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Quantitative exposure assessment and risk characterization for fipronil residues in laying hen eggs

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Abstract: Poultry production is linked to veterinary drug use to treat diseases. Few ectoparasitic compounds are approved for poultry. Fipronil is a pesticide widely used in agriculture. It is also a drug authorized to control ectoparasites in small animals and, in some countries, in cattle. There has been evidence of fipronil extra-label use in laying hens, mainly to control the red mite *Dermanyssus gallinae*. Fipronil's popularity is due to its high toxicity to invertebrates. It could be metabolized to more toxic metabolites that potentially damage human health. In the present study, we carry out a quantitative exposure assessment and risk characterization for fipronil residues in laying hen eggs for local consumption in five cities of Buenos Aires province in Argentina, namely, Azul, Balcarce, Juarez, Chaves, and Tandil. Consumption surveys and egg sampling were conducted for three summer periods. Eggs were analyzed by UFLC-MS-MS. Fipronil prevalence, residue concentrations, residue stability to cooking methods, egg consumption, among the most important variables were modeled. The results indicated that 20.7% of samples contained fipronil residues. The highest residue was fipronil sulfone metabolite. Fipronil concentrations quantified ranged between 10 and 2510 ppb (median value = 150 ppb). When eggs were cooked, fipronil residues were stable. The exposure assessment and risk characterization revealed that the highest probability of consuming eggs with fipronil residues above the admissible limits was for young adults (20.8%), followed by babies (16.9%), young children (16.4%), children (13.4%), teenagers (10.3%), older adults (9.41%), and adults (8.65%). These results suggest an unacceptable risk associated with egg consumption with fipronil residues for all age groups.

KEYWORDS
exposure assessment, fipronil egg residues, fipronil sulfone egg residues, laying hen eggs, risk characterization

Practical Application: Fipronil is widely used as an extra-label way on laying hens since its use is prohibited in poultry production both in Argentina and in most countries. This molecule has been classified as Class II, a moderately hazardous pesticide because it could damage various human organs. Fipronil

residues in eggs could be one of the exposure pathways for consumers. Monitoring residual levels and carrying out the health risk assessment in eggs are thus in an urge.

1 | INTRODUCTION

Eggs are the most complete, accessible, and economical source of protein for human consumption (Farrell, 2013). Therefore, egg production is an activity that takes place in most countries of the world. Considering all the Latin American countries, Mexico and Brazil are the ones that most contribute to world egg production, followed by Argentina, Peru, and Chile (FAO, 2015). Argentina is also an important egg consumer, due to intake amounts to roughly 284 eggs per person each year, a number that positions the country among one of the five largest consumers in the world (CAPIA, 2020).

Dermanyssus gallinae, the poultry red mite or chicken mite, is the most important ectoparasite affecting egg layers in many countries. It can cause irritation, anemia, lowered egg production, stained eggs with a lower commercial value, and in extreme situations, death (Kilpinen et al., 2005). Under favorable conditions (hot and humid weather), they can complete their cycle in just 7 days. Consequently, *D. gallinae* are attracted by warm temperatures since the mite's ideal conditions are 35°C and relative humidity over 70% (Cafiero et al., 2019; Flochlay et al., 2017). Moreover, when heat treatment is used in combination with chemical treatment, excellent control efficacy has been achieved (Sparagano et al., 2014).

In general, few drugs have been approved for their use in laying hens, as a result, producers frequently resort to extra-label drug use (Marmulak et al., 2015). In this context, although fipronil (FIP) is not licensed to treat laying hens, we have evidence from our relationship with different local poultry producers that it is being used for ectoparasites control, mainly *Dermanyssus gallinae*, particularly during the summer season. In Argentina, it would correspond to the period between November to March.

FIP is a new generation, broad-spectrum, and highly effective insecticide drug. It belongs to a member of the phenylpyrazole class and was developed by Rhone-Poulenc Ag company (now Bayer Crop Science) in 1987 (Tingle et al., 2003). It is used to prevent fleas and ticks in cats and dogs (Gupta & Anadón, 2018) as well as to control numerous insects in crops and houses (Salgado et al., 2012). FIP is not approved for its use in any food animal species in most countries (Stafford et al., 2018). However, in Argentina, there is a pour-on formulation licensed for cattle (Ectoline®, Merial Argentina S.A., fipronil 1%). FIP can be metabolized into different metabolites, depending on the conditions. The metabolism of FIP involves a reduction to the sulfide and oxidation to the sulfone (FIP-SO_2). FIP metabolites are 6–10 times more toxic and more persistent than the parent drug. The major metabolite detected in the tissues of treated animals is FIP-SO_2 (Hainzl & Casida, 1996). Several studies using rodent models have shown that this metabolite is more persistent in organisms than the parent drug, with an estimated half-life of 6–10 days; it is slowly eliminated, which is attributed to its high distribution to adipose tissue (FAO/WHO, 2016). Moreover, the FIP-SO_2 has been reported to be more toxic to insects, mammals, fish, and birds than the parent compound itself (EPA, 1996; Hainzl & Casida, 1996).

Regarding human health, the World Health Organization (WHO) in their "Recommended Classification of Pesticides by Hazard" classified FIP as Class II, a moderately hazardous pesticide (WHO, 2020). FIP is moderately toxic to rats and mice, highly toxic to aquatic invertebrates, fish, and game birds (EPA, 1996). Rat organs affected by chronic FIP exposure include thyroid, liver, and kidney (Tingle et al., 2003). Studies in rat showed reproductive toxicity at higher doses (EPA, 1997) and thyroid cancer (Hurley, 1998).

Considering hens could consume FIP accidentally in food or drinking water and they also could absorb FIP residues from the environment to ensure the health of human beings, the Codex Alimentarius Commission defines the maximum residue limit (MRL) values for FIP (sum FIP + FIP metabolites) in different tissues and the lowest MRL is 0.02 mg/kg for eggs and poultry muscle (CODEX, 2018). Furthermore, the European Commission sets a more rigorous limit of 0.005 mg/kg in those tissues (European Commission, 2005).

Focusing on FIP residues that could appear in laying hen eggs, the Codex Alimentarius (CAC) states that any food safety regulations should be based on a "risk analysis" or in the central component of risk analysis, the "risk assessment." It is a very useful tool to evaluate the food safety of products of animal origin for different age groups (Dorne & Fink-Gremmels, 2013), which consists of four steps: hazard identification, hazard characterization, exposure assessment, and risk characterization (WHO, 2008). Particularly in the present study, we will focus on the last two steps.

Numerous studies have shown the presence of FIP residual levels in eggs after FIP administration to laying hens

(Gerletti et al., 2020; Stafford et al., 2018). The availability in the market of eggs with FIP residues above the MRLs could constitute a risk to consumer health. In this context, considering the scarce available information on the subject, this study aimed to develop a quantitative exposure assessment and risk characterization for FIP residues in laying hen eggs for local consumption in Argentina.

2 | MATERIALS AND METHODS

2.1 | Sampling and chemical analysis

The study was carried out in the warmest months (between November and March) from 2017 to 2019 in five cities of Buenos Aires province in Argentina, namely, Azul, Balcarce, Benito Juarez, Gonzales Chaves, and Tandil. The sampling was executed in this specific period time since *D. gallinae* incidence is higher in warm months, consequently FIP residues could potentially appear in the eggs. A total of 350 eggs were sampled for laboratory analysis. Sampling was carried out at supermarkets, markets, poultry houses, and greengrocers of the five cities mentioned. These stores were provided by different poultry farms of each city, so most poultry farms of the different cities were monitored. Once in the laboratory, eggs were opened and placed individually in plastic tubes (mixing white and yolk) and mechanically shaken for several minutes. Finally, they were frozen at −18°C until high-performance liquid chromatography (HPLC) analysis.

2.1.1 | Chemicals and reagents

Reference standards (99% purity) of FIP and FIP-SO_2 were purchased from Toronto Research Chemicals Inc (20 Martin Ross Ave, North York, Canada). Each standard was dissolved in 10 ml of methanol to prepare the stock solutions with a concentration of 1000 μg/ml. The stock solutions were stored in a volumetric flask and could be kept stable for 6 months at −18°C. Working standard solutions were prepared weekly (or daily) by diluting the stock standard solutions in methanol. Acetonitrile and methanol were HPLC grade and supplied by Baker; n-hexane was obtained from Sigma–Aldrich; HPLC grade water was doubly distilled and purified using a water purification system (Simplicity; Millipore).

2.1.2 | UFLC-MS/MS system and chromatographic condition

All egg samples were analyzed by UFLC-MS-MS equipment (Shimadzu) following the methodology previously described by Canton et al. (2021). The equipment was composed of a SIL-20AC HT Prominence injector, two UFLC-LC-20AD Prominence pumps, an LCMS-8050 triple quadrupole mass spectrometer, and a CTO-20AC Prominence column oven. A Shim-pack HR-ODS C18 analytical column (15 cm·3 mm i.d., 2.6 mm particle size) at 40°C in the column oven was used for separation. The mobile phase was composed of water (A) and acetonitrile (B) at the flow rate of 0.4 ml/min, using a gradient program. First, B was 60% (0 min), increased linearly to 80% (2 min), followed by a linear increase to 90% (5 min), decreased to 60% (5.5 min), and thus remain constant until reaching the stop time at 7 min. The injection volume was 5 μl.

The analysis was performed in the negative ion electrospray ionization mode (ESI). Monitoring was done in multiple reaction monitoring, with a dwell time of 0.08 ms. Two transitions were followed for each molecule, the first being the quantifier and the second the qualifier. The temperature parameters for the heated ESI were 300°C (interface), 250°C (desolvation line), and 400°C (heat-block). The flow rate parameters for heating (air), nebulizing (N_2), and drying gas (N_2) were 10, 3, and 10 L/min, respectively. In the optimization procedure of individual compounds' MRM transitions, the best quantifier, qualifier ion, and collision energies (eV) were selected for MRM analysis optimized by injections (0.1 mg/ml).

2.1.3 | Method validation

Complete validation for FIP and FIP-SO_2 extraction and quantification in eggs was carried out and previously described by other authors (Canton et al., 2021). Calibration curves were prepared in the range between 10 and 2500 ppb. The linear regression for FIP and FIP-SO_2 showed correlation coefficients ≥0.998. Recovery percentages ranged between 78.4% and 85.8% for FIP and FIP-SO_2, respectively. The long-term stability of each compound was tested at 30 and 1000 ppb and stored at −20°C. Samples ($n = 3$) were analyzed at 0 and 90 days postfreezing. Stability is given by CV after analyses were between 4.4% and 17.6%. The LOQ values were established at 10 ppb for both molecules. The matrix effect values were −9.2 and −2.1 for FIP and FIP-SO_2, respectively. No endogenous chromatographic peaks, which could interfere with the resolution of drugs, were observed. FIP and FIP-SO_2 chromatographic peaks were well separated; besides, a good peak shape was obtained for the different molecules. Representative chromatograms obtained after analysis are shown in Figure 1.

2.1.4 | Sample analysis

First, an aliquot of 0.5 g of raw or cooked homogenized egg samples was deproteinized with 1 ml cold

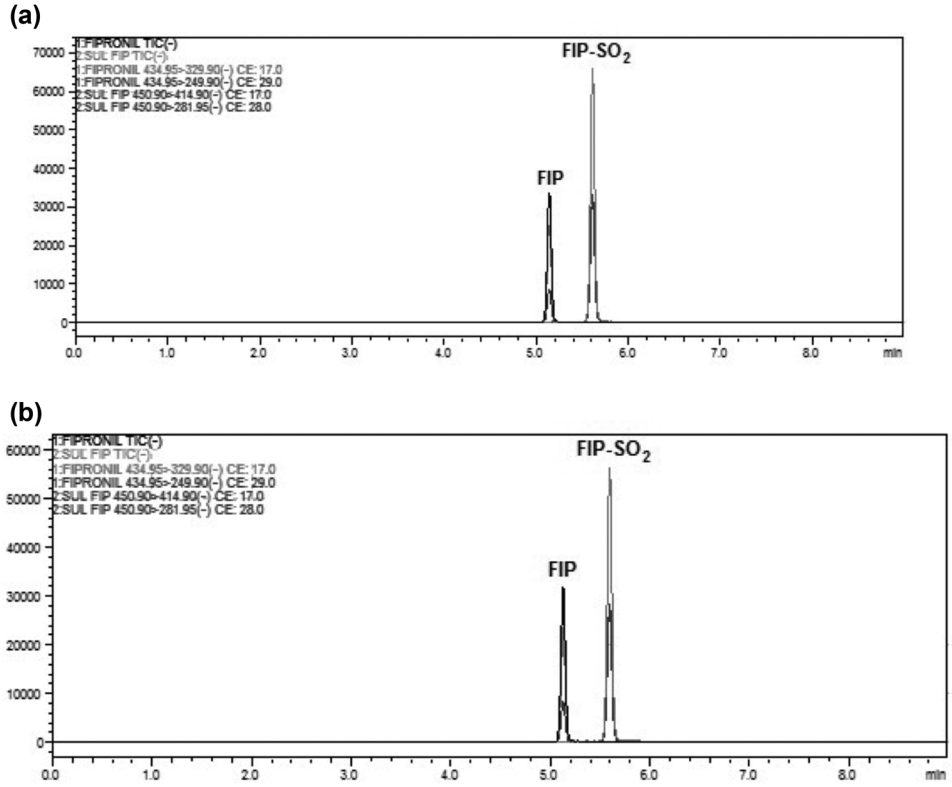

FIGURE 1 Chromatographic separation of fipronil (FIP) and its metabolite fipronil sulfone (FIP-SO_2). Chromatograms include (a) FIP and FIP-SO_2 analytical standards at 10 ppb and (b) blank egg sample fortified at 10 ppb with FIP and FIP-SO_2

acetonitrile and was mechanically homogenized with an ultraturrax. Then, the egg sample was stirred for 10 min in a multi-tube vortexer. The mixture was sonicated in an ultrasonic bath (Transonic 570/H, Elma) for 10 min. Finally, the sample was centrifuged at 3500 g for 10 min (4°C). The resulting supernatant was transferred into a plastic tube and 2 ml of HPLC grade water was added. The second step consisted of solid-phase extraction. The total supernatant was transferred to preconditioned C18 cartridges (100 mg/ml, Strata C18-T, Phenomenex), using a manifold vacuum. Then, they were washed with 1 ml HPLC grade water, 1 ml methanol/water (4:1), and 1 ml n-hexane, allowed to dry for 2 min, and eluted with 2 ml of acetonitrile. Eluents were collected in 5 ml glass tubes and evaporated to dryness under vacuum (Speed-Vac®, Savant, Los Angeles, CA, USA) at 40°C. Finally, the dry residue was dissolved in 2 ml acetonitrile: water (60:40) by shaking (10 min) and sonication (10 min). The samples were filtered with 0.22 μm nylon filters and 5 μl were injected into the chromatographic system.

2.2 | Food consumption data

A survey was carried out to identify behavior patterns of egg consumption in all city areas, including both the center and the periphery of Azul, Balcarce, Benito Juarez, Gonzales Chaves, Tandil, in order to include the different social strata. The survey contemplated different aspects such as age, sex, body weight, social stratum, daily egg consumption frequency, egg amount, and egg cooking methods. The survey was "in-person interview" and anonymous (the person surveyed was never asked their name). It was conducted to adults acquainted with the consumption patterns of their family members. A total of 312 respondents were enrolled in the study. Seven groups of interest were identified: babies (6–23 months), young children (2–5 years), children (6–12 years), teenagers (13–18 years), young adults (19–40 years), adults (41–70 years), and older adults (over 70 years).

2.3 | Model development

The model used to simulate the presence of FIP (FIP + FIP-SO_2) residues in eggs and thus estimate the exposure assessment and risk characterization of consuming eggs with FIP residues above the allowed limits was developed in Microsoft Excel with the @Risk software (version 5.5, Palisade Corporation, Newfield, New York, USA). Data obtained from the survey and FIP concentrations found in sampled eggs were used as inputs to develop the

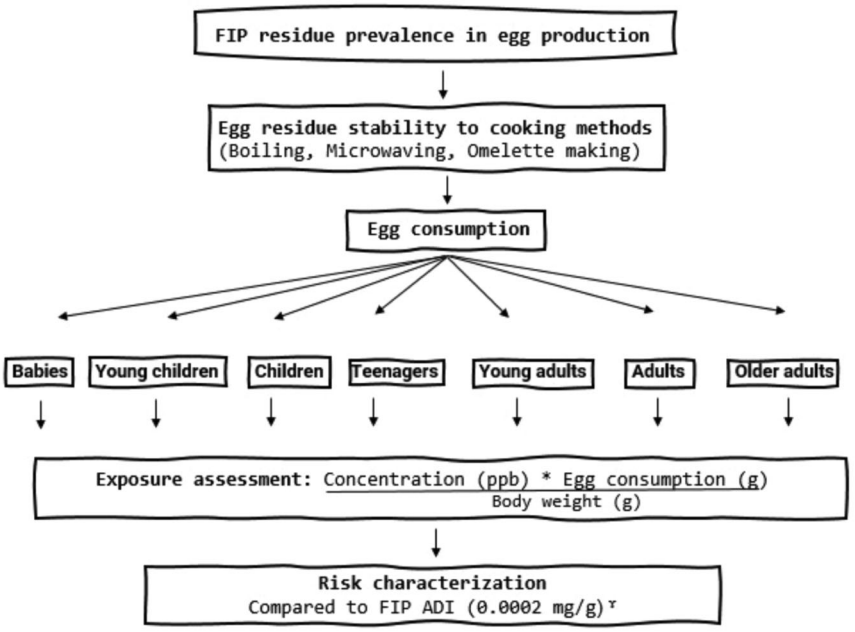

FIGURE 2 Flow diagram of the mathematical model for exposure assessment and risk characterization of fipronil + fipronil sulfone (FIP + FIP-SO_2) residues in laying hen eggs in Argentina ᵞ (FAO/WHO, 2016)

model. Nonetheless, when some information was missing, national and international data from literature were consulted to improve the model. The main output of the model was the associated probability of a person consuming eggs with FIP residual levels above the allowed limits. This probability was estimated through 5000 iterations with Latin hypercube sampling. This number of iterations provided adequate convergence of the simulation statistics (<1%).

The model was divided into five modules: FIP residue prevalence in egg production, egg residue stability to cooking methods, egg consumption, residue exposure, and risk characterization. From different inputs, it generated outputs for the next module. The conceptual model is shown in Figure 2.

All the variables used in this quantitative exposure assessment and risk characterization model and their probability distribution and parameters are presented in Table 1.

2.3.1 | FIP residue prevalence in egg

In this module, the FIP residue concentration and prevalence were estimated. This information was obtained from sampled and analyzed eggs as previously described. A total of 350 egg samples were collected for FIP residue quantification in the five cities involved in the trial. First, the FIP concentrations quantified in experimental samples (FIPcon) could be characterized with a Normal distribution. To calculate FIP prevalence (FIPprev), the beta distribution was used. This information was served as an input for the Binomial distribution, to decide if the person had consumed eggs with FIP residues (ECfip).

2.3.2 | Egg residues stability to cooking methods

The drug residue stability was evaluated by comparing the residue concentration quantified in raw egg samples with the concentration measured in cooked egg samples. The cooking procedures used were boiling, microwaving, and omelette making. Egg samples with FIP residual levels used in the essay were taken from a previous trial where hens were experimentally treated with FIP (Canton et al., 2021). A total of 36 eggs were used in the present study (four eggs per cooking time and process).

Boiling: Egg samples (10 g) were placed into plastic tubes, immersed in a water bath (Dubnoff, Vicking, Buenos Aires, Argentina) and cooked for different time periods (15, 30, and 45 min) at 100°C. Subsequently, egg samples were removed from the water and cooled to room temperature, and three aliquots (0.5 g) were obtained from each sample. Particularly, for boiling, we proposed long cooking times (30 and 45 min) in order to observe FIP concentration behavior.

Microwaving: Egg samples (10 g) were placed into plastic tubes and cooked under full power (500 W) in a microwave (EM NA 203 D1PW; Electrolux, Buenos Aires, Argentina). The samples were cooked for 0.5, 1, and 1.5 min. After each of these periods, the cooked egg samples were removed and cooled to room temperature, and three aliquots (0.5 g) were obtained from each sample.

TABLE 1 Model inputs parameters used in the exposure assessment and risk characterization of fipronil residues in laying hen eggs in Argentina

Symbol	Description	Unit	Distribution/model
FIPcon	FIP concentration in egg	μg/g	Normal (0.15; 0.40)
FIPprev	FIP prevalence in egg		Beta (93 + 1; 350–93+1)
ECfip	Eggs consumed with FIP residues		Binomial (1; FIPprev) 1 = consumed/0 = not consumed
EA	Egg amount consumed		LogNormal
		g	Babies (18.5, 18.6)
		g	Young children (25.8, 26.9)
		g	Children (29.8, 29.4)
		g	Teenagers, young adults, adults and older adults (33.9; 31.8)
CM	Cooking method used for consumption		Discrete (1, 2, 3; 134, 66, 20)
RIb	Rate of increase achieved after boiling	%	Normal (107, 8.2)
FIPb	FIP concentration obtained after boiling	μg/g	(Rib/100) × FIPcon
RIm	Rate of increase achieved after microwaving	%	Normal (119.9, 12)
FIPm	FIP concentration obtained after microwaving	μg/g	(Rim/100) × FIPcon
RIo	Rate of increase achieved after omelette making	%	Normal (100.6, 8)
FIPo	FIP concentration obtained after omelette making	μg/g	(Rio/100) × FIPcon
FIPc	FIP consumption	μg	EA × FIPb or FIPm or FIPo
BW	Body weight		Pert
		g	Babies (8000, 10200, 12900)
		g	Young children (12900, 15100, 19910)
		g	Children (19910, 30000, 56000)
		g	Teenagers (30000, 60000, 90000)
		g	Young adults (40000, 60000, 120000)
		g	Adults (48000, 70000, 130000)
		g	Older adults (55000, 65000, 90000)
FIPdi	FIP daily intake	μg/g	FIPc/BW
EAc	Egg amount consumed by each age group		Discrete
			Babies, young children and children (0, 1, 2, 3, 4,5, 6; 8, 32, 64, 128, 24, 24, 0)
			Teenagers (0, 1, 2, 3, 4, 5, 6; 0, 16, 40, 68, 8, 4, 0)
			Young adults (0, 1, 2, 3, 4, 5, 6; 4, 48, 122, 200, 32, 8, 4)
			Adults (0, 1, 2,3, 4, 5, 6; 4, 28, 104, 140, 20, 12, 4)
			Older adults (0, 1, 2, 3, 4, 5, 6; 4, 4, 16, 12, 4, 4, 0)

Abbreviation: FIP, fipronil.

Omelette making: Samples (10 g) of whipped egg were placed in a traditional teflon frying pan and cooked on a burner at 130°C for 1, 2, and 3 min. They were cooled to room temperature, and three aliquots (0.5 g) were obtained from each omelette.

Due to the potential water losses suffered during boiling, microwaving, or omelette making, the cooked samples were reweighed, and concentration was corrected according to the new weight ("expected concentration"). Egg samples obtained following each cooking method were processed in the same way as the raw egg samples. This is detailed in Section 2.1.4.

Reduction/increase rates after each cooking process were calculated by comparing the "expected concentration" for raw egg and the measured concentrations in cooked eggs.

First, the average rates and the standard deviation of the different cooking times for each cooking procedure were determined. These data were then used in a Normal distribution to obtain the final rate per cooking method (Rib—Rim—RIo). Then, new FIP concentrations were calculated (FIPb—FIPm—FIPo), associating the concentration in raw egg and the final rates. Finally, the final FIP consumption (FIPc) was calculated as the product of egg amount consumed (EA) and *FIPb* or *FIPm* or *FIPo*.

Information about frequency and consumer preferences (CM) in egg cooking methods was obtained from surveys. A Discrete uniform distribution was included in the model so that all cooking procedures had the same probability of being selected.

2.3.3 | Egg consumption

For each of the age groups (babies, young children, children, teenagers, young adults, adults, and older adults), the egg consumption frequency data were obtained from our survey, whereas the egg portion size consumed data were obtained from the National Nutrition and Health Survey of the Ministerio de Salud of Argentina (Ministerio de Salud, 2012). The egg amount consumed (EA) was assumed to be LogNormal distributed with a mean of 18.5 g (±18.6) for babies, 25.8 g (±26.9) for young children, 29.83 g (±29.4) for children, 33.9 g (±31.8) for teenagers, young adults, adults, and older adults. Additionally, the Discrete distribution was used to model the preferences and frequency regarding egg consumption (Table 1).

In this module, information about body weight (BW) was necessary as input for the following step. A Pert distribution was used to model the body weight, with the minimum, most likely, and maximum values: 8, 10.2, and 12.9 kg for babies; 12.9, 15.1, and 19.9 kg for young children; 19.9, 30, and 56 kg for children; 30, 60, and 90 kg for teenagers; 40, 60, and 120 kg for young adults; 48, 70, and 130 kg for adults; and 55, 65, and 90 kg for older adults. All of these data were obtained from the surveys performed in the cities involved in the study.

2.3.4 | Residue exposure

The potential exposure to FIP residual levels was estimated to assess the risk to human health associated with the consumption of eggs. CODEX proposed the following equation for calculating chronic dietary exposure (Reuss, 2014):

*Dietary exposure = Concentration (ppb) * Egg consumption (g) / Body weight (g)*

All the information achieved in the previous modules was used in the equation raised for each age group.

2.3.5 | Risk characterization

Risk characterization is the estimation of the probability of consumed eggs with FIP residues above the allowed limits in the different age groups based on hazard characterization and exposure assessment. The values obtained after carrying out the exposure assessment were compared with the admissible daily intake (ADI), which is the international toxicological reference value recommended for chronic exposure. The established ADI for FIP is 0.0002 mg/kg (FAO/WHO, 2016).

The FIP daily intake (FIPdi) was determined by making the quotient between the probabilities of FIPc and BW. Finally, the probability of exceeding the allowed ADI was obtained from the model.

2.4 | Sensitivity analysis

Sensitivity analysis is a procedure that improves the information obtained from a risk assessment. This tool allows identifying those variables that produce a greater or lesser impact on risk. The best-known technique for this analysis is the *Pearson correlation*. It consists of determining the correlation degree between the variables involved and the result. The correlation coefficient (r) takes values between −1 and +1. When the relationship is directly proportional, the coefficient takes positive values. On the contrary, when the relationship is inversely proportional, the coefficient acquires negative values. When Pearson's correlation coefficient is zero or closely zero, it indicates that the correlation is non-existent or weak; therefore, the variable does not affect the result (Patil & Frey, 2004).

2.5 | Statistical analysis

To carry out the exposure assessment and risk characterization, the model was developed using Microsoft Excel 2010 with the add-on @Risk 5.5 software (Palisade Corporation, Newfield, New York, USA). In chronic dietary, exposure assessment is preferred to use the median of the residues in the tissues of the target species (Arcella et al., 2019). Therefore, FIP + FIP-SO_2 concentrations are shown as median and range.

Statistical parameters for the validation and calibration line settings for FIP and FIP-SO_2 quantification were performed using the Instat 3.0 software (Graph Pad Software Inc., San Diego, USA). Student's t-test was used to compare the drug residue in raw eggs with residues found after the different cooking procedures in the stability assay. A value of $p < 0.05$ was considered statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | FIP prevalence in egg

After the analysis of all the samples collected, 69 of them quantified FIP-SO_2 residual levels above the LOQ, while three of these samples also contained FIP parent drug. Those samples that contained FIP or FIP-SO_2 residues below the LOQ (n = 24) were also considered for the

prevalence calculation. Based on these results, the mean prevalence of FIP + FIP-SO_2 residues in eggs was 26.6% (95% confidence interval [CI]: 18,6 to 35.6%).

3.2 | FIP residues concentrations in egg

The main residue found in the analyzed eggs was FIP-SO_2. For residue control, both molecules are considered as marker residues, the sum of both residues (FIP+FIP-SO_2) was contemplated for exposure assessment and risk characterization in the present work. The median was 150 ppb in a range between 10 and 2513 ppb.

Figure 3 shows FIP concentrations (FIP + FIP-SO_2) in raw and egg cooked after different cooking times by boiling (A), microwaving (B), and omelette making (C). For each cooking time, four different eggs were used, resulting in 12 eggs per cooking treatment.

Surprisingly, cooking resulted in a striking increase of egg FIP residues concentrations, due to an evident process of dehydration of the egg sample and clear drug stability. Microwaving showed the greatest differences in concentrations between raw and cooked eggs, reporting the highest increase percentages. The boiling process also presented an increasing trend at almost all cooking times, except after 30 min. No clear trend was observed during omelette making since FIP residue concentrations between raw and cooked eggs increased at 1 min but decreased at 2 and 3 min. However, every increment/decrement exhibited during boiling, microwaving, or omelette making was not statistically significant.

3.3 | Egg consumption

The egg amount (g) consumed by different age groups was determined. The average values were between 18.5 and 33.9 g for the different age groups. Data about egg consumption by teenagers, young adults, adults, and older adults were scarce, so it was assumed that these groups consumed the same egg amount (Table 1). Additionally, the most used cooking method was boiling (89.3%), followed by omelette making (44%) and microwaving (13.3%).

3.4 | Prediction model

Each iteration predicted the probability of consuming eggs with FIP residues above the allowed limits in the different age groups. The average probability was $16.9 \pm 0.2\%$, $16.4 \pm 0.3\%$, $13.4 \pm 0.3\%$, $10.3 \pm 0.3\%$, $20.8 \pm 0.1\%$, $8.65 \pm 0.3\%$, and $9.41 \pm 0.3\%$ for babies, young children, children, teenagers, young adults, adults, and older adults, respectively. The main variables to estimate this probability were FIP concentration quantified in experimental eggs, egg amounts consumption, and bodyweight. Considering the disparity in the values of the same variable for the different age groups, it explained the obtained differences among the probabilities.

The sensitivity analysis (Figure 4) indicated that the variables: "eggs consumed with FIP residues" (r-values between 0.56 and 1), "FIP concentrations found in eggs" (r-values between 0.10 and 0.21), and "egg amount consumed" (r-values between 0.10 and 0.22) were positive correlation variables in almost all groups studied, namely, they influence directly proportional on the risk of consuming eggs with FIP residues above ADI.

Additionally, the variable "bodyweight" seemed to have a protective effect in young children ($r = -0.02$), children ($r = -0.03$), teenagers ($r = -0.02$), young adults ($r = -0.02$), adults ($r = -0.05$), and older adults ($r = -0.04$). In other words, the variable value decreases; thus, the risk also decreases. Nonetheless, r-values were not significant. Regarding the variable "cooking method used," it had a positive correlation in most age groups although the correlation is very weak since r-values were close to zero.

3.5 | Discussion

Few drugs have been approved to treat ectoparasites in laying hens. In fact, only two active drugs, phoxim and fluralaner, are licensed for red mite control in some countries (European Commission, 2017; Prohaczik et al., 2017). The scarcity of drugs to control the red mite in laying hens has led to the illegal extra-label use (Marmulak et al., 2015). Even though FIP is not licensed to treat laying hens, it is widely used in this production (Stafford et al., 2018). This background led to carry out the present study to quantitatively carry out an exposure assessment associated with the presence of FIP residues in laying hen eggs.

As expected (Guo et al., 2018; Kitulagodage et al., 2011; Lopez-Antia et al., 2015), a significant difference in concentrations between the parent drug and its metabolite was found in egg samples. The FIP concentration represented only 4.3% of the total amount of FIP-SO_2. These results agree with those previously reported in red-legged partridge (Lopez-Antia et al., 2015). The authors reported higher concentrations of FIP-SO_2 residues than FIP concentrations in the liver and brain after feeding the animals with maize containing this pesticide. Kitulagodage et al. (2011) have shown a similar behavior between FIP and FIP-SO_2 residues in eggs after treating female zebra finches with a single dose of FIP at different levels. Similar results were reported by Guo et al. (2018)

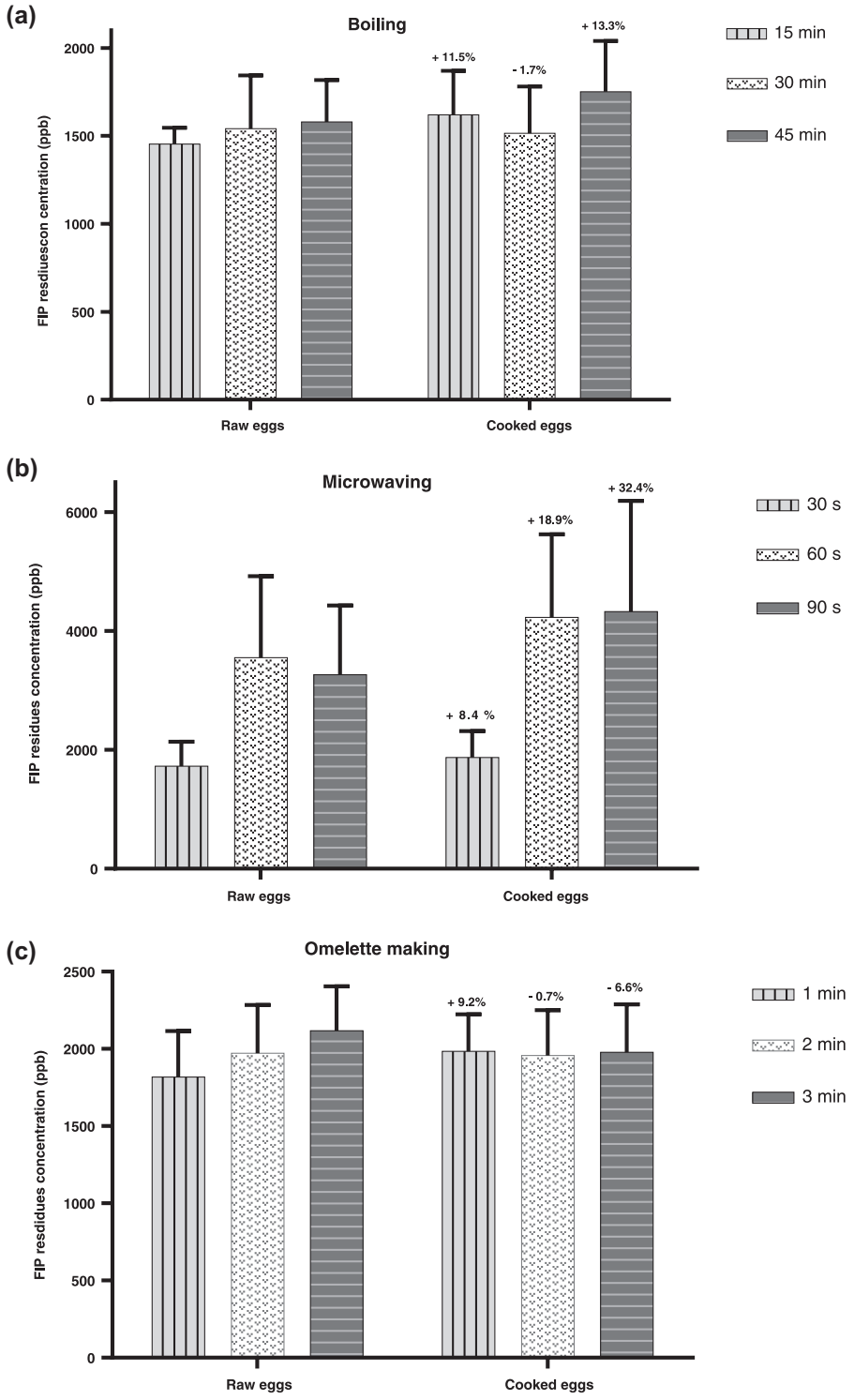

FIGURE 3 Fipronil + fipronil sulfone (FIP + FIP-SO_2) concentrations (mean ± SD) (ppb) in raw eggs cooked by boiling (a), microwaving (b) and omelette making (c) during different times. Change percentages are reported as an indicator of stability. *Significant differences with p-value < 0.05. ** Very significant differences with p-value < 0.01

who analyzed FIP, FIP desulfinyl, FIP-SO_2, and FIP sulfide concentrations in chicken eggs, muscles, and cakes randomly purchased from a local supermarket. Among the tested samples, FIP-SO_2 was present in a higher concentration compared to the parent drug. However, in contrast with the findings of the present study, these authors reported that FIP-SO_2 concentrations ranged from 0.002 to 4.17 μg/kg, which were below the MRL of the European Union (EU) (European Commission, 2005).

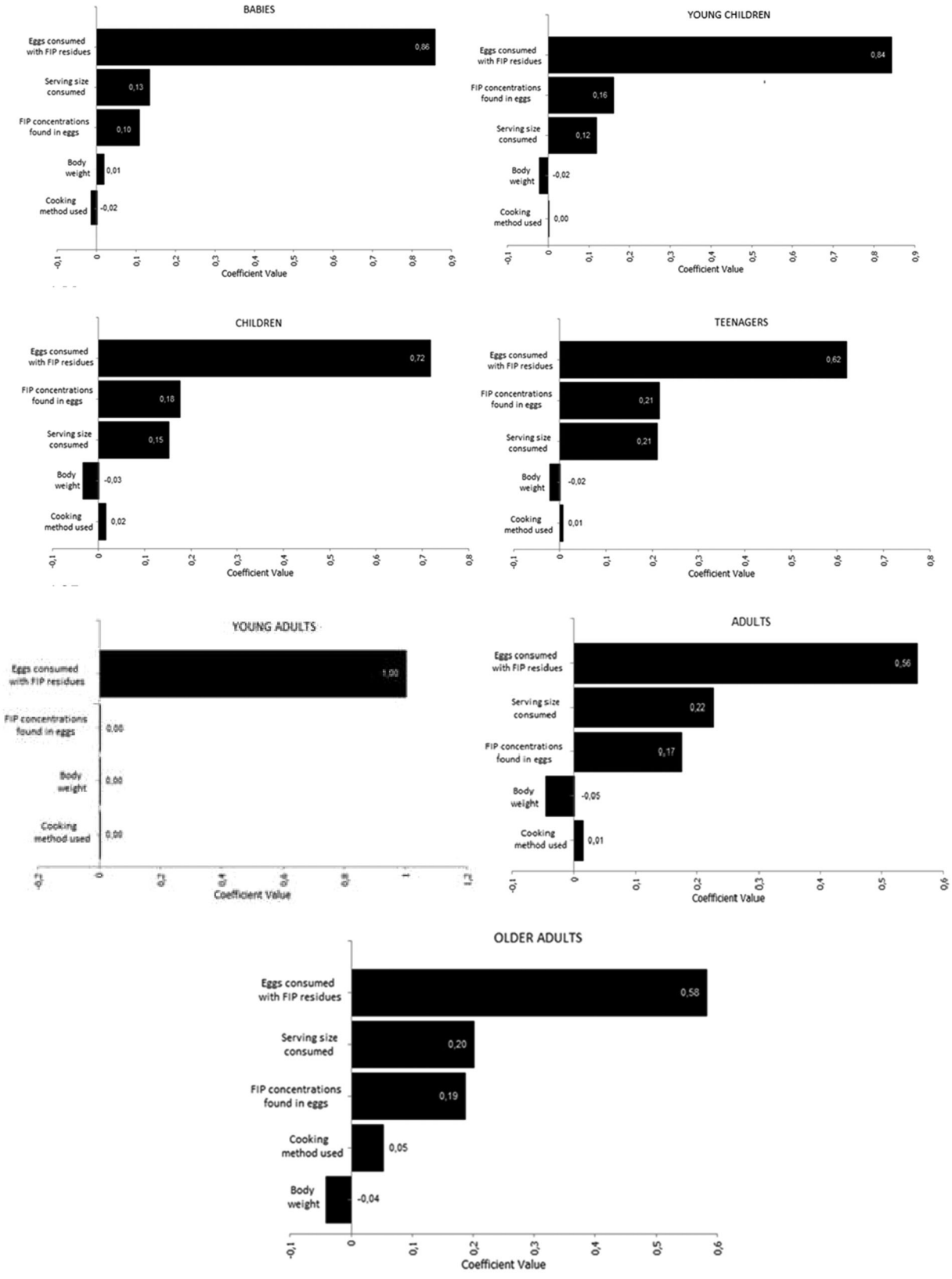

FIGURE 4 Correlation coefficient between the estimated probability of consuming eggs with fipronil + fipronil sulfone (FIP + FIP-SO_2) residues above the allowed limits and the most important predictive factors

The European Commission and the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) established an MRL of 0.005 mg/kg (European Commission, 2005) and 0.02 mg/kg (CODEX, 2018) for FIP/metabolites in egg, respectively. Considering these MRLs, the percentage of analyzed eggs that exceeded them was calculated in the present study. All FIP (FIP + FIP-SO_2) residues quantified in the experimental samples exceeded the EU MRL, and 87% of the samples presented concentrations above the FAO/WHO MRL. In Argentina, the National Plan for Residue Management and Food Safety (CREHA) monitors various raw materials of animal origin for residues of veterinary drugs, pesticides, and mycotoxins. From 2015 to 2018, FIP was analyzed exclusively in raw chicken/hen samples (CREHA, 2018). Among a total of 969 samples, only two of them showed FIP residues but did not exceed the MRL (FAO/WHO levels). In contrast, the results observed in the present study showed few egg samples below this limit.

The development of the present study was coincident with the EU alert regarding FIP residues in eggs in 2017. Precisely, since that year, the presence of FIP residues in eggs has been more relevant because the European Rapid Alert System for Food and Feed reported the distribution of eggs possibly contaminated with FIP, as well as egg products, processed products, and chicken meat, in 19 countries of the EU. The origin of the contamination was located in the Netherlands, where some laying hen farms had used a formulation containing FIP to sanitize facilities (EFSA, 2018). In this context, EFSA requested EU member states to take samples of laying hen eggs. They collected 1244 suspected samples and 2246 random samples, which resulted in 434 and 167 samples with FIP residues above de MRL, respectively. Therefore, the average prevalence of FIP residues in laying hen eggs was 17.2% including suspected and random samples. This is in agreement with the findings of the present study, where the prevalence was 20.7% ± 2.2. Additionally, FIP values up to 0.92 mg/kg were found in egg samples from some Dutch farms (EFSA, 2018). These results are consistent with those obtained in our study in which alarming FIP concentrations were detected, as one of the analyzed eggs reached 2.51 mg/kg.

Regarding dietary exposure assessments, obtaining accurate information on drug residue levels in food is as important as getting accurate information on food consumption. The most accurate data on residue concentration levels can be obtained when these are quantified in food as consumed, due to cooking or processing spoilage (Heshmati, 2015), and other sources of waste and additions from subsistence practices could occur (WHO, 2008). In fact, some stability studies reported changes in veterinary drug residue levels after different cooking procedures (Alaboudi et al., 2013; Canton et al., 2019; Heshmati, 2015). Several authors have studied FIP/metabolites stability in different tissues. H. S. Kim and Hur (2018) mixed FIP in egg whites and yolks, and concentrations were unchanged after cooking for 10 min at 100°C. These results are consistent with those obtained in the present study since the FIP/metabolites residues did not disappear after cooking; moreover, the residues were concentrated. This is a very relevant factor since it has a direct incidence of the risk of consuming eggs with FIP residues. As the model's results evidenced, FIP residual levels were not reduced by cooking but rather this process concentrated them due to the dehydration of the samples. In contrast, Hingmire et al. (2015) applied FIP in okras by a sprayer and evidenced a reduction after washing with boiling water (blanching).

Data available on exposure assessment and risk characterization of FIP residual levels, particularly regarding its presence in laying hen eggs are very scarce or, rather, null. Liang et al. (2019) investigated the residual level and potential chronic and acute risk of FIP in marketed fruits and vegetables for adults and children. The results showed that 0.2% of fruits and 0.8% of vegetables contained FIP residues. The risk assessment found that the intake of fruits and vegetables did not entail a chronic risk for adults and children. Although it was carried out in another matrix and with another technique, these results differ from those obtained in the present study, as the probability of consumed eggs with FIP residues above the allowed limits was, on average, 7.3% and 13.4% for adults and children, respectively.

Information about toxicity caused by FIP chronic exposure in the human population is limited; nonetheless, the US Environmental Protection Agency (EPA) has classified FIP as a possible human carcinogen, since it showed an increase in thyroid follicular cell tumors in rats after long-term exposure (Hurley, 1998). In this context, Kim et al. (2019) determined FIP and FIP-SO_2 serum levels in the general and sensitive human population (parent–infant triads) in Korea. They only detected FIP-SO_2 in the serum of mothers, fathers, and infantile cord blood. Although FIP-SO_2 levels were higher in the paternal samples (geometric mean = 1.163 ± 0.797 ng/ml) than in the maternal (0.744 ± 0.426 ng/ml) and infant samples (0.525 ± 0.240 ng/ml), maternal and paternal FIP-SO_2 levels were strongly correlated with infantile levels. This suggested two remarkable points: first, mother and father share similar exposure routes, including lifestyle and diet; and secondly, maternal FIP-SO_2 can be placentally transferred to the fetus and subsequently to newborn infants. Like the results reported by Kim et al. (2019), in the present study, the main residue detected was FIP-SO_2. Additionally, they found that the presence of this metabolite was mainly associated with consumers' diet, which would pose a potential

health risk. Although we did not measure FIP levels in consumer serum, modeling data from the consumption survey and FIP concentrations in egg samples showed differences in FIP exposure for certain population groups.

According to our sensitivity analysis, the most important variables that were directly proportional to the risk associated with consuming eggs with FIP residues were "eggs consumed with FIP residues," "FIP concentrations found in eggs," and "egg amount consumed.". It is very important to highlight that a risk exists, greater or lesser depending on the age group, associated with egg consumption since they may contain FIP residues. In addition, given that *D. gallinae* develop at warm temperatures, during the autumn-winter season, the probability of consuming eggs with FIP residues above the allowed limits decreases since it would be unlikely that poultry producers use FIP during those months. Considering that toxicological studies were carried out on this molecule, and thus ADI and MRL values were established, if FIP residues above the established levels are consumed over a long period, they could have negative consequences on consumer health.

Eggs are one of the most complete and healthy foods. It provides proteins with the highest biological value. Eggs have been catalogued by FAO as the most nutritious resource that exists in nature (Abad, 2007). However, if this wonderful food carries a risk, either physical, biological, or chemical to human health, this would be seriously disqualifying to its suitability. For this reason, the risk associated with veterinary drug residues, such as FIP, in eggs deteriorates its quality and undermines all its benefits. To avoid the presence of FIP residues in eggs, some actions should be taken, such as monitoring FIP residues in eggs and poultry products at a national level, developing educational campaigns for small, medium, and large farmers about good farming, and veterinary practices, among others.

4 | CONCLUSIONS

Concerning the available information on exposure assessment and risk characterization for FIP residues in eggs is scarce, the original findings of the present study make an important contribution to public health. Moreover, this study provides data on FIP prevalence in egg and FIP/metabolites stability in eggs cooked by different methods, namely, boiling, microwaving, or omelette making.

Results demonstrated that FIP residues in eggs presented unacceptable exposure risk for people, which varies according to consumer age in Argentina. This information could be extrapolated to the rest of the countries that resort to extra-label use of FIP. Since FIP use is prohibited in laying hens whose eggs are intended for human consumption, it is indispensable to control the misuse in this production, to avoid dietary exposure from FIP residual levels in eggs. This is crucial to safeguard both the consumer health and the profitability of food producers. Implementation of quality assurance programs to protect public health is a major challenge for developing countries.

AUTHOR CONTRIBUTIONS

Formal analysis, investigation, writing—original draft, and writing—review and editing: Lucila Canton. Formal analysis, methodology, software, and writing—review and editing: Marcelo Signorini. Writing—review and editing: Candela Canton. Investigation and project administration: Paula Dominguez. Project administration: Cristina Farias. Resources: Luis Alvarez. Funding acquisition, resources, writing—review and editing: Carlos Lanusse. Conceptualization, funding acquisition, and writing—review and editing: Laura Moreno.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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