Received: 07 August 2022, Accepted: 28 August 2022

Original Research

Journal of Advanced Veterinary Research
(2022) Volume 12, Issue 4, 456-461

Assessment of Microbial Safety and Quality of Market Raw Milk and Pasteurized Milk Sold in Dakahlia Governorate, Egypt

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Abstract

In this study, assessing the microbial quality and safety of the market raw milk and pasteurized milk is the basal objective of this study. One hundred and twenty (120) milk samples (60 of each) were collected randomly from different supermarkets and retailer shops in Dakahlia governorate, Egypt. Samples were analyzed for Total bacterial count (TBC), total coliforms (TCC), Total *Staphylococcus* count (TSC), and Total yeast and mould count. All market milk samples, and pasteurized milk were found to be contaminated and judged poor. *Listeria* spp. were isolated on Oxford agar and then subjected to biochemical and molecular identification. The overall isolation rate of *Listeria* spp. from the market raw milk was 28.33%. The prevalence rates of *Listeria monocytogenes* were 13.33% in raw market milk. The prevalence of *Listeria* innocula and other *Listeria* species were 3.33% and 11.67%, respectively. Polymerase chain reaction (PCR) was used to detect the virulence genes (16SrRNA, *hylA*, and *prfA*) of 8 biochemically identified *L. monocytogenes* strains recovered from raw market milk using specific primers. 16SrRNA, *hylA*, and *prfA* genes are considered the best indicator for virulence determination of *L. monocytogenes* isolated from market raw milk. A high microbial load of market milk and pasteurized milk may present a public health hazard to the consumers and emphasizes the need for improved hygienic standards.

KEYWORDS

Microbial quality, Safety, Market raw milk, pasteurized milk, TBC, TCC, TSC, Yeast-mold count, *Listeria monocytogenes*

INTRODUCTION

Milk is considered a complete food as it contains abundant nutrients required for growth and development in infants, adults, and elder ones. It is a good source of proteins, fats, sugars, vitamins, and minerals (Lahankar *et al*., 2019). Since milk is a complex biological fluid, many microorganisms can thrive well in it. The microbiological content of milk is a key factor in assessing its quality since it is hard to avoid microbial contamination due to the specific production methods (Singh *et al*., 2011). Raw milk contamination can come from a variety of places, including the air, milking equipment, feed, soil, human waste, and grass (Coorevits *et al*., 2008).

This makes it vital to keep these products wholesome, safe, fresh, clean, and free from contamination with spoilage as well as pathogenic microorganisms at all times (Bille *et al*., 2009). In the dairy industry, as well as in the fields of medicine and public health, milk quality remains a contentious issue. Every dairy operation should strive to produce as much milk as possible in the highest possible quality. The bacterial count of raw milk is more significant to the final product's quality as the dairy industry pushes toward increased production of milk and milk products with longer shelf life (Boor *et al*., 1998).

In recent years, many dairy processors have increased high temperature, short time (HTST) pasteurization temperatures over the minimum requirements stipulated by the Pasteurized Milk Ordinance (72°C for 15s; enhance fluid milk shelf life) (Gandy *et al*., 2008). During production, processing, and handling, dairy products are susceptible to contamination with various bacteria from various sources, rendering them unfit for consumption and posing a risk to the public's health (Todaro *et al*., 2013).

To eliminate such dangerous microorganisms, pasteurization is used. If milk is not adequately pasteurized, there will be a higher number of bacteria present in the milk. To make fluid milk safe and increase its shelf life, the dairy industry uses a heat treatment procedure. Most of the milk processed in Egypt is ultra-heated (UHT) sterilized, which is costly for most consumers and encourages them to purchase retail raw milk (Metwally *et al*., 2011).

Foodborne infections should not be present in raw milk. Foodborne pathogens like *Campylobacter jejuni*, Shiga-producing *Escherichia coli* (STEC), *Listeria monocytogenes*, *Salmonella* spp., enterotoxigenic *Staphylococcus* aureus, Yersinia enterocolitica, *Mycobacterium bovis*, *Brucella* spp., *Coxiella burnetti*, and others have been found in raw milk in various surveys. Some of

ISSN: 2090-6277/2090-6269/

these foodborne pathogens can be found on the skin and gastrointestinal tracts of animals that produce food as well as in the farm environment (Oliver *et al*., 2005).

In the agricultural and food industries, *Listeria* species are common, and they regularly contaminate milk and dairy products (Meyer-Broseta *et al*., 2003). *Listeria monocytogenes* is a significant pathogenic microbe among the genus of *Listeria*, which causes the infection of Listeriosis in both animals and humans, and the more infrequently occurring bacteria *Listeria* ivanovii is pathogenic for humans (McLauchlin *et al*., 2004).

MATERIALS AND METHODS

Samples collection and preparation

One hundred and twenty (120) samples of raw and pasteurized milk (sixty samples of each), raw milk samples were collected in clean, sterile sampling bottles with a capacity of around 500 ml each and pasteurized milk samples were collected in their containers. Samples were obtained (May 2019 to April 2020) from dairy stores and supermarkets in Dakahlia governorate, Egypt. Collected samples were transported directly to the Microbiology and Food Hygiene laboratories of Animal Health Research Institute (Dokki, Giza, Egypt) in an insulated icebox (3±1°C) to be evaluated instantly.

Physical examination

All samples were examined for color and odor before being subjected to bacteriological examination to ensure that the samples were normal.

Microbiological evaluation

Preparation of ten-fold serial dilutions of milk samples (APHA, 2001): One ml of well-mixed sample was added aseptically to nine ml sterile distilled water to form (10^{-1}) dilution and so on (10^{-2}, 10^{-3}, 10^{-4}). Bacteriological counts were conducted according to the established methods: Total aerobic bacterial count (APHA, 2001), total coliform count (Swanson *et al*., 2001), total *Staphylococcus* count (Roberts and Greenwood, 2003), total yeast and mould count (Roberts and Greenwood, 2003), isolations and identification of *Listeria* spp. (Hitchins, 1998).

All used media were obtained from Oxoid (Oxoid Ltd., Hampshire, UK). Each sample (25 ml) was added to 225 ml of sterile *Listeria* Selective Enrichment Broth (Oxoid) and incubated at 30°C for 48 h. A loopful of broth was streaked on surface of Oxford agar (Oxoid). The Oxford plates were incubated at 35°C for 48 h under aerobic conditions. All colonies surrounded by black halo zoon were suspected *Listeria* spp.

Five suspected *Listeria* spp. colonies from each plate were chosen and purified on tryptic soy agar (Oxoid CM 131) with 0.6% yeast extract (Oxoid L 21) and incubated at 30°C for 24-48 h for further biochemical characterization. Presumptive *Listeria* isolates were confirmed and identified to species level based on Gram staining, typical umbrella motility in SIM medium (Oxoid CM 435), H2S production, indole, urease, catalase, oxidase reaction, B-hemolysis, nitrate reduction, methyl-red/Voges Proskauer (Oxoid CM 43), CAMP tests and fermentation of mannitol, L-rhamnose, D xylose, sorbitol, dextrose, maltose, esculin, dulcitol and salicin 4,20,21. Serotyping of isolates was performed with Bacto-*Listeria*-O-antisera types 1 and 4 and poly (Difco Laboratories, Detroit, MI) by the slide agglutination test.

Molecular identification of the species-specific for *L. monocytogenes* and some virulence genes in isolated *Listeria monocytogenes* by polymerase chain reaction (PCR).

Extraction of DNA

Genomic DNA from biochemically identified *L. monocytogenes* strains was extracted by using QIAamp DNA Mini Kit Catalogue no. 51304 (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's guidelines. Emerald Amp GT PCR mastermix (Takara) with Code No. RR310A.

Amplification reaction of L. monocytogenes (Kaur et al., 2007).

The PCR condition has a specific sequence and amplifies a specific product as shown in Table 1. Temperature and time conditions of the primers during PCR are shown in Table 2 according to specific authors and Emerald Amp GT PCR master mix (Takara) kit 3.

Agarose gel electrophoresis (Sambrook et al., 1989).

Amplified DNA fragments were analyzed by 1.5% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBE buffer stained with ethidium bromide and captured as well as visualized on UV trans illuminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

RESULTS

Table 3 represents that the total bacterial count (TBC) in raw market milk ranged from 1.2×10^4 to 5×10^8 cfu/ ml with a mean value of $5.46\times10^7\pm1.57\times10^8$ cfu/ ml, and the total Coliforms count (TCC) ranged from 5×10^2 to 6×10^7 with a mean value of $8.42\times10^6\pm1.91\times10^7$cfu/ ml, total staphylococci count (TSC) ranged from 3×10^2 to 9×10^5 with a mean value of $1.21\times10^5\pm2.75\times10^5$ cfu/ml, the total yeast count ranged from 6×10^2 to 6×10^7 with a mean value of $1.29\times10^7\pm2.48\times10^7$ cfu/ml and the total mold count ranged from 2.8×10^2 to 8×10^6 with a mean value of $1.04\times10^6\pm2.52\times10^6$ cfu/ml.

Table 4 represents that the total bacterial count(TBC) in pas-

Table 1. Oligonucleotide primers sequences.

Primer	Oligonucleotide sequence (5′-3′)	Amplified Product	References
16S rRNA(F)	GGA CCG GGG CTA ATA CCG AAT GAT AA	1200 bp	Wiedmann *et al*. (1993)
16S rRNA(R)	TTC ATG TAG GCG AGT TGC AGC CTA		
hlyA (F)	GCAGTTGCAAGOGCTTGGAGTGAA	456 bp	Swetha *et al*. (2013)
hlyA (R)	GCAACGTATOUTOCAGAGIGATCG		
prfA(F)	CTGTTGGAGCTCTTCTTGGTGAAGCAATCG	1060 bp	Kaur *et al*. (2007)
prfA(R)	AGCAACCTCGGTACCATATACTAACTC		

teurized milk ranged from 4×10^1 to 2×10^4 with a mean value of $1.55\times10^4\pm2.25\times10^4$ cfu/ml., the total Coliforms count (TCC) ranged from 1.5×10^1 to 2×10^2 with a mean value of 9.96×10 ±9.52 ×10 cfu/ml., total staphylococci count (TSC) ranged from 1.2×10^1 to 1.5×10^2 with a mean value of 1.98×10±4.57×10 cfu/ml., the total yeast count milk ranged from 120 to 5×10^2 with a mean value of $1.46\times10^2\pm1.64\times10^2$ cfu/ml. and the total mold count ranged from 1×10 to 52.6×10^2 with a mean value of $1.54\times10^2\pm3.06\times10^2$ cfu/ml.

Table 5 showed that the prevalence of *Listeria* species isolated from raw market milk, results showed that *Listeria* Species were isolated from 17 samples recovered from 60 samples (28.33%) where 8 *L. monocytogenes* (13.33%), 2 *L. innocua* (3.33%) and 7 Other *Listeria* Species (11.67%).

Table 6 represents that the PCR results for *L. monocytogenes* showed that (16S rRNA; *hlyA* and *prfA*) were detected in 8 studied strains, and all studied strains were *L. monocytogenes*. The species- spesific16SrRNA gene was amplified in 8 (100%), *L. monocytogenes* strains to give a product of 1200 bp, and the *hlyA* gene was amplified in 3 (37.5%). *L. monocytogenes* strains gave a product of 456 bp and the *prfA* gene was amplified in 5 (62.5%) *L. monocytogenes* strains giving a product of 1060 bp.

Table 2. Temperature and time conditions of the primers during PCR.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
16S rRNA (F)	94˚C 5 min	94˚C 30 sec	60˚C 45 sec	72˚C 45 sec	35	72˚C 10 min.
16S rRNA (R)	94˚C 5 min	94˚C 30 sec	60˚C 45 sec	72˚C 45 sec	35	72˚C 10 min.
hlyA (F)	94°C 5 min	94°C 30 sec.	50°C 30 sec.	72°C 30 sec.	35	72°C 7 min
hlyA (R)	94°C 5 min	94°C 30 sec.	50°C 40 sec.	72°C 45 sec.	35	72°C 10 min
prfA (F)	94°C 5 min	94°C 30 sec.	50°C 1 min	72°C 1 min	35	72°C 12 min
prfA (R)	94°C 5 min	94°C 30 sec.	50°C 1 min	72°C 1 min	35	72°C 12 min

Table 3. Microbial quality of raw market milk (cfu/ml)

Tested parameters	Min.	Max.	Mean ± SD
TBC	1.2×10^4	5×10^8	$5.46\times10^7\pm1.57\times10^8$
TCC	5×10^2	6×10^7	$8.42\times10^6\pm1.91\times10^7$
TSC	3×10^2	9×10^5	$1.21\times10^5\pm2.75\times10^5$
Total yeast count	6×10^2	6×10^7	$1.29\times10^7\pm2.48\times10^7$
Total mold count	2.8×10^2	8×10^6	$1.04\times10^6\pm2.52\times10^6$
P value		P≤0.001*	

Table 4. Microbial quality of Pasteurized milk (cfu/ml)

Tested parameters	Min.	Max.	Mean ± SD
TBC	4×10^1	2×10^4	$1.55\times10^4\pm2.25\times10^4$
TCC	1.5×10^1	2×10^2	$9.96\times10\pm9.52\times10^1$
TSC	1.2×10^1	1.5×10^2	$1.98\times10\pm4.57\times10^1$
Total yeast count	120	5×10^2	$1.46\times10^2\pm1.64\times10^2$
Total mold count	1×10	52.6×10^2	$1.54\times102\pm3.06\times10^2$
P value	P≤0.001*		

Table 5. Prevalence of *Listeria* species in raw market milk and Pasteurized milk.

Isolates Milk &dairy roduct	Total no.of samples	No. of suspected samples		*Listeria monocytogenes*		*Listeria* innocua		Other *Listeria* species	
		No.of samples	%	No.of samples	%	No.of samples	%	No.of Samples	%
Raw market milk	60	17	28.33	8	13.33	2	3.33	7	11.67
Pasteurized milk	60	0	0	0	0	0	0	0	0

Table 6. Distribution of genus species-specific 16S rRNA, *hlyA*, and *prfA* genes in isolates of *L. monocytogenes* from raw market milk were molecularly confirmed by PCR.

No. of positive Samples	16S rRNA	*hlyA*	*prfA*
8	8/8	3/8	5/8
	-100%	(37.5%).	-62.50%

DISCUSSION

The total bacterial count, which is typically made up of spoilage and lactic acid bacteria, is an excellent way to keep track of how hygienically raw milk is produced, collected, and handled (Chambers, 2002). Therefore, teaching milk handlers about hygiene can greatly lower the number of bacteria in milk. All market milk samples exhibited bacterial loads that were higher than the permitted limit of 105 cfu/ml of milk in the majority of European nations (IFCN, 2006). As a result, the microbial content of milk directly affects both its quality and safety.

Milk samples were examined for their microbiological quality and safety in the current study. The results revealed that the TBC for randomly collected raw market milk samples ranged from 1.2×10^4 to 5×10^8 cfu/ ml with a mean value of $5.46\times10^7\pm1.57\times10^8$ cfu/ ml, These results were higher than the results obtained by El-Diasty and El-Kaseh (2009) and EL-Sebaey (2016), while lower results were reported by El Zubeir and Ahmed (2007) and Abdelhamid (2022) who found mean values of 5.63×10^9 cfu/ ml and $7.32\times10^7\pm1.12.\times10^7$ respectively.

In the authors opinion, poor sanitary conditions during milking, collection and transport are the most frequent source of increased bacterial burdens. The high bacterial load may also be caused by unclean milk handling procedures in supermarkets, a lack of knowledge about hygienic milk processing, and a lack of chilling equipment used in milk production, handling, and shipping.

Concerning the pasteurized milk, the results revealed that TBC ranged from 4×10^1 to 2×10^4 with a mean value of $1.55\times10^4\pm2.25\times10^4$ cfu/ml. This means that the TBC from pasteurized milk had a higher counting of bacterial load. Authors believes that this high level of contamination could arise from contaminated equipment and personnel; delayed pasteurization; and substandard heat treatments. Nearly similar results were reported by EL-Naenaee. *et al*. (2014) and Kumala *et al*. (2021) (2.10 $x10^1$ and $2.94x10^4$ cfu/ml respectively). On the other hand, the current study finding contradicted El-Ziney (2018) who revealed that the TBC of pasteurized milk was $2.95\ x10^1\pm0.62$ cfu/ml.

Related to the total coliforms count (TCC) of raw market milk, the result of the current study showed that the TCC ranged from 5×10^2 to 6×10^7 with a mean value of $8.42\times10^6\pm1.91\times10^7$ cfu/ ml.

This study's findings were remarkably similar to those of Hasan *et al*. (2015), who concluded that the TCC (MPN/ ml) of market milk samples examined in Cairo and Giza governorates-Egypt, ranged from 7.5×10^2 to 2.1×10^7 with a mean value of $1.8\times10^6\pm4\times10^5$ and from 2.1×10^2 to 2.3×10^6 with a mean value of $2\times10^5\pm7.7\times10^4$, respectively. Additionally, this result was inconsistent with a study by Talukder *et al*. (2019) and Kumala *et al*. (2021) who found lower results of TCC of pasteurized milk (6.4×10^5cfu/ml and 5.63×10^5 cfu/ml respectively).

Concerning TCC of pasteurized milk, results of the current study were varying from1.5×10^1 to 2×10^2 with a mean value of $9.96\times10\pm9.52\times10$ cfu/ml. These findings disagree with the study of Nawabi *et al*. (2019) and Limbu *et al*. (2020) who found higher results (>4.9×10^5 cfu and 14×10^3 cfu/ml respectively).

Additionally, this result was inconsistent with a study by Aglawe and Wadatkar (2012) and Singh *et al*. (2015) who reported lower TCC in pasteurized milk samples. On contrary, these findings disagreed with Hussaini *et al*. (2014) who reported absence of coliforms.

From the authors' point of view, the reason for this result is that the detection of the coliform group of bacteria is more important than other bacteria because these are indicators of fecal contamination and imply the possible presence of other gastrointestinal pathogens. These are destroyed during pasteurization treatment and therefore, a positive coliform test of pasteurized milk indicates either inadequate pasteurization or post pasteurization contamination.

Staphylococcal food poisoning (SFP) is one of the most prevalent causes of gastroenteritis worldwide. Symptoms of SFP have a rapid onset (2 to 6 hours) of abdominal cramps, nausea, and vomiting, sometimes followed by diarrhoea. Patients become symptomatic after ingestion of thermostable staphylococcal enterotoxins (SE) of an approximate dose of 0.1 to 1.0 mg/kg of body weight (Bendahou *et al*., 2008).

Regarding total staphylococcal count (TSC), the present study displayed that the TSC of raw market milk ranged from 3×102 to 9×10^5 with a mean value of $1.21\times10^5\pm2.75\times10^5$ cfu/ml. In contrast with finding from the current study, Hasan *et al*. (2015) reported lower results ($5.9\ x10^2$ to $7.9\ x10^2$ cfu/ml). On the other hand, Hossain *et al*. (2011) reported higher results (5.7×10^4 to 1.48×10^6 cfu/ml). Additionally, (Uddin *et al*., 2011) reported higher results of TSC in the raw market milk ($4.7\ x10^7$ cfu/ml).

Concerning TSC in pasteurized milk, the current study results ranged from1.2×10^1 to 1.5×10^2 with a mean value of $1.98\times10\pm4.57\times10$ cfu/ml. Nearly similar findings were obtained by (Hasan *et al*., 2015) who reported that TSC in pasteurized milk was 2.8 x10 to 8.6 $x10^2$ cfu/ml. On contrary, the finding of this study contrasts with the study of (Hossain *et al*., 2011) who reported higher results (1.4×10^3 to 8.1×10^4 cfu/ml). The authors believe that the reason for high TSC in pasteurized milk may be attributed to defective pasteurization machinery, survival of pasteurization, and post-pasteurized contamination such as poor processing and handling conditions and/or poor worker hygiene.

The ability of foodborne molds and possibly yeasts to produce poisonous byproducts known as mycotoxins makes them potentially dangerous to human or animal health. Even though the producing organisms may not survive food preparation, the preformed toxin may still be present because the majority of mycotoxins are stable molecules that are not destroyed. Some foodborne moulds and yeasts can also cause infections or allergic reactions (El-Kholy *et al*., 2016). Yeasts and moulds commonly enter dairy products via airborne pollutants, inappropriate storage conditions, or packing materials, leading to several defects in dairy products (Tamime and Robinson, 2007).

Regarding the Results of total yeast and mould counts of raw market milk, the present study showed that the total yeast count of raw market milk ranged from 6×10^2 to 6×10^7 with a mean value of $1.29\times10^7\pm2.48\times10^7$ cfu/ml, while the total mold count of raw milk ranged from 2.8×10^2 to 8×10^6 with a mean value of $1.04\times10^6\pm2.52\times10^6$ cfu/ml.

The current study finding contradicted El-shinawy *et al*. (2018) who revealed lower findings and reported that the yeasts in raw milk with a mean value of $6.22X10^2\pm3.62X10^2$, while The mean value of molds was $2.23X10^2\pm9.3X10^1$ cfu/ml. Additionally, this result was higher than that of Talukder *et al*. (2019) who found that the yeast-mold count of raw milk was 3.48×10^2 cfu/ml and 4.85×10^2 cfu/ml respectively.

According to the results of the current investigation, the total yeast count in pasteurized milk ranged from 120 to 5×10^2 with a mean value of $1.46\times10^2\pm1.64\times10^2$ cfu/ml. On the other hand, Roostita *et al*. (2011) who stated that it was 1.2×10^6 cfu/g, disagree with these findings.

Also, the results of total mould in pasteurized milk in the present study showed that it ranged from 1×10 to 52.6×10^2 with a mean value of $1.54\times10^2\pm3.06\times10^2$ cfu/ml. On the other hand, the obtained finding is in contradiction with Jodral *et al*. (1993) who reported that it was 25 cfu/ml.

Public health, as well as dairy products, is greatly endangered by the high frequency of *Listeria* spp. in milk, feces, and dairy products. Most listeriosis outbreaks around the world are thought to be caused by milk and dairy products. Pasteurized milk intake caused the first report of listeriosis in the USA in 1983

(Mansouri-Najand *et al*., 2015).

The present study showed the prevalence of *Listeria* species isolated from raw market milk, results showed that *Listeria* species were isolated from 17 samples recovered from 60 samples (28.33%) where 8 *L. monocytogenes* (13.33%), 2 *L. innocua* (3.33%) and 7 Other *Listeria* species (11.67%), but *Listeria* species were not detected in pasteurized milk. Similarly, the prevalence of *Listeria* spp. was reported by Shamloo *et al*. (2015) and Hamidiyan *et al*. (2017).

Also, these findings are in the same line with Akrami-Mohajeri *et al*. (2018) who evaluated the prevalence of *Listeria* species in milk and traditional dairy products and found that 29.2% of examined raw milk samples were positive for *Listeria* species where 7.8% were *Listeria monocytogenes*, while *Listeria innocua* could be isolated from 15% of the examined samples.

In this study, 8 isolates of *L. monocytogenes* were molecularly identified to the species level using PCR amplification of 16S rRNA gene was amplified in eight (100%). Similarly *L. monocytogenes* from different raw milk samples based on 16S rRNA gene were recorded in Egypt (Khedr *et al*., 2016).

Results of PCR for amplification of Listeriolysin O, hemolysin (*hlyA*) gene in *L. monocytogenes* showed that the *hlyA* gene was amplified in three (37.5%). Similar findings were recorded by Ciolacu *et al*. (2015) and El-Gohary (2018) and the *prfA* gene was amplified in five (62.5%). These findings may be supported by the findings of Abd El Tawab *et al*. (2015).

CONCLUSION

According to the current study's findings, Dakahlia governorate still has a poor microbiological quality for both raw and pasteurized milk. The discovery of pathogenic organisms, high levels of coliforms, and Staph. spp. and *Listeria monocytogenes* in raw milk suggested that milk suppliers did not practice good hygiene and sanitation. The presence of coliforms and Staph. spp in pasteurized milk raises several questions, from improper handling to contamination after pasteurization. Regular monitoring of dairy industries and raw milk vendors, awareness programs, and the promotion of proper hygiene practices should all be done to improve the quality of raw and pasteurized milk.

ACKNOWLEDGMENTS

The authors are grateful to the head of the Department of Food Hygiene, Faculty of Veterinary Medicine, Mansoura University for providing the necessary facilities for the study.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related.

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