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Assessment of antibiotic residues in commercial and farm milk collected in the region of Guelma (Algeria)

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Abstract

Background: In an attempt to enhance the quality and quantity of food production (especially milk) and in order to prevent, or treat,animal diseases, the use of antibiotics in Algeria follows an increasing trend. The increased use evidently contributes to the emergence of increased contamination levels of antibiotic residues.

Results: In this work, two methods were used to detect presence of antibiotic residues in raw and fermented cow's milk collected in Guelma's farms (in Algeria). The screening comprised different points of sale in Guelma province. In a first step a widely used prescreening method based on microbial inhibition assay; Delvotest SP-NT; was used to analyze 131 milk samples. In a second step a liquid chromatography coupled to mass spectrometry (LC-MS/MS) was used. The latter was first optimized for extraction of 36 veterinary drugs of penicillins, quinolones, macrolides, tetracyclines, sulfonamides, and trimethoprim from the collected milk. After simple extraction and dilution, the 194 samples, including those previously tested by the Delvotest SP-NT, were analyzed by LC-MS/MS. Results obtained by both methods were compared. Among the LC-MS/MS findings, 65.46 % of non-conform samples contained authorized residues at levels higher than the MRL, residues without set MRL, or non-authorized residues.

Conclusion: The comparison of both methods showed that Delvotest SP-NT is less trustworthy due to number of false negative results. This was further confirmed by LC-MS/MS pointing out the traces of antibiotics in numerous samples. In 65.46 % of milk samples residues of antibiotics were found suggesting a lack of public health controls as well as an evidence of the negligent use of antibiotics in the livestock industry, which both form a risk to public health. This indication should be confirmed by a nationwide study with in-depth analyses of antibiotic's presence in food chain originated from animals. The study offered an LC-MS/MS based analytical method ready to be used in Algerian National Residues Control Plan as a versatile analytical tool to monitor and determine the occurrence of antibiotic multi-residues in milk.

Keywords: Antibiotic residues, Delvotest SP-NT, LC-MS/MS, False positive, False negative, Fermented milk (lben)

Background

Antibiotics are widely used in livestock production for many purposes, such as: animal disease treatment (therapeutic application), animal disease prevention (prophylactic application), and feed efficiency (as growing promoters) (Jank et al. 2015). Their presence as residues in food products especially milk implicates certain damages in the public health like: the development of allergic reactions in some hypersensitive individuals, increased risk of carcinogenicity (Petrović et al. 2008; Hou et al. 2014), the growth of resistant bacterial strains, and imbalances in intestinal microflora (Wang et al. 2006; Borràs et al. 2011). Low concentrations of antimicrobial drug residues create problems in the production of milk products by inhibiting the starter cultures (Petrović et al. 2008; Stead et al. 2008). Moreover, the risk of contamination with antibiotic residues of farms milk is higher if inappropriate practices are applied. For these reasons, control measures must be implemented to prevent drug residues

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from entering into the food chain. In this regard, and to ensure the safety of the consumer, worldwide regulatory authorities have set Maximum Residue Limits (MRLs) for several veterinary drugs (Kassaify et al. 2013). Since 2006, these substances are forbidden by the European Union (EU) to be used either in sub-therapeutic doses for prophylaxis or as growth promoters in veterinary medicine (Council Regulation No 1831/2003). Consequently, the presence of antibiotic residues in products that are targeted for food consumption has to be controlled. Similarly, various analytical methods have been described to analyze milk; especially microbiological and immune assays which are widely used because of their low cost and short time of analysis (Ramirez et al. 2003; Beltran et al. 2015). However, most current microbial screening tests have been initially developed to detect β-lactams in cow's milk. They are based on the inhibition of *Geobacillus stearothermophilus var. calidolactis* which is highly sensitive to these substances (Beltran et al. 2015). Delvotest SP-NT is considered as one of the most commonly used microbial inhibition tests (Stead et al. 2008). However, the limited sensitivity and selectivity of the method demand further confirmation of the results obtained using more sensitive technique (Ferrini et al. 2015).

Instrumental techniques such as liquid chromatography (LC) coupled to UV- VIS spectroscopy or mass spectrometry (MS), are widely used in food control for either screening or confirmation of positive findings of less specific test within the following studies: Hermo et al. (2008), Li et al. (2012), Boix et al. (2014), Hou et al. (2014), Martins et al. (2014), and Cepurnieks et al. (2015). Furthermore, LC-MS/MS is nowadays the most frequently used analytical tool for detecting a large number of multiclass veterinary drug residues in food and decreasing the rate of false negative and false positive results with high selectivity and sensitivity (Martins et al. 2014). Many papers have tackled the analysis of different classes of veterinary antibiotics in milk using LC-MS/MS (Bogialli et al. 2008; Hermo et al. 2008; Han et al. 2015, and Meng et al. 2015). But recent research themes have enlarged their interests to the development of multiclass veterinary drug residues methods which facilitate the discrimination of antibiotics in milk and other matrices; such as, the study of Martins et al. (2014), which has established two screening methods that can analyze 24 antibacterial and one metabolite residue in milk and liver using LC-MS/MS.

Very few studies in Algeria have been conducted on the potential presence of antibiotic residues in raw cow's milk samples and that was by using less specific microbiological methods. Of the few researches that are available, studies made by Tarzaali et al. (2008), Aggad et al. (2009), and Titouche et al. (2013) have described high levels of milk contamination by using Delvotest SP-NT. They indicated that among all tested milk samples 89, 29, and 47 % were found to contain residues, respectively. This appears significantly high prevalence of positive samples in comparison to results from any European Union country where a regular system for antibiotic residues control in milk is many decades-old. On contrary in Algeria similar milk control appears to be immature. Consequently, there are no data available on occurrence of antibiotic residues in milk produced in Guelma province located in North East of Algeria. Moreover, in any of the published reports contamination of fermented milk was not considered. The fermented milk - called lben- and cow's milk are important components of Algerian's diet (Belhadia et al. 2011; Zoubeidi and Gharabi 2013). Lben is traditional cultured milk widely consumed in North Africa and in Middle Eastern countries. It is produced by spontaneous souring of cow's milk followed by churning in order to separate lben from butter. Whereas only one report about a similar milk type (raibi milk) is available (Zinedine et al. 2007), no other data on the specific products are available or known.

In this work, the analyses of two types of milk (raw and lben) collected in Guelma province (in the north of Algeria) is presented. The samples were collected directly from the farms, and further in the value chain from dairy markets. Two methods were used to detect the presence of antibiotic residues in those samples. The first one was Delvotest SP-NT, which is most commonly used test for this purposes in Algeria. The second one was an optimized LC-MS/MS multi-residues screening method. The results obtained by both tests were compared and correlated to evaluate the feasibility of the use of Delvotest SP-NT as a prescreening test in Algerian setting. The optimization and validation of multi-residue LC-MS/MS screening method was performed following the international norms.

Methods

Samples and accompanying data

A total of 194 cow's milk samples were collected. Among them, 156 samples were collected from two farms (A and B), whereas 38 (including fermented and raw cow's milk) were purchased from 16 various points of sale in Guelma province during the period of January 2013 to July 2014. Of 156 farm samples 53 were collected from farm A located in the centrum of Guelma province. The samples were gathered as such: 8 samples were taken from individual cows which have been treated less than a month prior to the collection, 28 from individual untreated cows, and 17 samples were collected from bulk tank milk at the farm. Similarly, 103 milk samples were collected from farm B located more than 7 Km far from Guelma center-. Their collection and number were as follows: milk from individual cows

treated less than a month prior to the collection were 18, milk from individual untreated cows were 70, and bulk tank milk were 15. In addition to sampling for the assessment of antibiotic residues, questionnaires were made to collect data on the cow's treatment like: the kind of antibiotic administered to the cow, the day of treatment, and the withdrawal time of each antibiotic used. The latter may vary from 2 to 7 days depending on the administered antibiotics (penicillins, tetracyclines, macrolides and sulfonamides) and this either intermammary or intramuscular.

Out of 38 milk samples purchased at various points of sale in Guelma province, 22 were fermented milk "lben" and 16 were raw cow's milk. Seven points of sale were situated in the province's capital whereas the others were situated in separate regions surrounding the state. For this part of the value chain a short questionnaire was used in order to collect more specific information about the milk origin. Those data specified milk as collected either from a farmer, private collection, milk factories, or from farms in Guelma municipalities and Souk Ahras state. Due to missing data, other collections were indicated as unknown origin.

Approximately a volume of 140 mL of each milk sample was collected and conserved in sterile flask and transported to the laboratory at 4 °C. Ten millilitre were used to be analyzed by a microbial test Delvotest SP-NT for the prescreening of antibiotics in milk samples. The remaining 130 mL were frozen and kept at −20 °C prior to further analysis by LC/MS-MS. Two blank milk samples were assigned in the collection after a negative test for antibiotics by Delvotest SP-NT and one purchased from Belgian supermarket for LC-MS/MS analysis.

Chemicals

The following reference standards were from Sigma-Aldrich (Bornem, Belgium). The sulfonamides (sulfapyridine, sulfamethoxypyridazine, sulfadoxine, sulfadimidine, sulfamonomethoxine, sulfaquinoxaline, sulfamoxole, sulfachloropyridazine, sulfadimethoxine, sulfathiazole, sulfaguanidine, sulfamerazine, sulfamethoxazole, sulfadiazine, sulfacetamide, sulfisoxazole, sulfamethizole), trimethoprim, penicillins (amoxicillin, ampicillin, penicillin V, penicillin G, dicloxacillin, cloxacillin, oxacillin, nafcillin), quinolones (flumequine, difloxacin, sarafloxacin, ciprofloxacin, enrofloxacin, danofloxacin, nalidixic acid, marbofloxacin, norfloxacin, ofloxacin, oxolinic acid), macrolides (erythromycin, spiramycin, josamycin, clindamycin, lincomycin, neospyramycin, tilmicosin, tylosin, tylvalosin, tulatrhomycin), and tetracyclines (doxycycline, oxytetracycline, 4-epi- chlortetracycline, 4-epi-oxytetracycline, 4-epi- tetracycline, tetracycline, chlortetracycline) were with a purity of 95 to 100 %. These standards were used to prepare stock standards solutions. Similarly, the internal standards sulfadimidine C^{13}, flucloxacilline, norfloxacin_D_5, roxythromycine, and demeclocycline, were from Sigma-Aldrich (Bornem, Belgium). Besides, stock standards solutions of sulphonamides, penicillins, macrolides, and tetracyclines were prepared by dissolving 10 mg of each substance into 10 mL of methanol, except for penicillins which were dissolved in 10 mL of Milli-Q water (Millipore corp., Bedford, MA, USA). For the quinolones, 5 mg of each substance were dissolved into 10 mL of methanol. In addition, mixed intermediate standard solutions at 5MRL (Commission Regulation 2377/1990) were prepared from diluting stock standard solutions to obtain a specific final concentration for each substance then conserved them at −20 °C. Actually, solvents acetonitrile and methanol used for mobile phase and extraction were of UPLC-MS grade. They were purchased from Biosolve (Valkenswaard, the Netherlands). Yet, Formic acid, oxalic acid 10 mM, Ethylene-diaminetetraacetic acid (EDTA) 100 mM, and sodium sulfate anhydride were purchased from Merck (Darmstadt, Germany). The analytical liquid chromatography column (Waters, Millford, MA, USA) C18 2.1 × 100 mm, 1.7 μm was used.

Microbial inhibitor test (Delvotest SP-NT)

Delvotest SP-NT, which is a non-specific microbial inhibitor test, was used to detect the presence of antibiotics in milk samples. In short this is an agar diffusion test that contains a standardized number of *Bacillus stearothermophilus* spores, selected nutrients, and pH indicator bromocresol purple. Four kits of Delvotest SP-NT (DSM, Netherlands) were used whereas three were provided by DSM Food Specialties located in Spain, and the fourth was purchased. After adding milk sample directly to the agar bed (ampoules), an incubation step was conducted for 3 h at 64 °C. During incubation, microbial metabolism resulted in a change in pH, and hence in a change of color from purple to yellow. By contrast, if the sample contained sufficiently high concentrations of inhibitory substances, the color would remain purple (Stead et al. 2008). Except fermented milk, 10 mL of each raw milk sample was heated at 80 °C for 10 min to destroy natural inhibitors lysozyme and lactoferrin (Hillerton et al. 1999). In parallel, 0.1 mL was decanted into Delvotest SP-NT ampoules using a specific pipette for each sample. The ampoules were incubated in water bath at 64 °C ± 2 °C within 3 h. Test and data interpretation were performed according to the manufacturer's instructions.

Extraction optimization

The extraction procedure was based on an existent method for screening of antibiotics in meat. This method was previously developed and validated in accordance with the European commission (Commission Decision 2002). In this study, only 36 antibiotics were

selected to be followed in milk samples during method optimization instead of 59 ones targeted in the initial method. The selected antibiotics had either the same level of MRL both in milk and meat, or lower MRL in milk than in meat as prescribed in European commission (Commission Regulation 37/2010). With required scrutiny, the selection of the appropriate method for the extraction of antibiotics residues in milk was based on the comparison between four modified extraction protocols as briefly presented in Table 1. All protocols were tested on a set of a blank milk sample and two control samples spiked with mixture of antibiotic standards. Out of the comparative overview of the average recoveries obtained by four extraction protocols (Table 2), method 3 was selected as the optimal. Further details are explained in the section of Optimization of the LC-MS/MS screening method results. The following list includes the steps of the extraction procedure. Ten grams of one blank and two control milk samples were weighed in 50 mL falcon. To these 500 μL of mixed internal standard solution at 1MRL, 667 μL of EDTA 0.1 M, 23 mL of the mixture (methanol/acetonitrile) and 3 g of sodium sulfate anhydride were added. The spiked samples were fortified with 250 μL of each mixed standard solutions at 1MRL as above in order to obtain levels corresponding to 0.5 MRL. Samples were vortexed for 1 min, and centrifuged at 10,000 rpm for 10 min at 4 °C. Subsequently, 5 mL of the supernatant was decanted into 10 mL tubes and evaporated to dryness at 40 °C. Three-hundred microlitre of Milli-Q water was added into tubes after evaporation, vortexed for a few seconds and transferred to eppendorfs tubes to be centrifuged at 12,000 rpm for 30 min. Supernatant was filtered through a 0.2 μm filter directly to injection vial prior to LC-MS/MS analysis.

LC-MS/MS analysis

UPLC analysis was performed using an Acquity sample and solvent manager (Waters, Milford, MA, USA). Chromatographic separation was achieved using an Acquity UPLC column C18 2.1 × 100 mm 1.7 μ (Waters, Millford, MA, USA) at 30 °C with the mobile phase composed by 0.1 mM oxalic acid, 0.1 % formic acid (solution A) and acetonitrile 0.1 % formic acid (solution B) at a constant flow of 0.4 mL min^{-1}. The gradient elution program used

Table 1 Comparative overview of four extraction methods parameters

Extraction steps	Method 1	Method 2	Method 3	Method 4 (Martins et al. (2014))
1) Sample amount	3g	10g	10g	0.5g
2) Type of container	50mL Falcon tube			2mL Eppendorf tube
3) Mixed internal standard solution, 1MRL (EU2377/90)	150μL	150μL	500μL	12.5μL
4) EDTA, 0.1M	200μL	200μL	667μL	30μL
5) Mixture methanol/acetonitrile	7mL	7mL	23mL	/
6) Sodium sulfate anhydride	3g	3g	3g	/
7) Mixed standard solution*	75μL	75μL	250μL	12.5μL
8) Vortexing	1min			10sec and equilibration for 10min from light
9) Centrifugation	10000rpm, 10min at 4°C			
10) Deprotonization				0.6mL Ethanol/acetic acid (96/4) Followed by short vortexing and 30min at -18°C incubation
11) Supernatant	5mL evaporated to dryness at 40°C followed by addition of 300 μL of water MilliQ			/
12) Vortex and centrifugation	Few seconds 12000rpm during 30min			
13) Supernatant	All supernatant			0.75mL with addition of 0.25mL Formic acid 0.1% in water/ formic acid in acetonitrile (98:2)
14) Filtration	0.2μm filter			/

*mixed standard solution contained: Sulfonamides (sulfapyridine, sulfamethoxypyridazine, sulfadoxine, sulfadimidine, sulfamonomethoxine, sulfaquinoxaline, sulfaquinoxaline, sulfamoxole, sulfachlorpyridazine, sulfadimethoxine, sulfaquinoxaline, sulfathiazole, sulfaguanidine, trimethoprim, sulfamerazine, sulfamethoxazole, Sulfadiazine, sulfacetamide, sulfisoxazole), penicillins (amoxicillin, ampicillin, penicillin V, penicillin G, dicloxacillin, cloxacillin, oxacillin, nafcillin) quinolones (flumequine, difloxacin, sarafloxacin, ciprofloxacin, enrofloxacin, danofloxacin, nalidixic acid, marbofloxacin, norfloxacin, ofloxacin , oxolinic acid), macrolides (erythromycin, spiramycin, josamycin, clindamycin, lincomycin, neospyramycin, tilmicosin, tylosin, tylvalosin, tulatrhomycin), tetracyclines (doxycycline , oxytetracycline ,4-epi- chlortetracycline, 4-epi-oxytetracycline, 4-epi- tetracycline, tetracycline, chlortetracycline)

Table 2 All parameters used for the comparison between four methods (M1, M2, M3 and M4) and the final selection of the test suitable method

Group and type of antibiotic	Spiked Concentration (μg/L)	Average Rec% ± STDEV M1	Average Rec% ± STDEV M2	Average Rec% ± STDEV M3	Average Rec% ± STDEV M4	MRL in milk (μg/L)	MRL in meat (μg/L)	MRL milk/MRL meat
Macrolides		35.65 ± 2.72	24.50 ± 8.54	73.66 ± 8.33	79.20 ± 6.51			
Erythromycin	2000	3.40^{ab} ± 0.98	0.67^{a} ± 0.35	20.10^{b} ± 3.77	0.00^{a} ± 0.00	40	200	0.2
Neospyramycin	2500	18.78 ± 1.33	22.14 ± 1.10	70.31 ± 11.95	102.99 ± 3.17	200	200	1
Spiramycin	2500	25.96 ± 3.06	29.07 ± 1.18	53.17 ± 4.80	37.45 ± 2.02	200	200	1
Tilmicosin	500	96.42 ± 2.34	63.54 ± 39.90	171.49 ± 11.93	245.02 ± 27.02	50	50	1
Tylosin	1000	33.69^{a} ± 5.89	7.08^{b} ± 0.16	53.23^{a} ± 9.22	10.55^{b} ± 0.35	50	100	0.50
Penicillins		34.36 ± 2.10	30.36 ± 1.87	72.33 ± 9.17	75.42 ± 7.91			
Amoxicillin	500	57.08^{a} ± 4.54	69.32^{a} ± 0.38	143.26^{b} ± 25.38	107.28^{c} ± 35.83	4	50	0.08
Ampicillin	500	78.19^{a} ± 7.09	104.03^{a} ± 7.99	204.73^{b} ± 26.17	273.58^{c} ± 7.65	4	50	0.08
Cloxacillin	30,000	1.43^{a} ± 0.12	1.31^{a} ± 0.03	3.05^{a} ± 0.21	5.76^{a} ± 0.49	30	300	0.1
Dicloxacillin	3000	9.13^{a} ± 0.44	6.96^{a} ± 0.09	21.14^{ab} ± 2.80	60.03^{b} ± 5.54	30	300	0.1
Nafcillin	3000	8.23^{a} ± 0.20	10.69^{a} ± 0.12	18.07^{a} ± 0.39	10.23^{a} ± 0.31	30	300	0.1
Oxacillin	3000	10.99^{a} ± 0.86	11.72^{a} ± 0.27	28.44^{ab} ± 1.39	53.30^{b} ± 2.03	30	300	0.1
Penicillin G	500	75.48^{a} ± 1.44	8.46^{b} ± 4.25	87.64^{a} ± 7.85	17.76^{b} ± 3.53	4	50	0.08
Quinolones		37.05 ± 2.20	26.58 ± 1.62	76.52 ± 1.74	83.07 ± 13.94			
Enrofloxacin	500	53.28 ± 3.60	40.40 ± 1.90	110.21 ± 1.22	95.99 ± 19.05	100	100	1
Flumequine	1000	9.05^{a} ± 1.45	0.00^{b} ± 0.00	24.33^{a} ± 0.70	52.72^{c} ± 9.48	50	200	0.25
Marbofloxacin	750	48.80^{a} ± 1.55	39.32^{a} ± 2.94	95.02^{b} ± 3.29	100.49^{b} ± 13.30	75	150	0.5
Sulfonamides		34.80 ± 1.32	29.49 ± 2.52	94.58 ± 6.43	99.73 ± 9.82			
Sulfacetamide	1000	18.54 ± 1.36	14.02 ± 1.57	35.42 ± 8.03	20.53 ± 2.80	100	100	1
Sulfachloropyridazine	1000	38.38 ± 0.48	26.78 ± 3.87	108.43 ± 0.11	132.90 ± 3.87	100	100	1
Sulfadiazine	1000	25.49 ± 2.58	24.09 ± 0.89	58.88 ± 0.63	65.94 ± 3.54	100	100	1
Sulfadimethoxine	1000	14.31 ± 1.46	7.10 ± 0.40	41.56 ± 1.33	85.52 ± 2.47	100	100	1
Sulfadimidine	1000	39.33 ± 0.21	38.29 ± 1.55	114.46 ± 7.31	123.00 ± 9.00	100	100	1
Sulfadoxine	1000	30.31 ± 0.74	16.97 ± 2.60	82.33 ± 0.96	153.84 ± 1.11	100	100	1
Sulfaguanidine	1000	18.64 ± 0.12	14.25 ± 0.14	39.51 ± 7.71	26.60 ± 10.07	100	100	1
Sulfamerazine	1000	9.70 ± 1.57	7.65 ± 0.11	39.23 ± 1.08	89.31 ± 11.19	100	100	1
Sulfamethizole	1000	42.44 ± 0.80	55.31 ± 7.74	92.82 ± 1.55	84.97 ± 4.78	100	100	1
Sulfamethoxazole	1000	43.95 ± 6.02	18.70 ± 3.82	127.40 ± 4.92	161.97 ± 15.02	100	100	1
Sulfamethoxypyridazine	1000	43.94 ± 0.02	51.72 ± 1.76	140.44 ± 5.21	165.70 ± 12.54	100	100	1
Sulfamonomethoxine	1000	42.22 ± 1.56	50.56 ± 0.46	148.67 ± 9.67	174.34 ± 7.26	100	100	1
Sulfamoxole	1000	38.61 ± 1.46	19.51 ± 2.34	80.30 ± 10.71	100.31 ± 23.38	100	100	1

Table 2 All parameters used for the comparison between four methods (M1, M2, M3 and M4) and the final selection of the test suitable method *(Continued)*

Sulfapyridine	1000	29.02 ± 0.68	31.62 ± 2.64	89.32 ± 6.45	101.35 ± 18.40	100	100	1
Sulfaquinoxaline	1000	13.64 ± 0.32	10.28 ± 2.07	28.72 ± 5.89	79.34 ± 14.84	100	100	1
Sulfathiazole	1000	36.39 ± 1.95	39.05 ± 0.88	88.64 ± 5.73	41.14 ± 21.28	100	100	1
Sulfisoxazole	1000	55.57 ± 0.80	29.05 ± 2.59	168.29 ± 12.57	62.20 ± 4.43	100	100	1
Trimethoprim	1000	86.00 ± 1.57	75.98 ± 9.94	217.95 ± 25.88	126.27 ± 10.81	50	50	1
Tetracyclines		25.73 ± 0.54	21.89 ± 17.13	73.50 ± 12.79	52.61 ± 7.69			
Chlortetracycline	1000	18.87 ± 0.49	21.77 ± 9.36	30.30 ± 6.29	35.67 ± 6.17	100	100	1
Oxytetracycline+ 4-Epi-Oxytetracycline	1000	32.79 ± 0.77	22.24 ± 23.07	108.49 ± 15.90	48.16 ± 2.89	100	100	1
Tetracycline+ 4-Epi-Tetracycline	1000	25.53 ± 0.36	21.67 ± 18.97	81.72 ± 16.19	74.02 ± 14.00	100	100	1

Means with the same letter do not differ significantly at the level of 0.05
STDEV Standard Deviation, Rec Recovery
*the estimated concentration was based on the calibration curve produced with the five different standard concentrations (250, 500, 1000, 1500 and 2000 μg/L) after screening the quantifier ion in the MS spectra. Four methods used ($n = 2$) in milk samples spiked each antibiotic of 500 μg/L as a final concentration

was initially 98 % of A decreasing to 2 % within 10.5 min (0–10.5 min). After that, the composition was set back to the initial levels A: B (98:2) (10.5–12.5 min). The total run time was 12.5 min. XEVO TQ MS triple quadrupole mass spectrometer (Waters, Millford MA, USA), operating in positive Electrospray ionization (ESI) MS/MS mode was used for detection. Data was controlled and evaluated by MassLynx software (version 4.1). The selected reaction monitoring (MRM) mode was used and the following tune parameters were applied: capillary, 3 kV; cone 15 V; extractor, 3.00 V; source temperature, 150 °C; desolvation temperature, 500 °C, cone gas flow, 80Lh^{-1}; desolvation gas flow, 1000Lh^{-1}; collision gas flow, 0.16 mL min^{-1}, resolution (LM1, HM1, LM2, HM2 where LM is low mass and HM is high mass), 2.7, 15, 2.8, 14.8; ion energy (1 and 2), 0.3, 0.6; multiplier 546.52 V. Cone voltage (V), collision energy (eV) and transition mass parameters for all antibiotic residues analyzed in milk samples are presented in Table 3.

Table 3 Mass spectrometry parameters used for the screening of antibiotic residues in milk

Analyte	Transition	Cone voltage (V)	Collision energy eV
Amoxicillin	366 > 114	10	20
Ampicillin	350 > 106.1	30	20
Chlortetracycline	479.1 > 302.8	30	40
Cloxacillin	436.24 > 160.0	30	10
Dicloxacillin	469.9 > 160.1	30	10
Enrofloxacin	360.4 > 341.9	30	20
Erythromycin	734.4 > 576.6	30	20
Flumequine	262.1 > 244	30	20
Marbofloxacin	363.26 > 72	30	20
Nafcillin	415 > 199	30	10
Neospiramycin	699.4 > 174.2	30	20
Oxytetracycline + 4-epi-Oxytetracycline	461.1 > 443.1	30	10
Oxacillin	402.2 > 243.3	15	10
Penicillin G	335.1 > 176.1	30	10
Spiramycin	843.6 > 174.4	30	40
Sulfacetamide	215.2 > 156	30	10
Sulfachloropyridazine	285 > 155.8	25	15
Sulfadiazine	251 > 108	30	20
Sulfadimethoxine	311.1 > 156	30	20
Sulfadimidine	279.2 > 155.9	30	20
Sulfadoxine	311.2 > 155.9	30	20
Sulfaguanidine	215.1 > 156	25	15
Sulfamerazine	265.1 > 155.9	30	20
Sulfamethizole	271.1 > 107.8	30	20
sulfamethoxazole	254.1 > 108.1	30	20
sulfamethoxypyridazine	281 > 155.7	30	15
Sulfamonomethoxine	281.2 > 155.9	30	20
Sulfamoxole	268.2 > 155.9	30	10
Sulfapyridine	250.1 > 184	30	20
Sulfaquinoxaline	301.2 > 107.7	30	20
Sulfathiazole	256.1 > 107.7	30	30
Sulfisoxazole	268.1 > 156.2	30	10
Tetracycline + 4-epi-tetracycline	445.1 > 410.1	30	20
Tilmicosin	869.8 > 174.1	65	45
Tylosin	916.6 > 127.2	30	40
Trimethoprim	291.3 > 230.3	60	20

Validation of the LC-MS/MS screening method

Validation of the selected method was performed on all antibiotic substances cited in Table 4 and performed following the international norm (Commission Decision 2002) through determining: specificity/selectivity, detection capability CCβ, linearity, and applicability. Milk samples bought from a Belgian supermarket were analyzed, and after confirming the absence of antibiotics were used as blank samples in the validation studies.

To determine the specificity of the proposed method, a set of extract of blank milk samples ($n = 20$) were injected into the chromatographic system on the same day. The process included also an analysis of the spiked samples on three levels. This permits the specificity to be evaluated through the average and standard deviation of the noise amplitude (S/N), expressing relative to the internal standard signal amplitude. When a ratio of signal to noise (S/N) of blank sample is higher than 3, its relative retention time (RRT) is equivalent to RRT of the spiked milk sample and its response area is higher than 1 % of that of the spiked milk sample, then, the result is deemed false positive. Moreover, the selectivity was guaranteed by following up the multi-reaction monitoring (MRM) transitions per substance on LC-MS/MS and the relative retention time. The detection capability (CCβ) which is the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β = 5 %. Probability of false non-compliance ≤5 % was tested with 20 milk samples. The latter, had been spiked at 0.5 MRL and analyzed according to two criteria. Firstly, relative retention time (RRT) of the suspect sample had to be in a range of ±2.5 % around the RRT of the standard solution. Secondly, S/N ratio of the daughter ion had to be equal or higher than 10. In order to get a linear calibration curve at 5 points, standard solutions series of 0.25, 0.5, 1, 1.5 and 2 MRL were injected with the series of the samples extracted with 4 different methods (Table 1). In particular, these solutions were prepared using three mixtures of antibiotic standards at

Table 4 Maximum residues limits (MRL*), CCβ and number of samples analyzed for each antibiotic used for the validation of the selected method

Family of antibiotics	Antibiotics	MRL in milk (μg/L)	CCβ (μg/L)	Detection	
				CCβ spiked	CCβ Blank samples
Penicillins	Amoxicillin	4	2	20/20	20/20
	Penicillin G	4	2	20/20	20/20
	Ampicillin	4	2	20/20	19/20
	Cloxacillin	30	15	20/20	20/20
	Dicloxacillin	30	15	20/20	19/20
	Nafcillin	30	15	20/20	20/20
	Oxacillin	30	15	20/20	20/20
Quinolones	Enrofloxacin	100	50	20/20	20/20
	Marbofloxacin	75	37.5	20/20	20/20
	Flumequine	50	25	20/20	19/20
Macrolides	Erythromycin	40	20	20/20	19/20
	Spiramycin	200	100	20/20	20/20
	Tilmicosin	50	25	20/20	20/20
	Tylosin	50	25	20/20	19/20
	Neospiramycin	200	100	20/20	20/20
Tetracyclines	Chlortetracycline	100	50	20/20	20/20
	Oxytetracycline+ 4-epi-oxytetracycline	100	50	20/20	20/20
	Tetracycline+ 4-epi-Tetracycline	100	50	20/20	20/20
Sulfonamides	Sulfadiazine	100	50	20/20	20/20
	Sulfapyridine	100	50	20/20	20/20
	Sulfamethoxypyridazine	100	50	20/20	20/20
	Sulfadoxine	100	50	20/20	20/20
	Sulfadimethoxine	100	50	20/20	20/20
	Sulfadimidine	100	50	20/20	20/20
	Sulfamonomethoxine	100	50	20/20	20/20
	Sulfamoxole	100	50	20/20	20/20
	Sulfaquinoxaline	100	50	20/20	20/20
	Sulfachloropyridazine	100	50	20/20	20/20
	Sulfathiazole	100	50	20/20	20/20
	Sulfamerazine	100	50	20/20	20/20
	Sulfamethoxazole	100	50	20/20	20/20
	Sulfacetamide	100	50	20/20	20/20
	Sulfisoxazole	100	50	20/20	19/20
	Trimethoprim	50	25	20/20	20/20
	Sulfaguanidine	100	50	20/20	20/20
	Sulfamethizole	100	50	20/20	20/20

*MRL (EU 37/2010)

1MRL: mix 1 (macrolides – tetracyclines), mix 2 (sulfonamides - quinolones), and mix 3 (penicillins). After adding specific volume of each internal standard to the mix, the intermediate solutions were evaporated to dryness at 40 °C. The specific volumes of penicillins and Milli-Q water were added in order to obtain final solutions at 300 μL with final concentrations of 250, 500, 1000, 1500, and 2000 μg/L, respectively. In order to evaluate applicability of the present multi-residue method, the collected milk samples (194) were analyzed. Additionally, the interpretation of results was based on five criteria (Commission Decision 2002) which

were: a S/N ratio of the ionic transitions greater than ten, the difference of the chromatographic retention time was within 2.5 % range of the retention time of the same peak in standard solution, area of the sample was higher than area of blank, area of sample was higher than area of spiked sample, and concentration of the sample analyzed was higher than MRL and LOD.

Stability of antibiotics in milk

Taking in consideration that lben is produced within 1 day of fermentation at temperature room; the stability of antibiotic residues was evaluated. Consequently, milk purchased in a Belgian supermarket was used either as a negative control ($n = 2$), or enriched ($n = 3$) as a test material. The enrichment was done at 0.5 MRL as prescribed for meat matrices (Commission Regulation 2377/1990) with mixtures of antibiotic standards of sulfonamides, penicillins, quinolones, macrolides, and tetracyclines. The samples were stored at different storage temperature (4 and 21 °C) and tested at various time periods (day 0, 2 and 7). The analysis was performed using LC-MS/MS after extraction (method 3). First and for most, the initial values were determined on the day of extraction (day 0). After that, samples stored at 4 and 21 °C were analyzed at day 2 and day 7 of storage. Some of these abused storage conditions also mimicked or overestimated the transport of the samples from Algeria to Belgium as well as possible practices in Algeria. These data also served for antibiotic stability evaluation.

Statistical analysis

In order to select the most suitable method extraction; the analytical methods were compared on the basis of the recoveries sufficient for the screening purposes, and only for the antibiotics having lower MRL in milk than in meat. The latter was defined as the ratio of MRL in milk to MRL in meat by being lower or equal to 0.5. In addition, Sstatistical analysis of the recovery results obtained with the four methods was performed using two-factor weighted ANOVA test with replication ($n = 2$) using S-Plus 8.0 for Linux (Tibco, Palo Alto, CA, US). The first factor was the method used and the second was the type of antibiotic. At the same time, the interaction between these two factors was also studied. Since the latter was significant, the comparison between the four methods was done per each antibiotic and corrected for simultaneous hypothesis testing according to Sidak (1967). Similar was done for comparison of storage effect on antibiotics. Statistical significance was tested at significance level of 0.05 and 0.01.

A generalized linear model for binomially distributed data was fit to model the frequency of positive LC-MS/MS and Delvotest SP-NT results using the logit link. These results were contrasted between different types of milk collected at the farms and points of sale. In particular, milk type was considered as a fixed factor, whereas, farm and points of sale were random ones. Also, the comparison between the different milk types was evaluated and *P*-values were corrected for simultaneous hypothesis testing, according to Tukey. For correlation purposes, results of the examined residues (without MRL, and non-authorized) using LC-MS/MS were considered as positive.

The agreement between the two tests was calculated using Cohen's kappa and its associated *P*-value. In addition, since the Delvotest SP-NT appeared to lack specificity, a list of antibiotics was set up containing the positive LC-MS/MS results and those for which there were more negative than positive Delvotest SP-NT.

Results

Optimization of the LC-MS/MS screening method

The main requirement for a reliable screening method is to detect authorized substances above the regulatory limits MRL and unauthorized at a level of MRPL; which is the minimum required performance limit, minimizing false negative results (Freitas et al. 2013). Before the final method validation, four methods were compared and the results presented in Table 2 were statistically evaluated to select the appropriate one. Comparison using ANOVA was limited to antibiotics (11 antibiotics) with lower MRL in milk than in meat (MRL ratio equal or lower than 0.5). Since highly significant interactions were observed between methods and antibiotics, methods were compared with each other for each of those selected antibiotics separately. Among the comparative results, the main differences were marked between two method groups. Particularly, out of 11 compared antibiotics, the recoveries of eight (method 1 as opposed to method 4) and seven (method 2 as opposed to method 3) were significantly different ($p < 0.05$). This result may be explained by the analytical differences of the methods. Method 3 presented recoveries that were higher than other methods and closer to 100 %. In a second step the lowest recoveries were compared among the methods to ensure that final method may detect all selected antibiotics. It was observed that only for erythromycin and nafcilin higher recoveries were obtained when applying method 3. Taking this in account and the fact that other compounds were acceptable for screening purposes by method 3, it was decided to use this method further in the study.

The optimized extraction method was validated for macrolides, penicillins, quinolones, sulfonamides, and tetracyclines. Except, ampicillin, dicloxacillin, flumequine, erythromycin, tylosin, and sulfisoxazole which were characterized with some interference peaked within

retention time range resulting in 5 % of false positive, the rest of the specificity compounds analyzed in 20 blank milk samples were negative. The detection capability levels were 50 % of MRL for all compounds in accordance to the guidelines for the validation of screening methods for residues of veterinary medicines (Table 4) (Community Reference Laboratories 2010). A linear calibration curve was obtained according to concentrations used. The correlation coefficient was ≥0.80 for all analyzed substances. One hundred ninety- four (194) milk samples were analyzed using this screening method. Within these, one hundred twenty-seven (127) were found non-conform. More details are presented in LC-MS/MS results section.

Delvotest

Out of 154 samples analyzed in this study 39 were positive forming 25.3 % of the total samples. According to the results presented in Table 5, the highest frequencies of positive results by Delvotest SP-NT prescreening were obtained for treated cow's milk (61.5 %), followed by untreated cow's milk (20.8 %), and market fermented cow's milk (20 %). Yet, market raw cow's milk (16.7 %) and bulk tank milk (10.3 %) were tested less positive than treated cow's milk. Correspondingly, a high percentage of positive results in the sample category of treated cow's milk were significantly different from untreated cow's milk and bulk tank milk ($p \leq 0.01$).

LC-MS/MS results

The comparison between different types of milk collected from distinct sources did not reveal any significant differences. As presented in Table 5, 127 out of 194 samples analyzed were found positive (65.5 %). The highest frequencies of positive results with LC-MS/MS analysis were obtained for milk collected from the market where each sample of collected raw milk was contaminated (100 % samples positive) and followed by fermented milk (85 % samples positive). High percentage of positive results was similarly found in samples collected from the farms. Hereby, the milk collected from treated cows contained the most antibiotic residues (68 %), followed by milk samples collected from untreated cows (58 %), and lastly bulk tank milk (54 %).

Correlation between LCMS/MS and Delvotest SP-NT results and occurrence of specific antibiotics

Only 131 milk samples were used for the comparative assessment of results obtained by Delvotest SP-NT and LC-MS/MS. Specifically, the comparison of the total numbers of results using Cohen's Kappa illustrated that there was no evidence of agreement between results obtained by LC-MS/MS and Delvotest SP-NT (Table 6). Furthermore, from 131 milk samples only 18 samples were negative by both LC-MS/MS and Delvotest SP-NT methods. In this study, 15 samples showed doubtful results and 13 samples presented positive results with Delvotest SP-NT. Both groups of samples (doubtful and positive results) were later confirmed negative by LC-MS/MS. Furthermore, results presented in Tables 6 and 7 also show that in 52 samples initially found negative by Delvotest SP-NT mutltiply antibiotics were detected by LC-MS/MS. The most abundant residues (β-lactams) were followed by macrolides. Sulfonamides, quinolones, and tetracyclines consecutively were present at similar low frequencies. Finally, 20 milk samples were confirmed positive by both methods and additionally 13 samples initially found questionable by Delvotest SP-NT were confirmed positive by LC-MS/MS. This all implies that LC-MS/MS method was more sensitive than Delvotest SP-NT.

Stability of antibiotics in milk during storage

The noticeable increase in measurable antibiotics was observed after storage. The followed antibiotics were spiked to blank milk samples which were stored during short (2 or 7 days) period. After 2 days there was no change of antibiotics independently of the storage conditions, but a significant increase in measurable antibiotics was noticed after 7 days both at 4° and 21 °C. These differences are shown in Fig. 1. For most of the antibiotics this difference was significant independently of the temperature at which milk was stored whereas for certain antibiotics it was insignificant ($p > 0.05$). This was for neospyramycine, sulfamethoxypyridazine, sulfaquinoxaline,

Table 5 Comparison of the results for different milk types obtained by Delvotest and LC-MS/MS

	Delvotest negative	Delvotest positive	%Delvotest positive	LC-MS/MS conform	LC-MS/MS non-conform	% LC-MS/MS positive
Bulk tank milk	26	3	10.3[a]	16	19	54[a]
Individual untreated cow's milk	57	15	20.8[a]	41	58	59[a]
Individual treated cow's milk	10	16	61.5[b]	7	15	68[a]
Market raw cow's milk	10	2	16.7[ab]	0	18	100[a]
Market fermented cow's milk	12	3	20[ab]	3	17	85[a]

Groups with the same letter behind the percentage of % Delvotest positive are not significantly different from each other at level of 0.05
Groups with the same letter behind the percentage of % LC-MS/MS positive are not significantly different from each other at level of 0.05

Table 6 Statistical agreement between results obtained by two techniques Delvotest SP-NT and LC-MS/MS

	Delvotest negative	Delvotest positive	Delvotest doubtful
LC-MS/MS conform	18	13	15
LC-MS/MS non-conform	52	20	13

Total number of results for each results category was compared and Cohen's kappa (−0.1042, *P* value: 0.1578) was calculated

OTC+epi-OTC2 and TC+epiTC1 at 4 °C; for penicilline G, trimetoprime at 21 °C and for: tylosine, dicloxacilline, sulfapyridine, sulfisoxazole at both temperatures. The results also shown lack of significant difference in antiobitic concentration in milk kept 2 days on either temperatures. However, the significant difference among some antibiotics was noticed after analyzing samples kept 7 days. At 21 °C after 7 days it was possible to measure significantly more antiobitics, in particular neospyramycine, spyramicine, cloxacilline, dicloxacilline, penicilline G, sulfacetamide, sulfadoxine, sulfamethoxazole, sulfamoxole.

Discussion

Monitoring large numbers of milk samples for the presence of residues in excess of the levels laid down under community legislation requires low cost screening methods. In practice, it is primarily performed using microbiological screening methods, because of their high cost-effectiveness compared to physical–chemical detection. In general, these assays can be operated: without special training, do not depend upon specialized equipment, and target a broad spectrum of antimicrobial residues within one test (Pikkemaata et al. 2009). The most widely used tests which are commercially available are microbial inhibitor tests with spores of *Bacillus stearothermophilus var. calidolactis* –Delvotest SP, Copan Test, Charm Farm-960 Test, and others (Žvirdauskienė and Šalomskienė. 2007). Within this study, Delvotest SP-NT was the selected one to be used for the assessment of antibiotic residues in milk samples collected in Guelma (Algeria). As the main limitation of this and other similar microbial assays non-specificity is usually assumed (Bilandzic et al. 2011). For a visual reading of in particular Delvotest SP-NT, clear yellow and clear purple colors are easy to determine. However, visual assessment of samples containing intermediate concentrations of antimicrobials is more difficult, even for experienced technicians which render the visual judgment of the colored reaction as in Delvotest SP-NT subjective (Suhren and Luitz. 1995; Stead et al. 2008). In such samples, the test medium often shows a cloud of purple in a yellow background indicating a suspect positive result. In addition, different types of milk and the different modes of action of antimicrobial compounds can lead to different colors in the test, making the interpretation more difficult (Stead et al. 2008). In turn, this makes Delvotest SP-NT less suitable for decisive analyses leaving spaces for disputable results (false positives, eg). Another explanation could be that false positive Delvotest SP-NT reactions may occur in samples from freshly calved cows due to the fact that natural inhibitors and incomplete milking could be responsible for positive reaction (Hillerton et al. 1999).

Table 7 Comparison of the results obtained by LC-MS/MS and Delvotest SP-NT given as frequency (%) of the measurement appearances

Antibiotic	(Frequency of results, %)		
	LC-MS/MS positive & Delvotest negative	LC-MS/MS positive & Delvotest positive	LC-MS/MS positive & Delvotest doubtful
Ampicillin	26.9	10	38.5
Oxacillin	9.6	/	/
Tylvalosin	11.5	5	/
Tilmicosin	13.5	10	/
Amoxicillin	42.3	40	/
Erythromycin	1.9	/	/
Tilosyn	1.9	/	/
Sulfathiazole	1.9	/	/
Sulfamerazine	1.9	/	/
Sulfamethizole	1.9	/	/
Enrofloxacin	1.9	/	/
Spiramicin	1.9	/	/
Nafcillin	1.9	/	/
Josamycin	1.9	/	7.7
Tulathromycin	1.9	/	/
Doxycyclin	1.9		/

To confirm the results obtained by those screening tests more specific, fast and sensitive techniques could be used. One such a method is LC-MS/MS technique which was employed in this study to verify the results of the Delvotest SP-NT. The comparative analyses pointed out several discrepancies between the methods, a number of false negative results using Delvotest SP-NT were identified and the sensitivity of Delvotest SP-NT for different groups of antibiotics as well as milk type was evaluated.

Whereas according to Delvotest SP-NT the most contaminated samples were those obtained from treated cows, LC-MS/MS revealed that the samples collected from the markets were mostly contaminated. Antibiotics in milk of treated cows could arise from a collection of milk shortly after administration of antibiotics. Subsequently, it may also reflect a certain disrespect of a prescribed antibiotic withdrawal time due to overdose usage, failure to observe withdrawal time, drug misuse or the bad hygiene (Zinedine et al. 2007; Petrović et al. 2008; Mensah

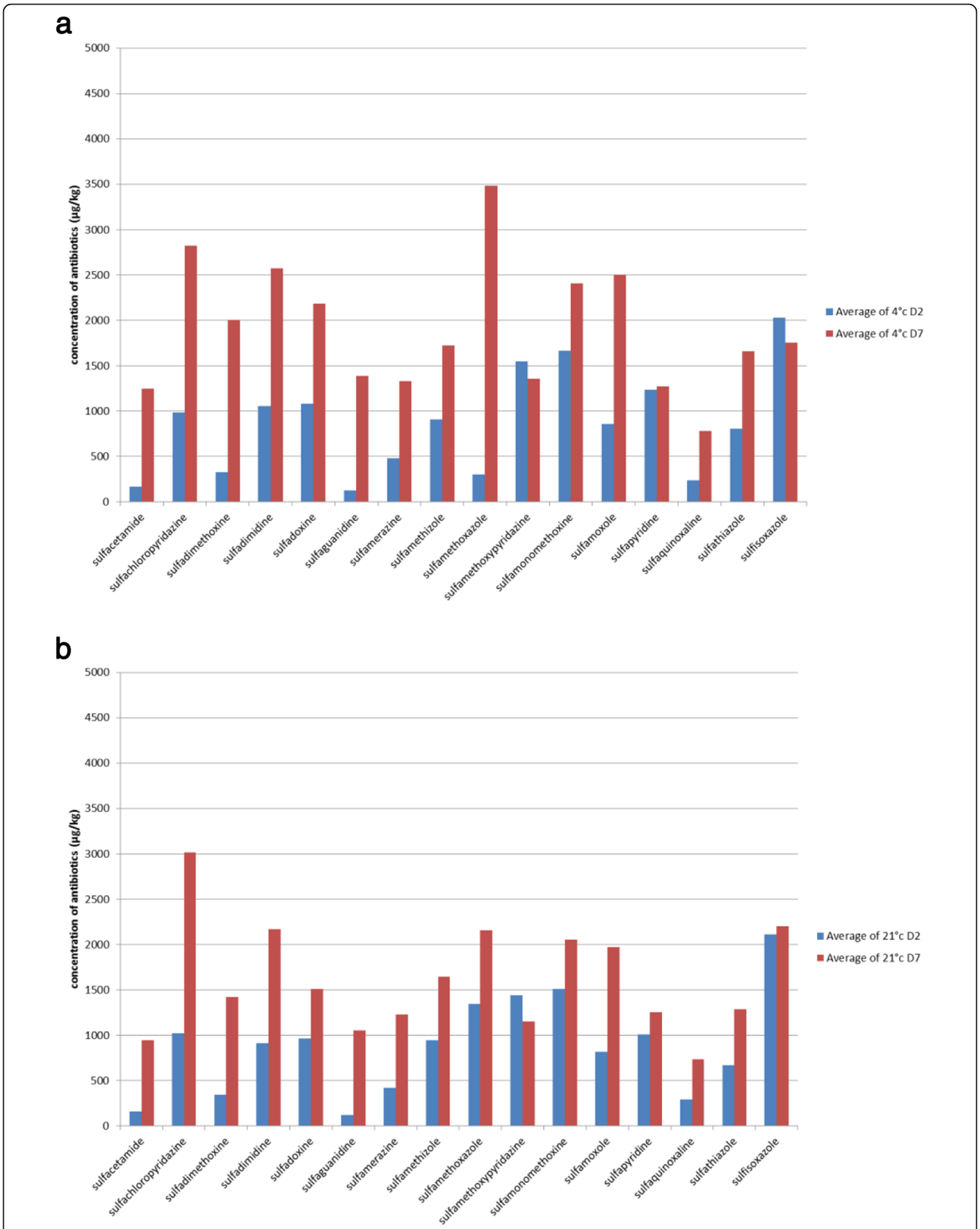

Fig. 1 Estimation of the antibiotic concentration in samples stored at two temperatures (4° and 21 °C) and during two periods (2 and 7 days). *The results present the estimated concentration of antibiotics measured in samples stored at 4 °C (**a**) and at 21 °C (**b**). The results are average of three measurements

et al. 2014b; Boultif. 2015). Establishment of a withdrawal time depends on different criteria (type of used antibiotic, quantity of given antibiotic, the way of applications, and as well age, health status, lactation stage and individual features of dairy animals) (Nikolić et al. 2011). Certain studies indicated that some African breeds may differ in terms of their genetic heritage making them more suited to local climatic conditions (water consumption, volume of distribution, and renal clearance) but non directly comparable to antibiotics withdrawal time as determined for breeds representative of a large-scale production in developed countries (Mensah et al. 2014a). Therefore, certain adaptation to local conditions could be envisaged.

Milk samples collected form the markets were provided by different private milk collectors that brought milk in isotherm tanks collected from private and/or governmental farms, and from small-scale cow's milk producers. The latter are widely dispersed in rural Algerian territories and located very far from urban areas and far away from any control by competent authorities. Actually, the accurate detection of low levels of antimicrobial drug residues in milk, as it was done in the study, is not only of great importance for governmental control laboratories and the dairy industry, but also for farmers to enable them to ensure that contaminated milk from individual cows is not consigned to the bulk tank (Stead et al. 2008) and prevent the further transfer of antibiotics in the food chain.

Algerian executive decree N°14-366 of December 15th, 2014 that regulates the conditions and the applicable modalities regarding contaminants tolerated in foodstuffs, does not mention sufficient information on the control plan and the dosage of the residues of antibiotics (Décret exécutif 2014). In fact, auto-control in dairy industries is commonly done following the European legislation. In contrast, quality assurance is rather low or not strictly followed in many African countries like in Algeria. Hence, the lack of statutory legal framework in Algeria may be a contributor to a high presence of the forbidden or regulated antibiotics residues in animal-derived foodstuffs (Mensah et al. 2014b).

In this study lben samples were highly contaminated. The fermented milk 'lben' is prepared by letting raw cow's milk to be naturally fermented within 24 h at room temperature. Fermentation of milk mainly involves lactic acid bacteria (LAB), but micrococci, coryneforms, yeasts and moulds can also occur (Zamfir et al. 2006). After that, it must be subjected to churning and removing of butter. Literature suggests that the presence of antibiotics in milk may affect fermentation processes in food production industries (Hsieh et al. 2011). Therefore, the fermentation during lben production may be affected if residues of antibiotics are present by possibly inhibiting the growth of the starter cultures (Nikolić et al. 2011). For this reason, the performance of Delovtest SP-NT was questioned. Whereas no specific limitations for analyzing antibiotic residues in fermented milk are neither mentioned by the manufacturer nor verified in some studies (Hennart and Faragher 2012), the fate of antibiotics in milk type as lben is not fully evaluated. Except a study on raibi milk from Morocco (Zinedine et al. 2007), there is a lack of scientific data on fate of antibiotic residues in fermented milk (lben). This milk type is sour milk and may be a product of mostly lactic acid bacteria fermentation (Ouadghiri et al. 2009) and therefore may contain some byproducts, certain natural antimicrobial substances of that fermentation type. In turn, results obtained may represent the reaction of the test to those antibiotics already present in raw milk (Zinedine et al. 2007).

The shelf life of 'lben' is about 3 days at 4 °C. However, it was reported that sometimes "lben" may be kept at room temperature in the countryside with limited electricity supply. In this conditions 'lben' reaches high acidity levels after 2–3 days (Benkerroum and Tamime 2004). The results from our study have shown that in these conditions the antibiotic concentration appears to increase. This was independent of the temperature conditions (both 4° and 21 °C). More studies would be necessary to reveal whether these antibiotics would be easier bioavailable increasing the exposure to the consumers.

Recently, few studies in Algeria were performed on the presence of antibiotic residues in milk. Using Delvotest SP NT, Hakem et al. (2012) had found no contamination of raw milk collected from two Dairies Mitidja's Farms. However, in the present study, around 10 % of bulk tank milk samples and 20 % of samples of untreated cow's milk were positive. The latter was higher than the contamination prevalence in Algiers (9. 87 %) indicated by Ben-Mahdi and Ouslimani (2009). Other authors, Zinedine et al. (2007) in Morocco Tarzaali et al. (2008) in Mitidja, Aggad et al. (2009) in the west of Algeria, Titouche et al. (2013) in Tizi-Ouzu, reported higher frequency of antibiotics positive milk samples (57, 89, 29, and 46 %, respectively). Similarly, frequency of positive farm bulk tank milk (40 %) was found to be higher in a study from Serbia (Petrović et al. 2008). Also results obtained for market raw cow's milk in the present study were comparable to those found in a study from Iran (19.78 %) (Aalipour et al. 2015). Nevertheless, contamination of marketed fermented cow's milk was lower than results attained for raibi milk (50 %) in the study of Zinedine et al. (2007). In general, 65.5 % of all analyzed samples using LC-MS/MS contained antibiotic residues at levels exceeding MRL. This positive frequency is much higher than 15 % (Li et al. 2012), 16.66 % (Meng et al. 2015) in China, 1.76 % (Martins et al. 2014) in Brazil, and 28 % (García et al. 2016) in Spain. Pereira et al. (2014) indicated that 47.17 % of analyzed samples were at

detectable concentration whereas Han et al. (2015) found 12 % at levels lower than MRL. Delvotest SP-NT has varying sensitivity to different antibiotics groups. According to the manufacturer and several reports (Althaus et al. 2003; Stead et al. 2008; Sierra et al. 2009a; DSM 2012, and Beltran et al. 2015), the sensitivity of Delvotest SP NT to β-lactams, as a main group of veterinary drugs used in therapy of cows in many countries is high except for cloxacillin. This one may be detected at levels higher than the MRL (Petrović et al. 2008). Delvotest SP-NT sensitivity to macrolides is limited to tylosin but erythromycin and spiramycin are detected at levels higher than MRL (DSM 2012). Althaus et al. (2003); Stead et al. (2008); Sierra et al. (2009b) and Beltran et al. (2015) also confirmed that the detection of erythromycin was at levels higher than MRL whereas, there are no data linked to Delvotest SP-NT sensitivity for the other macrolides (tilmicosin, tulathromycin, tylvalosin and josamycin). Furthermore, Delvotest SP-NT sensitivity to sulfonamides is limited only to sulfathiazole (DSM 2012). Enrofloxacin and doxycycline detection levels of this test were described by Sierra et al. (2009b). The latter argued that when the milk sample contained residues below the detection limit (LOD), the spores germinated and grew, thus, their metabolic activity made the indicator change the color. Althaus et al. (2003), Sierra et al. (2009b), Le Breton et al. (2007), and Comunian et al. (2010) noticed that Delvotest SP-NT demonstrated a lower ability to detect some other tetracyclines.

Both methods were in accordance for some milk samples that were found positive. The most frequently detected antibiotics were β-lactams. Mainly, amoxicillin (the most abundant residue in the studied milk) and ampicillin were found in half of the samples; which was still less than penicillin (97 %) and/or tetracycline (88 %) as reported by Ben-Mahdi and Ouslimani (2009), and Titouche et al. (2013), respectively where standard microbiological methods were used. The low cost of β-lactams in Algeria makes them easily available. The latter facilitated the use of penicillin by private farmers without veterinarian supervision in isolated places. Moreover, the presence of macrolides with low frequencies of tilmicosin and tylvalosin as -a residue without MRL - could be explained by their sporadic use. Aminoglycosides were not analyzed by LC-MS/MS method due to their relatively high MRL in comparison to meat. Fundamentally, Delvotest SP-NT manufacturer reported that the sensitivity of Delvotest SP-NT for dihydrostreptomycin and streptomycin is higher than MRL and lower than MRL for neomycin. Neomycin, in its turn, could be present within the 20 positive results of both methods. The high levels of contamination of milk samples by antibiotic residues can mainly be explained by massive and uncontrolled intermammary pharmaceutical preparations used for the treatment and prevention of bovine mastitis, while the withdrawal times after treatment were probably not correctly respected. Similarly, the voluntary addition of bacterial growth inhibitors (antibiotics, antiseptics) in order to stop microbial growth and stabilize the microbial quality of milk (Zinedine et al. 2007) may also be considered as a possible cause. Another argumentation is supported by the study of Reybroeck (2010) where it was stipulated that the main reason for antibiotic residues in milk was the accidental milking of treated cows that went unnoticed in 66 % of the cases and the non -compliance of withdrawal time deadlines in 41 % of the cases.

Conclusion

To conclude, the study findings preliminary revealed that the presence of antibiotics in raw and fermented cow's milk collected in Guelma region and intended for either direct consumption and/or fermentation was high. The results highlighted 65.5 % of non-conform samples contained authorized residues at levels higher than the MRL, residues without set MRL, or non- authorized residues. The occurrence of antibiotic starting from farm's milk and ending in milk purchased from markets in Guelma province indicated the necessity of further control of milk. Providing that there is a lack of data in this domain, the control and assessment might be considered on a national level. Additionally, to obtain results an extraction method with LC-MS/MS detection was validated following regulatory criteria and demonstrated satisfactory results for all parameters. The comparison of results of both methods showed that Delvotest SP-NT could not be accurately trusted under these circumstances. However, LC-MS/MS represented a better screening alternative with a possibility to further investigate each group of compounds applying the specific extraction to increase the sensitivity of the method and decrease the LOD. The present LC-MS/MS method could be used in Algerian National Residue Control Plan as a versatile analytical tool to monitor and determine the occurrence of antibiotic multi-residues in milk and other food matrices after optimization.

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Authors' contributions

SL and DEB conceived the study. SL performed the collection of the samples, al practical work, the interpretation of the results, and drafted the manuscript with supervision of MA. MA designed the stability study and the

LC-MS/MS analytical method approach. WC performed the statistical analysis of all the results. All authors took parts in drafting the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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