and their metabolites in milk pose public health risks. Antibiotic residues can be detected using different techniques. Detection of antibiotic residues is limited by complex pretreatment, high cost, time-consuming procedures, and required expertise. To overcome these limitations, the current research was conducted to establish a sensitive and time-saving detection kit containing *Bacillus subtilis* as an indicator to detect antibiotic residues in milk. The kit was validated using 14 antibiotic-spiked samples by measuring the limit of detection, specificity, and sensitivity. The kit successfully detected the antibiotic residues of ampicillin, oxytetracycline, streptomycin, spectinomycin, sulphadimidine, sulphadiazine, tylosin, lincomycin, enrofloxacin, ciprofloxacin, trimethoprim, spiramycin, chloramphenicol, and florfenicol. The limit of detection of the kit was lower than the maximum residue limit (MRL) for various antibiotics, including oxytetracycline, sulphadiazine, tylosin, enrofloxacin, ciprofloxacin, and trimethoprim. High specificity and sensitivity of 98.2% and 99.2%, respectively, were obtained. The kit showed accurate results for most antibiotics over 4 months, indicating good shelf life and suitability for regular use. The developed kit offers a rapid, cost-effective, and reliable method for detecting antibiotic residues in milk, ensuring routine monitoring and safeguarding public health.

Keywords: Antibiotic residues, *Bacillus subtilis*, public health, food safety, milk

INTRODUCTION

Antimicrobials are widely used in dairy animals as a primary means of controlling infectious diseases. They play a crucial role in treating and preventing mastitis, respiratory tract infections, and reproductive tract infections, which are common health challenges in dairy herds [1-3]. The use of antimicrobials helps in maintaining animal health, improve milk production, and reduce economic losses [4-6]. Improper or prolonged antibiotic use leaves residues in milk and dairy products, posing public health challenges, including allergies, disturbance of gut flora, and promotion of antibiotic resistance [7,8]. Furthermore, antibiotic residues also cause significant financial losses in the dairy industry by disrupting fermentation processes [9]. The presence of bacterial pathogens and antimicrobial residue in milk, poultry or other animal products poses a serious concern for public health, especially with reference to emergence of antimicrobial resistance [10-12]. The regulatory organizations such as Codex Alimentarius and European Union have set specific MRLs for each antibiotic in milk, to mitigate the public health concerns [13]. Veterinary drug approval requires the establishment of withdrawal periods (WPs) based on residue data. WPs define the minimum time after the last dose during which animals cannot be slaughtered, and milk or eggs cannot be consumed, ensuring that drug residues fall below MRLs and remain safe for human health [14].

Advanced physicochemical methods like mass spectrometry (MS) and high-performance liquid chromatography (HPLC) can detect antibiotic residues, but these methods are expensive, require expertise, and involve complex preparations [15]. Immunological methods such as ELISA allow rapid and specific detection but are limited to certain antibiotic classes [16]. Microbiological inhibitory approaches, conversely, provide a low-cost, broad-spectrum, and user-friendly solutions for identifying antibiotic residues in milk, but these methods face challenges such as milk matrix effects causing false positive results and labor-intensive procedures [17].

To address these limitations, a cost-effective and stable kit was developed using a 96-well microtiter plate containing minimal agar medium and a highly sensitive *Bacillus subtilis* spore suspension. This approach reduces the need for fresh bacterial cultures, as spores remain dormant under harsh conditions. It also permits processing of multiple samples, with results obtained within 6-7 h, and the kit remains stable for several months.

Material and Methods

Bioethical Statement

This research was conducted following approval from the Institutional Biosafety/Bioethics Committee (IBC) of the University of Agriculture, Faisalabad, Pakistan (D. No. 3275/ORIC, Dated: 24/05/2024).

Isolation and Identification of *B. subtilis*

A total of five soil samples were collected from agricultural fields of Faisalabad to isolate *B. subtilis*. Twenty-gram soil samples were collected 5 cm from the surface and placed in a sterile plastic container and sealed. The soil samples were cultured on LB agar using ten-fold serial dilution. Based on colony morphology, distinct bacterial colonies were isolated and sub-cultured repeatedly on nutrient agar to obtain pure cultures [18]. The purified colonies were identified using Gram staining, spore staining [19], and several biochemical tests according to Bergey's Manual and confirmed with molecular characterization by PCR and sequencing [20].

Molecular Characterization of *B. subtilis*

DNA Extraction and Polymerase Chain Reaction: The GeneJET PCR Purification Kit® (Thermo Scientific) was used for DNA extraction of pure culture. To differentiate *B. subtilis* from other *Bacillus* species, species-specific oligonucleotide forward and reverse primers for the pyruvate carboxylase *(pyrA)* and shikimate dehydrogenase *(aroE)* genes were used for PCR DNA amplification [21]. After PCR amplification, PCR products were subjected to gel electrophoresis on a 1.5% agarose gel [21]. Detection of the *aroE* (278bp) gene and *pycA* (233bp) confirmed the isolation of *B. subtilis*.

Sequence Analysis and Phylogenetic Relationship: The Gene JET Gel Extraction Kit® (Thermo Scientific) was used for the excision of DNA from agarose gels. The retrieved DNA was sequenced by the ABI PRISM® 3100 Genetic Analyzer at Macrogen Sequencing Facilities (Macrogen, Korea). The nucleotide sequences of isolates were submitted to GenBank NCBI for accession numbers. Phylogenetic tree analysis was conducted for sequenced *aroE* and *pycA* genes using MEGA 11[22].

Antibiotic Susceptibility Testing: The disc diffusion method was employed using Mueller-Hinton agar (MHA) plates and bacterial suspensions adjusted to 0.5 McFarland turbidity. Bacterial culture was swabbed onto MHA plates, followed by placement of antimicrobial discs. The plates were incubated at 37°C for 24 h [23]. The results of AST were based on literature confirmation and microbiology manuals. The bacterial isolates were classified as sensitive, intermediate, or resistant based on zone of inhibition *(Table 1)*, following CLSI guidelines [24].

Spore Suspension Preparation: Of the two isolated strains, the more sensitive *B. subtilis* strain was swabbed onto nutrient agar and incubated at 30°C for 10 days to induce spore formation. Spore production was confirmed by spore staining [19,25]. The spore suspension was subjected to viable count using serial dilution, and spore concentration was adjusted to 4.096 x 10^9 CFU/mL [26].

Preparation of the Kit Medium: The kit medium was prepared by dissolving 28 g of nutrient agar, 0.25 g K_2HPO_4, 5.25 g glucose, 0.006 g $MgSO_4$, 7 g carboxymethylcellulose sodium, 0.003 g $MnSO_4 \cdot H_2O$, 0.5 g NaCl, and 0.004 g $CaCl_2$ in 1 L dH_2O [27,28]. After autoclaving, the pH was adjusted to 6.6-7.0, and bromocresol purple (0.1 mg/L), along with *B. subtilis* spore suspension, was added. The mixture was vortexed, and 150 µL was dispensed into each well of microtiter plate, which was then sealed with a foil-wrapped lid and stored at 4°C until use [27].

Preparation of the Stock Solution and the Working Solution of Antibiotics: Fourteen pure antibiotics were used in powdered form: ampicillin, oxytetracycline, streptomycin, spectinomycin, sulphadimidine, sulphadiazine, tylosin, lincomycin, enrofloxacin, ciprofloxacin, trimethoprim, spiramycin, chloramphenicol, and florfenicol. Stock solutions (1 mg/mL) and working solutions (1 µg/mL) were prepared and stored at -20°C until use.

Blank and Spiked Milk Samples: Cow milk samples were collected from animals that had not received antibiotics during the previous 30 days. Working solutions of individual antibiotics were added to antibiotic-free milk to prepare spiked milk samples [29].

Microbiological Inhibition Test: Milk samples (blank and spiked) were first incubated at 80°C for 10 min in water bath. The treated milk samples were then added to the microtitration plate containing prepared culture media. Results were interpreted based on color change (purple or yellow). The experiment was repeated four times with each concentration tested in triplicate to ensure accuracy and consistency [27].

Validation Protocols

Limit of Detection: The ISO13969: 2003 criteria were followed in the establishment of the dose-response curves

for these drugs (ISO, 2003). Eight distinct concentrations of each drug were tested over different days, with 20 replicates of each concentration being examined. The lowest concentration that yielded 95% positive results was determined as the limit of detection (LOD) [30].

Specificity and Sensitivity: A total of 168 blank milk samples were tested with the kit to determine the false-positive rate. To assess the false-negative rate, 100 blank samples spiked at the LOD and MRL for each antibiotic were analyzed, as a method is considered reliable when false negatives remain ≤5% at both levels [31]. The specificity, sensitivity, positive predictive value and negative predictive value were calculated using standard formulae [32,33].

Stability: The stability of the kit was evaluated over four months (0, 30, 60, 90, and 120 days) at 4°C by monitoring appearance, odor, performance, incubation time, and detection capacity. Each month, the kit was tested with antibiotics at, above, and below their detection concentrations [29].

Statistical Analysis

Logistic regression was applied to assess the impact of antibiotic concentration on detection likelihood, with model fit evaluated by Hosmer-Lemeshow, Pearson, and Deviance tests. ANOVA was conducted using SPSS version 26 (Armonk, USA), and graphs were generated with SigmaPlot version 14.0 [34].

Results

Identification, Molecular Characterization and Phylogenetic Analysis

Initial soil samples yielded seven distinct bacterial colonies: five were Gram-positive rods and two were cocci. Spore staining revealed that all bacilli were spore-formers. Colony characteristics included circular, rough, opaque, fuzzy, white, or slightly yellow colored with jagged edges. After biochemical confirmation, PCR was performed on five bacterial cultures, of which four showed positive amplification with distinct bands at 233 bp *(pycA)* and 278 bp *(aroE)* to a 100 bp DNA ladder *(Fig. 1)*. Two strains (NF-S1, NF-S2) were further tested by AST, and the more sensitive strain (NF-S2) was sequenced. The verified sequences were submitted to GenBank (accession Nos. PP820929 and PP898198), and phylogenetic analysis confirmed the isolates as *B. subtilis (Fig. 2)*.

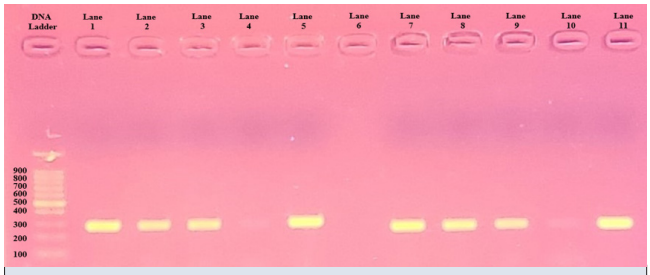

Fig 1. Species-specific PCR genes *aro*E and *pyc*A of *B. subtilis*. Lane 0 size marker (100 bp DNA ladder); lanes 1-5 *aroE* (278bp) gene fragments for *B. subtilis*, Lane 6 negative control, lanes 7-11 *pycA* (233bp) gene fragments

Antibiotic Susceptibility Testing

The susceptibility of two bacterial strains to 48 antibiotics revealed that all antibiotics posed a significant response against both strains (P<0.05). NF-S1 strain showed resistance to 24, intermediate resistance to 6, and sensitivity to 18 antibiotics. In contrast, the NF-S2 strain was resistant to only 14 antibiotics, with intermediate resistance to 3 and sensitivity to 31 *(Table 1; Fig. 3)*. This indicated that the NF-S2 strain was more sensitive than the NF-S1 strain. Therefore, NF-S2 strain was selected for sequencing and kit preparation. The number of sensitive antibiotics against the two strains was compared using Chi-square, suggesting that there was a non-significant relationship between the two strains (P>0.05). Therefore, there was a significant difference between the strains, the NF-S2 strain presented more sensitivity than the NF-S1 strain *(Table 1)*.

Microbiological Inhibition Test

Color changes in the kit were observed till 7 h: yellow indicated negative results (no antibiotic residues), while the purple indicated positive results (antibiotic residues present) *(Fig. 4)*. *Table 2* shows the mean limit of detection for the kit at different concentrations of each antibiotic using microbiological inhibition tests conducted four times. *Fig. 5* represents the mean LOD of all batches compared the MRL for each antibiotic. The concentrations determined in batches 3 and 4 were considered the true limit of detection of the kit, at which the results were stable and consistent *(Fig. 6)*. The results revealed that kit was highly sensitive for oxytetracycline (25 µg/L),

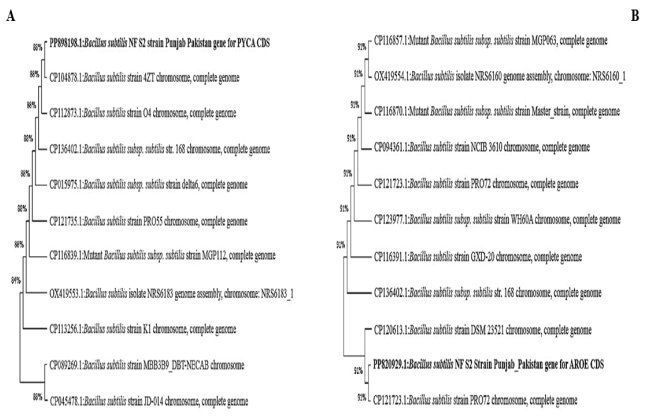

Fig 2. Phylogenetic tree of *Bacillus subtilis*. A- Phylogenetic tree of the *Bacillus subtilis* targeting species-specific gene *pycA*, pyruvate carboxylase, with 1000 bootstraps. B- Phylogenetic tree of the *Bacillus subtilis* targeting species-specific gene *aroE* shikimate dehydrogenase, with 1000 bootstraps

Table 1. *Mean zones of inhibition of antibiotics against NF-S1 and NF-S2 B. subtilis isolates*

Sr. No.	Antibiotic Discs	Zone of Inhibition (mm)			
		NF-S1	S, I, R	NF-S2	S, I, R,
1	Penicillin G	0	R	0	R
2	Ampicillin	8	R	29	S
3	Amoxicillin	12	R	25	S
4	Carbenicillin	0	R	13	R
5	Piperacillin	15	I	28	S
6	Piperacillin Tazobactam	14	R	14	R
7	Methicillin	0	R	0	R
8	Oxacillin	0	R	11	I
9	Tetracycline	15	I	22	S
10	Oxytetracycline	13	R	27	S
11	Tigecycline	9	R	19	S
12	Doxycycline	12	R	19	S
13	Gentamycin	18	S	19	S
14	Streptomycin	16	S	25	S
15	Lincomycin	25	S	18	S
16	Enrofloxacin	26	S	46	S
17	Amikacin	16	I	20	S
18	Norfloxacin	18	S	28	S
19	Bacitracin	9	I	0	R
20	Ciprofloxacin	24	S	40	S
21	Erythromycin	22	S	40	S
22	Vancomycin	10	I	15	S
23	Tobramycin	8	R	11	R
24	Azithromycin	30	S	34	S
25	Rifampicin	20	R	23	I
26	Clindamycin	28	S	22	S
27	Tylosin	32	S	38	S
28	Flumequin	20	I	38	S
29	Ceftriaxone	0	R	0	R
30	Meropenem	0	R	0	R
31	Imipenem	33	S	42	S
32	Entrapenem	8	R	0	R
33	Ceftazidine	0	R	0	R
34	Cephalexin	9	R	21	S
35	Trimethoprim	0	R	40	S
36	Sulphamethoxazole	30	S	32	S
37	Polymyxin B	8	R	8	R
38	Cefoxitin	0	R	0	R
39	Nitrofuratoin	20	S	30	S
40	Linezolid	26	S	40	S
41	Cefepime	0	R	0	R
42	Ceftiofur	0	R	0	R
43	Chloramphenicol	22	S	25	S
44	Florfenicol	34	S	38	S
45	Spectinomycin	18	S	40	S
46	Spiramycin	33	S	44	S
47	Sulphadiazine	12	R	22	S
48	Sulphdimidine	12	R	13	I

I = Intermediate, R = Resistant, S = Sensitive, 0 = No Zone of Inhibition

Fig 3. Antibiotic sensitivity of NF-S2 strains against various antibiotics. 1. enrofloxacin, 2. erythromycin, 3. linezolid, 4. azithromycin, 5. flumequine, 6. chloramphenicol, and 7. Imipenem

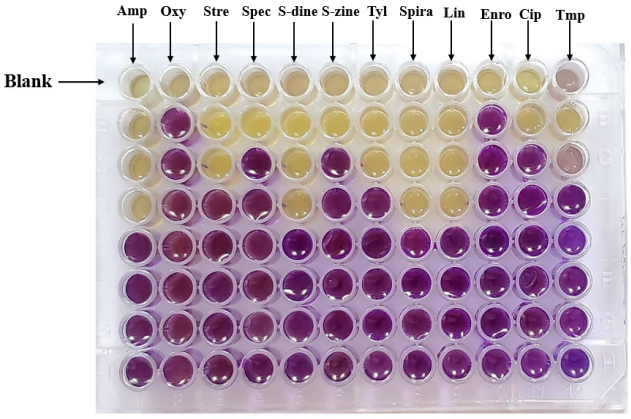

Fig 4. Results of batch 4 LOD concentrations to detect antibiotic residues. The first row A shows the blank samples, all the column shows the concentrations at which antibiotic residues were detected ampicillin (4 µg/L), oxytetracycline (25 µg/L), streptomycin (300 µg/L), spectinomycin (100 µg/L), sulphadimidine (100 µg/L), sulphadiazine (50 µg/L), tylosin (30 µg/L), spiramycin (250 µg/L), lincomycin (150 µg/L) enrofloxacin (50 µg/L), ciprofloxacin (25 µg/L), trimethoprim (30 µg/L)

Table 2. Estimation of the limit of Detection of the kit

Antibiotics	MRL EU (µg/L)	Mean of LOD of Batch 1 (µg/L)	Mean of LOD of Batch 2 (µg/L)	Mean of LOD of Batch 3 (µg/L)	Mean of LOD of Batch 4 (µg/L)
Ampicillin	4	10	6	4	4
Oxytetracycline	100	25	25	25	25
Streptomycin	200	700	300	300	300
Spectinomycin	100	50	150	100	100
Sulphadimidine	100	100	200	100	100
Sulphadiazine	100	80	20	50	50
Tylosin	50	20	40	30	30
Spiramycin	200	100	200	250	250
Lincomycin	150	200	100	150	150
Enrofloxacin	100	50	50	50	50
Ciprofloxacin	100	30	20	25	25
Trimethoprim	50	30	30	30	30
Chloramphenicol	N.A	0.6	0.6	0.6	0.6
Florfenicol	N.A	0.5	0.5	0.5	0.5

N.A.= Not Available

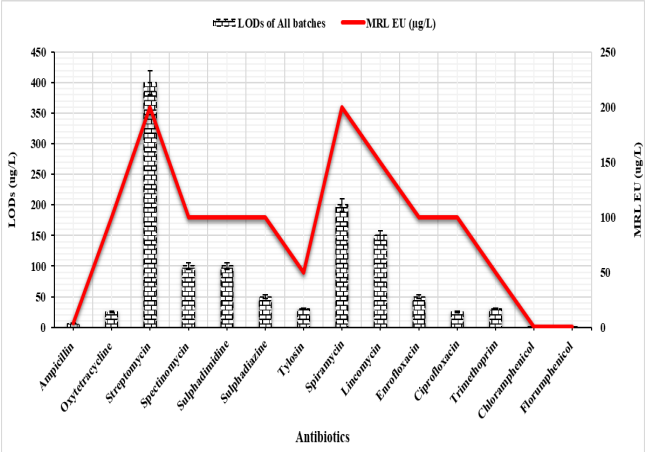

Fig 5. The overall mean of the limit of detection of 4 batches for antibiotic residues. The red line indicates the maximum residue limit of the European Union (EU) for the respective antibiotic residue's mean concentrations

sulphadiazine (50 µg/L), tylosin (30 µg/L), enrofloxacin (50 µg/L), ciprofloxacin (25 µg/L), and trimethoprim (30 µg/L). Except for streptomycin (300 µg/L), spiramycin (250 µg/L), chloramphenicol (0.6 µg/L) and florfenicol (0.5 µg/L), which showed that concentrations must be present at least 1-3 times higher than MRLs to detect the positive results through this bioassay. Furthermore, ampicillin (4 µg/L), spectinomycin (100 µg/L), sulphadimidine (100 µg/L) and lincomycin (150 µg/L) were detected at a level equivalent to MRL. Logistic regression revealed significant positive associations between antibiotic concentration

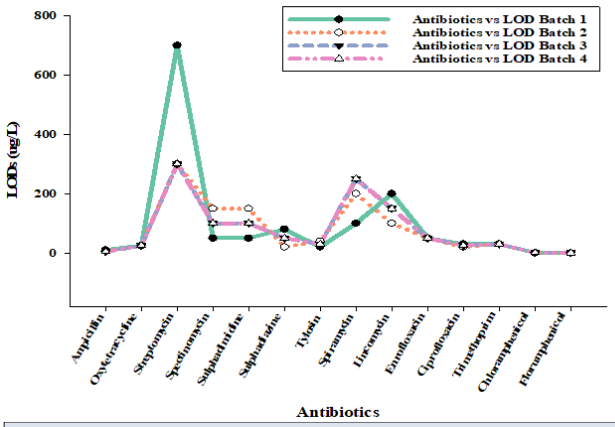

Fig 6. Four experiments were conducted to detect the LOD of the respective antibiotics mentioned. The trend showed that batches 3 and 4 reveal the final LOD of antibiotics

and probability of detection, having odds ratios that range from 1.0098 to 1.35E+06. Detection of quantities below allowable limits and goodness-of-fit tests demonstrated a good-to-acceptable model fit.

Validation Protocols

Limit of Detection: The final LOD values for the kit are summarized in *Table 3*, and the dose-response curve range for 14 antibiotics was generated. The limit of detection of the kit for oxytetracycline, sulphadiazine, tylosin, enrofloxacin, ciprofloxacin, and trimethoprim was lower than the MRL. On the other hand, streptomycin, spiramycin, chloramphenicol and florfenicol showed higher LOD than the MRL of the EU. Furthermore, ampicillin, spectinomycin, sulphadimidine

Table 3. Dose-response range and the LOD of the antibiotics at different concentrations according to the EU

Sr. No.	Antibiotics	LOD	MRL EU (µg/L)	Range of Dose-response Curve
1	Ampicillin	4	4	3-5
2	Oxytetracycline	25	100	25-50
3	Streptomycin	300	200	200-300
4	Spectinomycin	100	100	100-150
5	Sulphadimidine	100	100	80-100
6	Sulphadiazine	50	100	50-80
7	Tylosin	30	50	20-30
8	Spiramycin	250	200	250-300
9	Lincomycin	150	150	100-200
10	Enrofloxacin	50	100	50-100
11	Ciprofloxacin	25	100	25-50
12	Trimethoprim	30	50	20-40
13	Chloramphenicol	0.6	N.A	0.5-0.7
14	Florfenicol	0.5	N.A	0.4-0.6

N.A.= Not Available

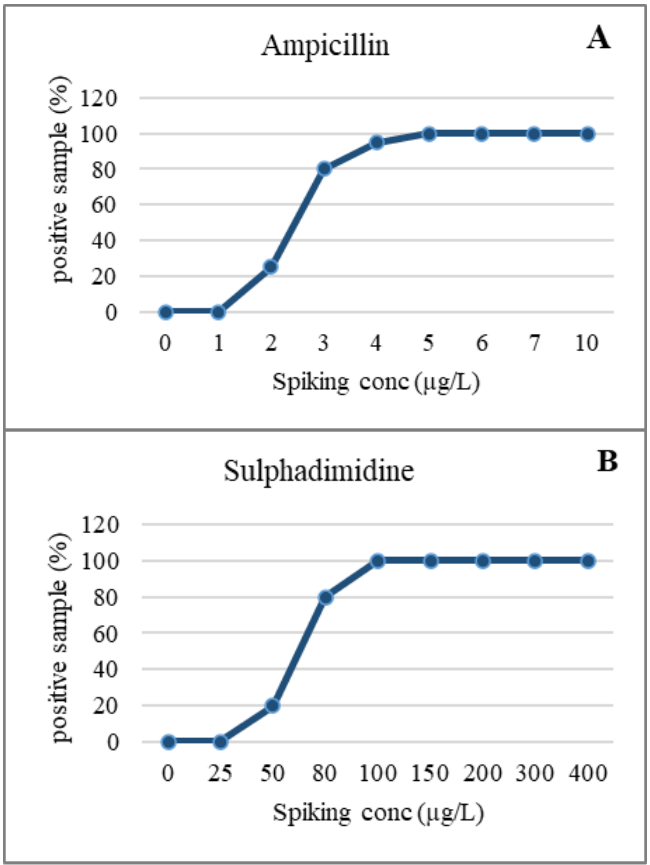

Fig 7. Dose response curve of ampicillin and sulphadimidine. A- Dose response curve of ampicillin, B- Dose response curve of sulphadimidine. The curves are generated by percentage of positive samples (y-axis) against spiking concentration (µg/L) (x-axis)

Table 4. *Results of the False-Negative Rate of the kit against 100 samples tested for each drug*

Sr. No.	Antibiotics	Total Samples	Positive (%)	Negative (%)	LOD	MRL	False Negative Rate
1	Ampicillin	100	98	2	4	4	2
2	Oxytetracycline	100	100	0	25	100	0
3	Streptomycin	100	97	3	300	200	3
4	Spectinomycin	100	100	0	100	100	0
5	Sulphadimidine	100	99	1	100	100	1
6	Sulphadiazine	100	100	0	50	100	0
7	Tylosin	100	100	0	30	50	0
8	Spiramycin	100	98	2	250	200	2
9	Lincomycin	100	100	0	150	150	0
10	Enrofloxacin	100	100	0	50	100	0
11	Ciprofloxacin	100	100	0	25	100	0
12	Trimethoprim	100	100	0	30	50	0
13	Chloramphenicol	100	99	1	0.6	N.A.	1
14	Florfenicol	100	98	2	0.5	N.A.	2

and lincomycin had LOD equivalent to MRL *(Fig. 7)*.

Specificity and Sensitivity: A total of 168 blank samples were analyzed using the kit: 165 were negative and 3 were positive. Therefore, the false positive rate of the kit was 1.78%. A total of 1400 samples were analyzed through the kit; 11 samples were negative and 1389 were positive. The results of the false negative rates of each of the 14 antibiotics were less than 5% at the MRL and limit of detection. Consequently, the kit's false-negative rates met ISO13969: 2003 requirements [30]. False-negative results are mentioned in *Table 4*. High specificity (98.2%) and sensitivity (99.2%) were obtained during kit evaluation.

Predictive Values: The positive and negative predictive values were evaluated using the specificity and sensitivity values of the experiment and concluded that the actual proportion of the positive rate was 99.8% while the negative rate was 93.9%.

Stability: The parameters (odor, performance, appearance, incubation time and detection capacity) of the kit did not change over 2 months *(Fig. 8)*. Odor and incubation time remained stable over 4 months, whereas the detection capacity for ampicillin, streptomycin, spectinomycin, sulphadimidine and lincomycin changed during the 3rd and 4th months, as shown in *Table 5*. Different correlations

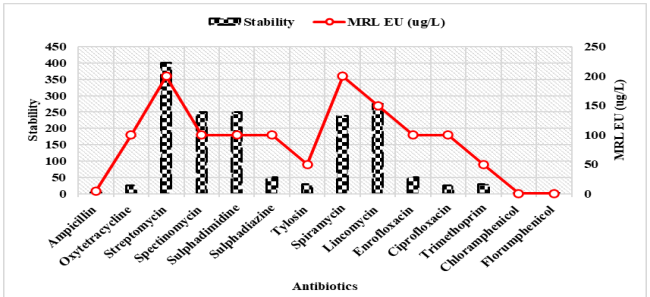

Fig 8. Four-month trend of stability study. The maximum residue limit and the mean results of the stability of the kit for four months of each antibiotic are shown. The red line indicates the maximum residue limit

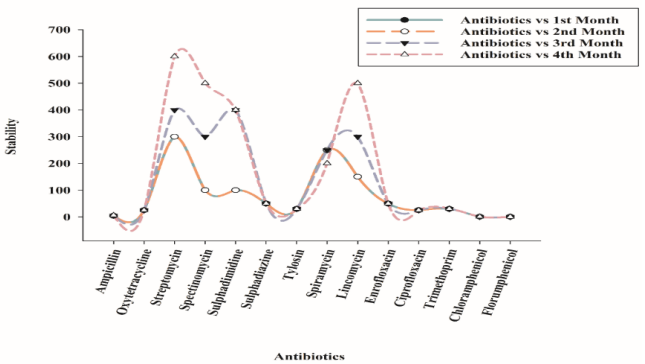

Fig 9. Month-wise antibiotic residues detected on the kit and their limit of detection. The trend showed that in the 1st and 2nd months, the antibiotic residue detection was highly stable, while in the 3rd and 4th months, changes in detection were observed

Table 5. Limit of detection of the four-month stability study

Antibiotics	MRL EU (µg/L)	Results (µg/L)			
		1st-month LOD	2nd-month LOD	3rd-month LOD	4th-month LOD
Ampicillin	4	4	4	5	6
Oxytetracycline	100	25	25	25	25
Streptomycin	200	300	300	400	600
Spectinomycin	100	100	100	300	500
Sulphadimidine	100	100	100	400	400
Sulphadiazine	100	50	50	50	50
Tylosin	50	30	30	30	30
Spiramycin	200	250	250	250	200
Lincomycin	150	150	150	300	500
Enrofloxacin	100	50	50	50	50
Ciprofloxacin	100	25	25	25	25
Trimethoprim	50	30	30	30	30
Chloramphenicol	N.A.	0.6	0.6	0.6	0.6
Florfenicol	N.A.	0.5	0.5	0.5	0.5

between concentration and detection were seen in the logistic regression analysis for 14 antibiotics. These odds range from modest (1.0089) to substantially high (1.24865E+06), accounting for 17.73% to 57.62% of detection variation. Tests for goodness-of-fit tend to validate practical applicability, even in the event of test inconsistencies. Effective detection at low concentrations (e.g, 25 µg/L) retains model utility, for antibiotics with non-significant associations (P=0.002 to 0.609), showing reliability in field applications for determining whether milk samples contain antibiotic residues. *Fig. 9* represents the mean LOD of four months of each antibiotic with their respective MRL.

Discussion

Various studies have highlighted the alarming extent of antibiotic resistance, often caused by antibiotic residues present in animals, food, and the environment [35-40]. Antibiotic residues in milk pose a significant public health risk because they contribute to antibiotic resistance and cause severe health issues. These residues occur when antibiotics administered to cattle are excreted in milk without proper metabolization or clearance [41]. These residues can cause allergic reactions in sensitive people and disturb gut microflora, leading to serious health issues. The detection of antibiotic residues is essential for ensuring food safety and safeguarding public health. It is crucial to monitor as well as control antibiotic residues in milk to preserve its quality and safety [42].

A kit was developed using an indigenously isolated *B. subtilis* strain. The isolated strain was confirmed using PCR amplification of two species-specific genes and sequenced for phylogenetic analysis. To ensure validity and consistency of results, strains used in assay development must be well-characterized, traceable, and maintained under controlled conditions [43,44]. Two isolated strains depicted sensitivity to several antibiotics. The NF-S2 strain was suitable for detecting various antibiotic residues. Its high sensitivity to various antibiotics and extensive use in microbiological assays demonstrates its efficacy and reliability. *B. subtilis* is the most suitable species for the development of this detection assay due to its robust genetic profile and established precedent. There are many studies conducted to detect antibiotic residues using *B. subtilis* [25,45-47]their presence in foodstuff derived from animals is a potential public health hazard. They pose a serious threat as they are implicated in direct toxicity; allergic reactions; disturbance of the normal gut microbiota, carcinogenesis, and emergence of antibiotic-resistant bacteria.

The bacterium in this study demonstrates distinct inhibitory zones, confirming its strong susceptibility to various antibiotics. Ji et al.[48] study from 2022 employed antibiotics that closely matched the antibiotics used in this research and produced similar findings, with the exception that ampicillin (20 mm) and piperacillin (20 mm) exhibited moderate sensitivity, but high sensitivity was observed in the current study. The sensitivity of the bioassay must also be validated by AST to identify low antibiotic residue concentrations and confirm the *B. subtilis* as a bioindicator for monitoring milk safety.

After confirming the sensitivity of the bacteria, the antibiotic residue detection kit was developed using various stabilizers, buffering agents, nutrients and color indicators. Every component has a vital function in maintaining pH stability, promoting bacterial growth, preserving spore integrity, and facilitating enzymatic reactions [46]. The remarkable sensitivity of *B. subtilis* spores to antibiotics enables accurate residue detection. The kit changed color from purple to yellow *(Fig. 4)* in 6-7 h, indicating negative results and vice versa. This rapid, easy-to-use test will enable dairy producers and regulators to monitor antibiotic residues in milk efficiently [49]. Comparison of the results from the present detection kit with those from studies by Nagel et al.[26] and Wu et al.[50] revealed that this kit demonstrated greater sensitivity in detecting antibiotic residues in milk. The assay for antibiotic residue detection concentrations in the Wu et al.[50] study kit showed oxytetracycline (200 µg/L), sulphadiazine (75 µg/L), sulphadimidine (150 µg/L), lincomycin (300 µg/L), streptomycin (500 µg/L) and tylosin (50 µg/L). Similarly, when comparing the results of Nagel et al. [26] for ciprofloxacin (150 µg/L), enrofloxacin (160 µg/L), and spiramycin (340 µg/L), the present kit performed better.

Due to the absence of several stabilizers, buffering agents, and specific additives in the Nagel et al.[26] Kit, there was a notable disparity in detection concentrations between present kit and their study.

The media composition for the kit was developed from Wu et al.[27] and Posada Uribe et al.[28] studies, with minor modifications to support the growth and sporulation of *B. subtilis* as well as the functionality and stability of the kit. A combination of additives, such as carboxymethylcellulose sodium, prevented milk from interfering with the nutritional aspects of the medium and ensured even distribution of spores, thereby improving detection assay clarity [51]. To enhance detection capabilities, components including $MnSO_4 \cdot H_2O$, $MgSO_4$, and glucose were added to stimulate bacterial growth, propagation, and acid production. As enzyme cofactors, manganese and magnesium ions enhanced enzyme activity, improving the kit's acid-producing capacity and sensitivity; however, lower bacterial concentrations increased detection time [52]. Nevertheless, the presence of nutrients such as glucose improved the detection process and time by enhancing bacterial proliferation and functionality. Comparing this detection kit to previous research shows its greater sensitivity in detecting lower antibiotic residual amounts in milk.

The validation of the detection kit was an essential aspect of the study, guaranteeing its dependability and precision for practical use. To determine the limit of detection (LOD), milk samples spiked with varying concentrations of antibiotics were analyzed, as performed by Yazdanpanah et al.[53]. The present kit identified traces of antibiotics at concentrations as low as below MRL *(Table 3)*, thereby confirming its high sensitivity. The sensitivity testing assessed the kit's capacity to accurately detect positive samples, resulting in a high sensitivity rate of 99.2%. This high sensitivity ensures that present kit can effectively detect antibiotic residues, thus reducing false-negative results. To ensure that the kit could correctly identify samples without antibiotics, specificity testing was conducted. The kit's specificity rate was 98.25%, indicating that it consistently discriminates negative samples, thus lowering the probability of false-positive results. These findings align with the study by Wu et al.[54].

There was no sample preprocessing except for heating the milk in a water bath for 10 minutes at 80°C before adding it to the detection system. A study by Andrew [52] used a similar approach for sample pretreatment. During the incubation process, antibiotics found in milk might contaminate the detection medium. Additionally, the detection medium can be contaminated by natural bacteriostatic compounds found in milk, including, fat, protein, somatic cells, bacteria, lysozyme, lactoferrin, and the lactoperoxidase system [52,55]. Inhibition of *B. subtilis* growth in the detection system by these natural bacteriostatic compounds might lead to false-positive results because they impede acid production and the color change of the pH indicator. Moreover, antibiotics may lose part of their efficacy if they encounter certain milk components. When testing for tetracyclines, false-negative results are common because calcium ions in milk may chelate with them [56]. Furthermore, during incubation, a significant amount of milk protein diffuses throughout the media, and white lactoprotein may mask the pH indicator's color change, complicating visual detection. In this study, milk samples were incubated in a water bath at 80°C for 10 min before being added to the kit's medium to inactivate natural bacteriostatic compounds and eliminate false positive results, as also demonstrated by Houali et al.[57]. A low false-positive results (1.78%) indicated the importance of this kit. The kit's carboxymethylcellulose sodium can also bind with milk's calcium ions, blocking their interference and ensuring that the test will not provide a false-negative result for tetracyclines. Furthermore, the complex network formed by carboxymethylcellulose sodium prevents macromolecules such as fat and protein from entering detection system and prevents white lactoprotein from obstructing the pH indicator's color change [58]. In conclusion, the sample pretreatment technique used in this study was simple to use, cost-effective, time-saving, and applicable in any setting.

Kit stability was assessed for four months, and consistent results were obtained for most antibiotics *(Fig. 9)*. However, for some antibiotics (streptomycin, ampicillin, spectinomycin, lincomycin, and sulphadimidine), LOD mildly increased after two months, which was possibly due to the lack of sensitizers in the kit. Therefore, to improve stability for more than six months, antibiotic sensitizers must be included for these antibiotics to increase bacterial susceptibility. Additionally, for the remaining antibiotics tested, the kit continued to function without changes the parameters such as color, odor, or detection time, indicating that the core medium, spores, and stabilizing additives/buffering remained functional, demonstrating its dependability for routine checking of antibiotic residues in milk. The study by Wu et al.[54] demonstrated that their kit remained stable for up to six months; however, their kit contained an antibiotic sensitizer. It is suggested that adding chloramphenicol and trimethoprim increases the sensitivity of the microbiological inhibition assay toward sulfonamide, tetracycline, and other antibiotics, including cephalosporin and penicillin. Another study by Nagel et al.[26] used trimethoprim as a sensitizer to increase the sensitivity and stability of the bioassay to detect antibiotic residues. The detection medium of this kit, along with the supplementary chemicals, microplates,

aluminized film cover, and other materials was aseptically treated. Moreover, sodium carboxymethylcellulose forms hydrogen bonds with water, further inhibiting water evaporation [54]. The kit's *B. subtilis* spores maintained their acid-producing abilities and antibacterial sensitivity for four months. In conclusion, the kit developed in this study demonstrated stability, with a shelf life of four months.

Growing concerns about antibiotic residues in milk and their connection to resistance and hazards to public health emphasizes the urgency of effective detection techniques. The detection kit utilized *B. subtilis* spore suspension to determine presence of antibiotic residues in milk. Altogether, this research established a practical, rapid microbiological screening method using *Bacillus subtilis* spores that enables dairy producers and regulators to detect multiple antibiotic residues in milk within 6-7 h with high accuracy, addressing critical food safety concerns while remaining accessible for routine field use.

Declarations

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Authors Contributions: NF, Concept Design, Experimental work, Data Collection and Analysis, Writing-original draft, MSM, Supervision, Concept Design, Review of the manuscript, MKK, Supervision, Critical review of the manuscript, SA, Supervision, Analysis and Interpretations, Writing, review and Editing, Correspondence. All authors approved the final version of the manuscript and accept responsibility for the scientific integrity of the work in accordance with the journal's Ethical Principles and Authorship Policy.

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