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Farmers' Knowledge, Attitudes and Practices on Antibiotics Usage and Antibiotic Residues in Dairy Milk in Districts of Boyolali and Malang, Indonesia

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ABSTRACT

The antimicrobial resistance issue has been raised as a global concern. This condition was triggered by the inappropriate usage of some antibiotics related to knowledge, attitude, and practice (KAP). A cross-sectional study was conducted in the Districts of Boyolali and Malang, Indonesia. This study aimed to assess farmer's KAP on antibiotic usage in dairy farms and investigate the presence of antibiotic residues in fresh dairy milk samples. Forty-nine dairy farmers were interviewed to assess the KAP on antibiotic usage. One hundred and twelve (112) milk samples were collected from the farm and examined for antibiotic residue (enrofloxacin, ciprofloxacin, and doxycycline) using high-performance liquid chromatography (HPLC). The interview revealed that 32.65% of the farmers were concerned about the withdrawal period and acknowledged commonly using antibiotics for sickness treatment or prevention. Farmers did not purchase antibiotics but obtained them from veterinarians/para-veterinary field staff, and the most used antibiotics were tetracycline (32.2%), sulfonamides (20.8%), and fluoroquinolones (9.8%). The residues of enrofloxacin, ciprofloxacin, and doxycycline were detected in 4.46, 8.93, and 33.03%, respectively, in the concentration range of 22.4 to 662.2, 15.0 to 38.6, and 44.8 to 557.2ng/g. The doxycycline mean concentration was 84.72ng/g, and 27 of these samples exceeded 100ng/g. This study revealed significant concerns regarding doxycycline residue. These findings contribute to improving knowledge about the use of antibiotics and the presence of residues in dairy products and suggest practical measures to reduce the associated risks.

Key words: Antibiotics, Usage, Dairy farmers, KAP, Milk, Residue

INTRODUCTION

Small-holder farms with less than six milking cows and a daily milk production of about 10 liters per head dominate the Indonesian dairy industry (Guntoro et al. 2016). Farmers sometimes use antibiotics as feed additives to enhance livestock growth and increase milk production, other than preventative and therapeutic purposes, however, some circumstances, including ignorance and overuse of antibiotics led to the antibiotic residue in milk affecting human health. Antibiotic resistance, allergic reactions, carcinogenicity, teratogenicity, disruptions in the natural intestinal environment, and disruptions throughout the dairy industry's manufacturing process are only a few of the detrimental consequences of antibiotic residue on the dairy industry and public health (Sachi et al. 2019).

Fluoroquinolone and tetracyclines are the most commonly used antibiotics by dairy farmers (Du et al. 2019). Fluoroquinolone residues have side effects on humans such as diarrhea, vomiting, nausea, and dyspepsia, as well as attacks the central nervous system (CNS) which causes headaches, dizziness and convulsions (Foo et al. 2023). It is noteworthy that most quinolones exhibit high stability under various storage conditions (Chen et al. 2016). Doxycycline is forbidden in European countries as no residues should be reported (European Commission 2010).

Like many other developing nations, Indonesia has poor enforcement of laws governing the use of antibiotics in cattle, and farmers have easy access to veterinary medications (Widiastuti et al. 2023). Currently, little information about antimicrobial usage in livestock is available in Indonesia. A study conducted in 2018 (Yusuf et al. 2018) concluded antimicrobial usage has also been widely employed to treat non-bacterial diseases due to a lack of information and a shortage of veterinarians.

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Lately, there has been a growing consciousness both domestically and globally regarding the risk associated with consuming food containing drug residues and preventing antibiotic resistance which are not reported in Indonesia, yet. Therefore, the purposes of this study were to evaluate the smallholder dairy farmers' knowledge, attitudes, and practices (KAP) about the use of antimicrobials in livestock production and to investigate the occurrence of antibiotics residues (fluoroquinolones and doxycycline) in raw milk samples in Indonesia.

MATERIALS AND METHODS

Ethical approval

This study does not require approval from animal ethics. The sampling was carried out by the owners of the dairy cows at regular milking time.

Samples questionnaire and collection

This cross-sectional study was carried out in April 2018 and conducted in dairy cattle farms located in two Districts on Java island which are the central for cow's milk production, namely Boyolali (covering 1,015 km^2 surface area) in Central Java Province and Malang (covering 3,530 km^2 surface area) in East Java Province, as seen in Fig. 1. Forty-nine dairy farmers were interviewed based on the questionnaire to determine the factors regarding their knowledge, attitude and practices (KAP) on the use of antibiotics and residue. The questionnaires were divided into two sections: (a) Personal data (5 questions); and (b) The animal handler response to KAP of antibiotic use (13 questions). All questions are presented in Table 1 and 2.

The samples were collected randomly, and 2 samples from different animals were taken 100mL from each dairy farmer, for a total of 112 fresh milk samples. All samples were transported aseptically in sterile bottles to the laboratory in cool boxes filled with ice and then stored at -18°C until it was time for further processing.

Chemicals, reagents and solutions

All chemicals used were of analytical grade from Merck (Darmstadt, Germany). Fluoroquinolone standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany), and doxycycline from Sigma Aldrich (Vetranal, USA). Stock standard solutions were prepared at 1000µg/mL in methanol, then diluted using the same solvent to make a standard working solution with various concentrations, and kept at -20°C. McIlvaine buffer solution was made of 11.8g of citric acid monohydrate, 13.72g of disodium hydrogen phosphate dihydrate, 33.62g of ethylenediaminetetraacetic acid disodium salt and solved in 1L of water.

Table 1: Respondent characteristics

Variables		Frequency	
		n	%
Gender	Men	44	89.80
	Women	5	10.20
Farmers ages (years)	≤ 30	2	4.08
	31-50	34	69.39
	≥ 51	13	26.53
Education	Elementary school	23	49.94
	Junior high school	7	14.29
	Senior high school	18	36.73
	University/college	1	2.04
Ownership of cattle	≤ 5	17	34.69
	6-20	26	53.06
	≥ 21	5	12.24
Business duration (years)	≤ 15	11	22.45
	16-25	29	59.18
	≥ 26	9	18.37

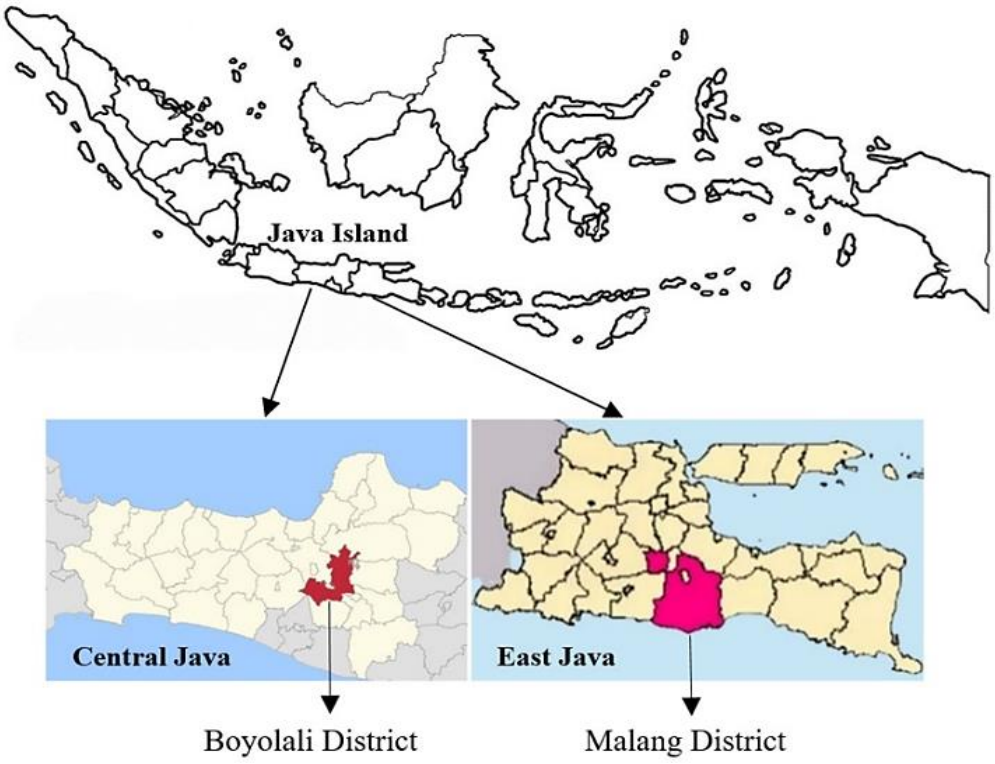

Fig. 1: Map of Indonesia and the locations of Boyolali District (Central Java Province) and Malang District (East Java Province), Indonesia.

Preparation and detection of enrofloxacin/ciprofloxacin residue in milk

The enrofloxacin and ciprofloxacin residues in milk were prepared following an adapted method described by (Widiyanti et al. 2022). A 5gr of milk was placed into a 15mL polypropylene tube, added with 2.5mL of trichloroacetic acid (20% in methanol), and then centrifuged at 3000rpm for 10min. A phosphate buffer solution (12.5mL, pH 7.4) was added and the mixture was re-centrifuged at 3000rpm for 15min. A solid-phase extraction (SPE) Oasis HLB cartridge (Waters, 200mg, 6mL) was used to purify the supernatant after it was conditioned with 6mL methanol, 6mL water, and 6mL phosphate buffer (pH 7.4). Following the extract's passage through the cartridge, 2mL of water was added, and the mixture was vacuum-dried for 3min. The fluoroquinolone in the cartridge was eluted with 2mL of 1% trifluoroacetic acid in acetonitrile and then dried under a nitrogen stream at 30ºC. The sample was then reconstituted with 200µL mobile phase and filtered using a 0.45mm PTFE filter. Samples were injected into the HPLC system (Shimadzu LC-20AD, Japan) equipped with a C_{18} column (Sunfire, 4.6x250mm, 5µm) (Waters, Ireland), and detected using a photodiode array (PDA) detector at 278nm wavelength. The mobile phase used was a mixture of 0.2M TCA-methanol-acetonitrile (74:4:22).

Preparation and detection of doxycycline residue in milk

The doxycycline was prepared using a method adapted by Widiastuti and Martindah (2023). A 5g milk sample in a centrifuge tube was added with 15mL ofMcIlvaine buffer, vortexed, and then centrifuged for 15min at 6000rpm. The supernatant was then re-extracted by adding 15mL of buffer solution and centrifuged for 20min at 4000rpm, then the extract was mixed with the previous and passed through to solid phase extraction (SPE) C_{18} cartridge (Bond Elut JR, 500mg), activated with 20mL of methanol, 15mL of purified water and 10mL of McIlvaine buffer. The cartridge was then flushed with 2mL of acetonitrile to remove impurities, and the doxycycline was eluted with 10mL of methanol and dried under nitrogen at a temperature of 30ºC. One mL of mobile phase was then added to the samples and passed through to a 0.45µm (Millipore) syringe filter, then injected into the HPLC system (Shimadzu LC-20AD) with a Shimp-pack VP-ODS C_{18} column (150x4.6mm) and a PDA detector equipment. The detection was performed at 355nm wavelength using a mixture of oxalic acid (0.0025mM) and acetonitrile (4:1, v/v) of mobile phase with 1.0mL/min flow rate.

Statistical analysis

The data were analyzed to determine the differences in antibiotic residue concentrations between milk samples collected from the districts of Boyolali and Malang. Enrofloxacin, ciprofloxacin and doxycycline concentration in the milk samples were expressed as mean ± standard error (SE). The t-test was applied to compare if there was a difference in the mean concentrations of these antibiotics between the two locations. This test was chosen because it compares the means of two independent groups and assumes that the data are normally distributed. The t-test was applied to each antibiotic separately, the significance level was set at $P<0.05$, and the analysis was conducted using SPSS–22 software.

RESULTS AND DISCUSSION

Farmers' socio-economic characteristics

The results showed that among 49 respondents, 89.90% were men, and the majority aged between 31-50. With regards to the level of education, only 1 person (2.01%) had attended the university/college, and the rest surpassed elementary and high school level. The majority of the respondents own about 6 to 20 dairy and cattle, therefore are classified as medium-scale dairy farmers (Shahjahan 2017) even though they have been experienced in the dairy cattle business for 16-25 years.

KAP on antibiotics usage by dairy farmers

Every farmer in both Districts collects their milk and sends it to the local cooperatives in the sub-district. The knowledge, attitudes, and practices on antibiotic usage among dairy farmers in the two Districts are summarized in Table 2.

It was discovered that the most common drugs belonged to various antibiotic families. The order of the drugs most used in this study based on the interview reports were tetracyclines (32.2%), sulfonamides (20.8%), fluoroquinolones (9.8%), macrolides (9.4%), polypeptides (8.0%), amprolium (6.5%), aminoglycosides (6.4%), trimethoprim (5.5%) and others (1.2%). The finding was closely similar to the baseline information on antimicrobial usage for cattle in Indonesia collected from 2014 to 2016 (Yusuf et al. 2018) which revealed the order of antibiotics used were tetracycline (62.0%), sulfonamides (22.9%), penicillin and aminoglycosides (7.1%), penicillin (3.5%), fluoroquinolones (2.5%), and others (0.6%). In contrast, a worldwide review conducted by (Sachi et al. 2019) discovered that the β-lactam group (which includes cephalosporins and penicillin) accounts for 36.54% of all antibiotic residue, followed by tetracyclines (14.01%), fluoroquinolones (13.46%), sulfonamides (12.46%), and aminoglycosides (10.44%).

The questionnaire consisted of questions to gain information about antibiotic usage in dairy farms. Farmers in Boyolali and Malang Districts typically use antibiotics to treat sick cows. Farmers had limited knowledge of antibiotic residue and resistance. However, some understood withdrawal time and followed the recommendation not to consume or sell the milk from cows three days after receiving the last injection. The choice of antibiotics given to dairy cows was made on the recommendation of veterinarians who carried out their application. This phenomenon was similar to the small-scale farmers in Peru who had low income, they relied on veterinarians to prescribe and administer antibiotics 95% and 59% of respondents respectively (Benavides et al. 2021). Farmers treated the animal directly by para-veterinary field staff from the dairy farmer cooperative or self-medicated it without the veterinarians' oversight. Veterinarians and para-veterinary field staff are the only officials authorized to administer antibiotics in Indonesia, following Act No. 18/2009 on Livestock and Animal Health. In the other part of Indonesia (Kupang, East Nusa Tenggara Province), farmers relied on paramedics to handle sick cows or relying on local herbal medicines (Muda et al. 2022).

Table 2: Animal handler's response to KAP usage of antibiotics

Animal handler's knowledge, attitude, and practice related to veterinary drug (antibiotic) usage		Response	
		Yes	No
Knowledge parameters	Heard about antibiotics	38	11
	Heard about antibiotic resistance or AMR	1	48
	Heard about antibiotic residues	6	43
	Antibiotics are used to treat the infected/sick animals	27	22
	Antibiotics are used as growth promoter	47	2
	Understand about veterinary drug withdrawal	16	33
Attitude parameters	Purchasing antibiotics from cooperative	49	0
	Antibiotic residues and AMR will occur when AM are not used prudently	12	37
	Milk should not consume/sell after the animals treated with antibiotics	30	19
Practice parameters	Treat animals with antibiotics (on their own) based on experience	9	40
	Treat animals with antibiotics (on their own) according to the instruction on the label	6	42
	Animals are treated with antibiotics by veterinarians/ para-veterinary field staff	35	14
	Treat animals with antibiotics based on advice from other farmers/breeders	8	41

Out of the whole respondents, only 16 (32.65%) considered a withdrawal period and kept records of antibiotic treatment, 35 out of 49 farms (71.43%) treated the animals with antibiotics under supervision by veterinarians/para-veterinary field staff and 38 out of 49 (77.55%) heard about antibiotics and did not know about residue and AMR. All interviewed participants admitted that antibiotics were used in dairy production for disease prevention or treatment. From the point of interviews, most of the farmers did not understand the danger of antimicrobial resistance and the presence of antibiotic residue in milk to human health. They did not purchase antibiotics but obtained those from veterinarians/para-veterinary field staff, and payment was made through a milk cooperative. It was common in Indonesia as well as in developing countries like India, majority of animal handlers in small-scale dairy households heard about antibiotics (83.31%), treated the animals on their own (51%), and bought antibiotics without prescription (23%) (Murugesan et al. 2023). In India, antibiotics use for therapeutic purposes was dominated by small farmers (98.21%), followed by medium farmers (83.92%) and large farmers (73.21%) while in reverse the use for sub-therapeutic purposes was dominated by large farmers and medium farmers (8.93%), and found low (1.79%) by small farmers (Kumar and Gupta 2018). Traditional medicine has been applied by farmers for a long time as an option that is thought to be more cost-effective and does not require additional costs for the purchase of the medication or veterinary care (Vijay et al. 2021).

Antibiotic residues in fresh milk samples

Antibiotic residues are frequently discovered to be hazardous for humans through animal-based food consumption which threatens public health. They have not received attention from farmers or the government as there are limited reports regarding antimicrobial residues found in cow's milk in Indonesia except for study on tetracycline residue (Widiastuti et al. 2023) and doxycycline residue (Widiastuti and Martindah 2023).

The analysis of antibiotic residues of 112 fresh milk samples from 2 Districts in this study is presented in Table 3. The statistical test results of the mean concentration for ciprofloxacin and doxycycline indicate a statistically significant difference (p-value <0.05) between the two locations (Boyolali and Malang). The concentration of enrofloxacin in the single positive sample from Malang was 38.10ng/g, which is higher than the mean concentration observed in Boyolali (19.73±16.98ng/g). However, for enrofloxacin, it is important to note that one positive sample was identified in Malang, preventing a t-test for this comparison. The results for enrofloxacin in Malang were therefore treated as an observational finding rather than subjected to statistical testing.

The analysis of milk samples indicated that the antibiotics' residual levels varied from 22.4-662.2, 15.0-38.6, and 44.8-557.2ng/g for enrofloxacin, ciprofloxacin and doxycycline, respectively. It was revealed most samples contained 2 types of antibiotics (enrofloxacin and/or ciprofloxacin, and doxycycline). Enrofloxacin and ciprofloxacin residues were found in less than 10% of the samples with mean concentrations of less than 10ng/g, and 1 sample in the District of Boyolali was exceeded 100ng/g which came from a cow that had just given birth, and two days before the cow was treated with an antibiotic by the para-veterinary field staff.

Even though doxycycline is prohibited for lactating cows, it was the most and the highest residue found compared to fluoroquinolones residues, especially for those collected from the District of Malang and 27 (72.97%) among those positive samples exceeded 100ng/g. A large number of samples having high residue levels require great attention and continuous monitoring programs to prohibit the toxicological effects on human health, especially children who are more vulnerable than adults. Lack of knowledge was the main factor contributing to the farmers' excessive use of antibiotics and disregard of the withdrawal time (Olasoju et al. 2021).

Doxycycline residue was incredibly uncommon in the samples examined in other nations. In Brazil, doxycycline was found in just one out of every 100 raw milk samples in 2015 (Prado et al. 2015). No samples examined in Kenya had doxycycline residue (Orwa et al. 2017) nor in Jordan (Muti et al. 2021). In commercial milk, doxycycline was not found among 20 brands of evaporated and powdered milk from Nigeria (Oluwafemi et al. 2018). Doxycycline was found in 2 out of 187 commercial milk samples from Iran (Bahmani et al. 2019) and in 6 out of 386 UHT milk samples in Brazil (Novaes et al. 2017).

The findings on enrofloxacin residue in this study were different from that found in Jordan where no enrofloxacin residue was found in 14 samples of long-term liquid milk and 3 samples of raw untreated milk (Muti et al. 2021) and (Nizamlıoğlu and Aydın 2012) among 50 milk samples analyzed. The percentage of enrofloxacin residue obtained was lower than that of Srinivasu et al. (2017) study, which

Table 3: Fluoroquinolones and doxycycline residues were detected in 112 fresh dairies milk samples from the District of Boyolali and Malang

Locations	Antibiotics	Mean ± SE concentration (ng/g)	n (%) positive samples	Concentration range (ng/g) of positive samples	n of samples ≥100ng/g
Boyolali	Enrofloxacin	19.73±16.98	4 (10.25)	22.4-662.2	1
(n = 39)	Ciprofloxacin	2.64±1.19	5 (12.82)	15.0-33.4	Nil
	Doxycycline	3.36±1.87	4 (10.25)	19.75-64.5	Nil
Malang	Enrofloxacin	38.1±0	1 (1.36)	38.1	Nil
(n = 73)	Ciprofloxacin	1.50±0.70	5 (6.84)	15.7-38.6	Nil
	Doxycycline	128.18±21.94	33 (45.20)	44.8-744.8	27
Total	Enrofloxacin	7.21±5.94	5 (4.46)	22.4-662.2	1
(n = 112)	Ciprofloxacin	1.90±0.62	10 (8.93)	15.0-38.6	Nil
	Doxycycline	84.72±15.36	37 (33.03)	44.8-557.2	27

discovered that 2 (10.52%) samples had residue levels above 100 ng/mL and that 19 (19%) out of 100 milk samples had detectable levels of ciprofloxacin and enrofloxacin residue. In contrast, Mohammed et al. (2016) found that 14 to 20.6% of 194 milk-type samples from the market, with a mean ciprofloxacin value of 2.94ng/mL, which was low for both bulk tanks and collecting centers. Priyanka et al. (2019) found that 8 out of 80 samples of raw milk were positive for quinolones. Different from the results obtained with the study of the dairy region of the state in Brazil (Valença et al. 2021) which reported that the fluoroquinolones and other antibiotic residues were below the current legislation for antimicrobial residues.

This study revealed that the presence of antibiotic residue in milk might be related to the non-enforcement of laws regulating antimicrobial usage, low education, and expertise combined with inadequate withdrawal period monitoring, similar to the study conducted by Alhaji et al. (2019). The majority of animal handlers are unaware of antibiotic residues and lack the withdrawal period and management of animals (Murugesan et al. 2023). It is hypothesized that imprudent or not logical use is more prevalent in low-income countries with limited access to veterinary care, poor animal husbandry practice, and poorly controlled antimicrobial access (Caudell et al. 2020). The use of antibiotics in Indonesia as growth promoters and drugs in animals continues to be used at high rates, despite the fact prohibiting their use unless for therapeutic purposes (Efendi et al. 2022).

Antibiotic residues in the environment have the potential ecological risk for the development of antibiotic resistance and human health risk (Hanna et al. 2018). The unmetabolized residues remain in manure, soil, and water and gain entry into the food chain resulting in antimicrobial-resistant pathogen dissemination (Groot and van't Hooft 2016). Concern should be taken for unexpected quinolone residues (94.4%) out of 90 mothers' milk in Ankara Turkey which had stated no use of quinolone for the previous year (Ergen and Yalçln 2019). Consequently, our study supported the suggestion to refrain from milking animals before the withdrawal periods.

Due to a lack of farmers' knowledge regarding the danger of animal products containing antibiotic residue, the government take action to increase public awareness and educate the farmers to improve their knowledge and practices on the appropriate use of antibiotics in livestock production and its impact on public health and environment (Ozturk et al. 2019). To ensure milk safety, monitoring of residues at village-level cooperatives by performing rapid tests is the preliminary step to reduce the burden of residues and prevent AMR. Management Livestock management by applying proper sanitation is essential for sustainable production without resorting to antibiotic use.

Conclusion

The KAP of smallholder dairy farmers in the District of Boyolali and Malang showed that they lacked knowledge on appropriate antibiotic uses and therefore needed intervention to improve the knowledge in reducing the usage level of antibiotics, which will decrease the residue level in dairy milk. Based on the interview report, the most common antibiotics used by farmers were tetracyclines, sulfonamides, and fluoroquinolones. Regarding the antibiotic residue in dairy milk examined, the results showed that fluoroquinolone had a low prevalence and residue level, and doxycycline had a high prevalence and residue level, which exceeded 100ng/g. It was recommended that the issue of antibiotic usage and residues in dairy farms be communicated to food safety and veterinary authorities, dairy farmers, and the public by improving their knowledge and practices on the appropriate use of antibiotics in raising dairy cattle.

Conflicts of interest: We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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Author contributions: RW and EM: conceptualization, sampling, methodology; draft writing, final editing. PMW and YA: methodology, reviewing, editing. RM and SW: reviewing, editing. All authors critically revised the manuscript and approved the final version.

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