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CICLO XXXVII

Tracing ivermectin residues in swine farms:

from treatment approaches to environmental impacts

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Summary

ABSTRACT

Pharmaceutical residues, particularly active pharmaceutical ingredients, have become significant environmental pollutants, with their impact extending across ecosystems and affecting public health. Veterinary pharmaceuticals are a major source of contamination, introduced through livestock excretion, manure application, and surface runoff, with different administration routes posing different environmental risks. Chronic environmental exposure to active pharmaceutical ingredients contributes to antimicrobial resistance, behavioral changes, reduced fertility, and biodiversity loss in wildlife. Efforts to mitigate pharmaceutical pollution align with global sustainability goals, such as the UN's Sustainable Development Goals, emphasizing responsible consumption and protection of ecosystems. Regulatory frameworks, including the EU Water Framework Directive and action plans on pharmaceuticals, aim to address this issue. Despite numerous advancements in therapy, animal welfare, wastewater treatment, and nature-based solutions, drugs may remain persistent in the environment, necessitating sustainable pharmaceutical practices and ecotoxicological research to safeguard environmental and public health. Ivermectin (IVM), a widely used antiparasitic drug in veterinary medicine, plays a critical role in managing parasitic infections in livestock. However, its widespread application raises concerns about its potential environmental impacts, particularly in swine farming systems. This PhD thesis investigates the detection, environmental fate, and ecological consequences of IVM residues in swine farms, as well as their effects on gut health, providing insights into sustainable livestock management practices and One Health implications.

An analytical method using HPLC-DAD and, subsequently, LC-MS/MS were developed and validated for detecting IVM in various matrices, including feces, slurry, wastewater, and soil. The method demonstrated high sensitivity, precision, and robustness, with limits of

quantification as low as 1.5 µg/kg, highlighting its utility in ecotoxicology and environmental monitoring. The findings showed that IVM residues predominantly accumulated in feces and soil, with no detectable concentrations in slurry or wastewater samples. This accumulation underscores the potential risks to non-target organisms. The research also explored the ecological pathways of IVM residues, revealing differences in residue excretion profiles between oral and injectable administration routes. Injectable formulations resulted in higher concentrations over a long period, while the oral treatment showed an initial higher peak in concentration, but the concentrations were significantly lower 10 days after treatment. These findings highlight the importance of administration routes in assessing the environmental impacts of veterinary pharmaceuticals.

Additionally, this research is the first to document the effects of IVM treatment on the gut microbiota of sows. The data demonstrated that IVM treatment significantly altered gut microbiota composition, raising concerns about potential long-term implications for animal health and antimicrobial resistance.

This thesis contributes to the growing understanding of veterinary pharmaceutical residues' environmental and ecological impacts. By addressing critical gaps in analytical detection, residue pathways, and microbial effects, it underscores the importance of sustainable livestock management practices. These findings advocate for integrated One Health approaches to mitigate the environmental footprint of veterinary drugs while safeguarding animal and ecosystem health.

ABBREVIATIONS

ADI – Acceptable daily intake

ADVeRSE – Analytical methoDs Validation Results Speedy Elaboration

APIs – Active pharmaceutical ingredients

b.w. – Body weight

CReAA – Centro di Referenza Nazionale per la Sorveglianza e il Controllo degli Alimenti per gli Animali (National Reference Centre for the Surveillance and Control of Animal Feed)

CV% – Coefficient of variation

DAD – Diode array detector

d.w. – Dry weight

EC_{50} – Effective concentration 50

EFSA – European Food Safety Agency

EMA – European Medicines Agency

ERA – Environmental risk assessment

ESI – Electrospray ionization

EU – European Union

FDA – Food and Drug Administration

FPA – Food-producing animals

HPF – Hours post fertilization

HPLC – High-performance liquid chromatography

HPLC-DAD – High-performance liquid chromatography with diode array detector

IVM – Ivermectin

LC-MS/MS – Liquid chromatography tandem mass spectrometry tandem mass spectrometry

LC_{50} – Lethal concentration 50

LC – Liquid chromatography

LOD – Limit of detection

LOQ – Limit of quantification

MDA – Mass Drug Administration

MRLs – Maximum residue levels

NFPA – Non-food-producing animals

RT – Retention time

SPE – Solid phase extraction

1. INTRODUCTION

1.1. Pharmaceutical pollution of the environment

Active pharmaceutical ingredients (APIs) have emerged as significant environmental pollutants over the past three decades. The issue gained prominence following reports of a sudden and drastic increase in vulture mortality in India and Pakistan, attributed to exposure to diclofenac residues from the carcasses of treated cattle [1, 2]. This event highlighted the far-reaching environmental impacts of pharmaceutical residues, which are an intrinsic aspect of treating and preventing diseases in both natural ecosystems and human health.

1.1.1. Pathways of pharmaceutical pollution

APIs can enter the environment during various stages of their lifecycle (Figure 1), including production, usage and disposal. The most significant source is excretion by treated individuals, while improper disposal of unused medicines and emissions from pharmaceutical manufacturing also contribute to the phenomenon [3].

In veterinary contexts, the terrestrial environment is primarily contaminated through the use of pharmaceuticals in livestock. Residues are introduced via slurry and manure application on agricultural lands or directly excreted by pasture-reared animals [4]. These APIs residues may subsequently enter aquatic systems through surface runoff or leach into groundwater. Due to their mode of application, topical treatments pose a higher risk of environmental release than oral or injectable administrations [5]. Grooming behaviors among animals can transfer pesticides from treated to untreated individuals. This secondary exposure can occur when animals groom each other or come into close physical contact,

particularly in social or herd settings. Such transfer has implications for both the efficacy of treatments and potential unintended effects on untreated animals, including non-target species within the same environment [6].

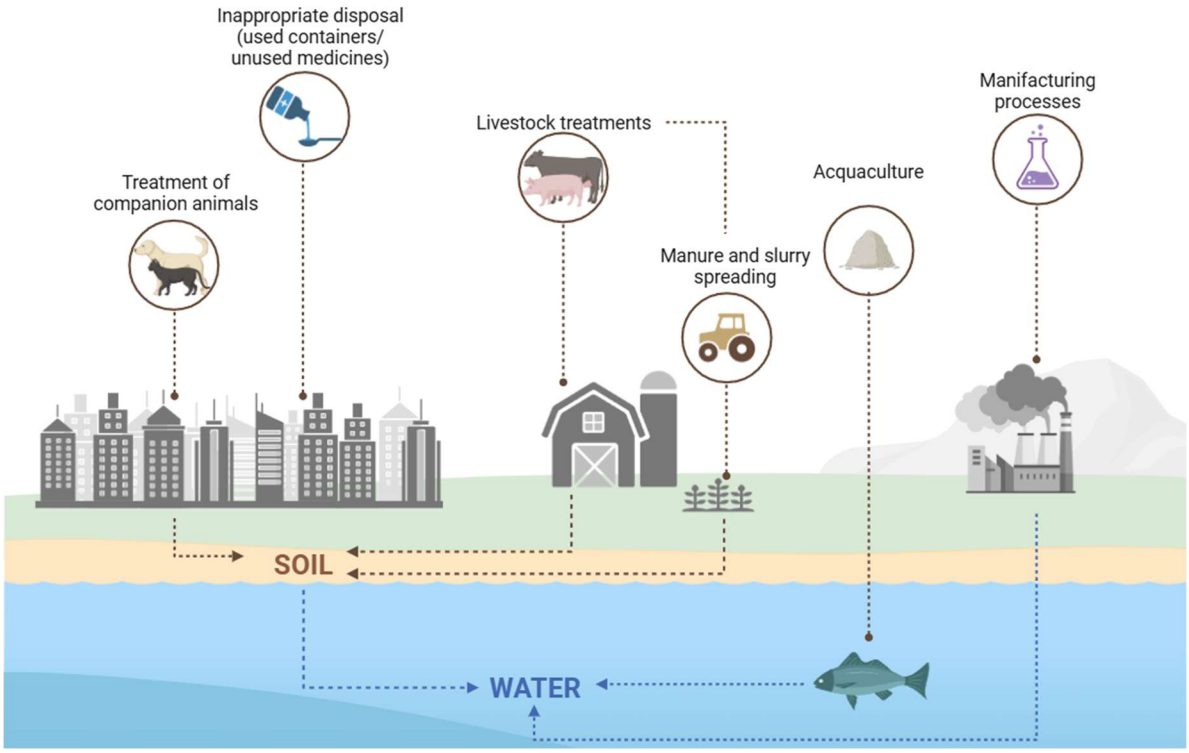


Figure 1 –Sources of active pharmaceutical ingredients into various environmental compartments. Key sources include human and veterinary pharmaceutical use, manufacturing processes, and improper disposal of unused or expired drugs. Pathways to the environment occur through excretion into wastewater, leaching from soil, run-off into aquatic systems, and direct discharge from manufacturing facilities. Once in the environment, APIs can accumulate in soil, water, and sediments, potentially impacting non-target species and ecosystems.

Additional pathways include discharging untreated waste from septic systems, pit latrines, unmanaged waste dumps, and emissions from pharmaceutical manufacturing facilities. APIs from these sources can migrate into drinking water or be absorbed by crops, creating indirect exposure pathways for humans. Although wastewater treatment plants can remove some APIs [3], many persistent compounds resist degradation and remain in treated effluents, particularly in regions with inadequate water treatment infrastructure [7].

The transformation of APIs in sewage and wastewater depends on several factors occurring in the administered subject and treatment systems, including the metabolic processes in

treated animals, the hydrolytic capacity of treatment plants, and the photosensitivity of the compounds [8, 9]. Oxidation processes during treatment may also contribute to the formation of transformation products. While some of these transformations lead to the deactivation of pharmaceutical molecules, others result in bioactive metabolites that continue to contribute to environmental pollution [10, 11]. Furthermore, certain APIs exhibit high stability, resisting degradation during treatment and persisting in wastewater as the parent compound, ultimately entering the environment in their original form [12, 13]. In low- and middle-income countries, poor water management, high pharmaceutical usage, and limited waste sorting exacerbate the problem [14]. Processed sewage used as fertilizer and wastewater effluents employed for irrigation further contribute to the accumulation of APIs in soils, leading to phytotoxicity in plants [3, 12].

The environmental presence of APIs is of concern for wildlife, domestic animals, and public health. Long-term exposure to mixtures of APIs raises concerns about health impacts, particularly the promotion of antimicrobial resistance and drug resistance [15, 16]. This issue is further complicated by limited knowledge of the threshold concentrations that drive resistance selection. Chronic exposure to APIs can lead to behavioral changes, reduced fertility, and population decline in wildlife [8, 17]. Reducing pharmaceutical pollution through more rational and judicious use of drugs aligns with the "One Health" goals, benefitting human, animal, and environmental health. By reducing drug residues in the environment, we protect ecosystems and indirectly enhance human health by supporting biodiversity. This holistic approach underscores the importance of sustainable practices in pharmaceutical use to promote global health and environmental resilience [18]. Nature-based solutions and advancements in wastewater treatment technologies have also been proposed to mitigate these effects.

1.1.2. Pharmaceuticals of concern

Over 2,000 APIs are currently used to treat and prevent diseases in humans, with approximately 40 new APIs introduced annually [19]. These compounds often exist not as isolated contaminants in the environment but as complex mixtures, including veterinary and human pharmaceuticals and other pollutants. Interactions among such compounds can amplify their environmental impact [12]. Within the European Union (EU) Water Framework Directive, several pharmaceuticals have been placed on a so-called Watchlist in 2015, and some of these are now flagged as potential priority substances for water quality [20]. The European Commission has also published a plan of action on pharmaceuticals in the environment [21]. As a result, environmental issues are one of the main themes of the upcoming revision of the EU Pharmaceutical legislation [22, 23].

While human pharmaceuticals contribute significantly to pollution, veterinary pharmaceuticals are of particular concern due to their systematic, widespread and large amounts application in animal husbandry [14]. Practices such as prophylactic treatments, pasture rearing, and manure spreading increase the risk of environmental contamination. Veterinary pharmaceuticals residues are released directly through animal excretion or indirectly via contaminated manure and slurry used as fertilizers. Due to their continuous use, these residues result in diffuse and persistent pollution [8].

Acute toxicity from drug pollution is typically associated with accidental drug discharges, such as poisoning incidents involving diclofenac or pentobarbitone [1, 2, 12, 24, 25]. However, exceptions exist, such as ivermectin and doramectin in dung, where environmental concentrations have exceeded levels harmful to certain species [6, 8, 26, 27]. Outside these cases, long-term and subtle environmental effects are generally more likely than acute impacts.

Numerous studies have identified APIs that persist in the environment despite treatment efforts [8, 11]. Establishing ecotoxicological safety thresholds for commonly encountered APIs has been a focus of research to mitigate their impact [28]. Addressing APIs pollution aligns with the United Nations' Sustainable Development Goals, particularly Goal 3 "Good health and well-being", Goal 6, "Clean Water and Sanitation", Goal 12 "Responsible consumption and production", Goals 14 "Life below water" and 15 "Life on land". Effective environmental awareness and management of pharmaceutical residues will play a critical role in ensuring sustainable development and protecting ecosystems [3, 14, 29].

1.2. Ivermectin

1.2.1. The molecule

Avermectins, discovered in the 1970s by Satoshi Omura in Japan and introduced to the international market in 1981, are naturally produced through the fermentation of *Streptomyces avermitilis*, a gram-positive bacterium named for its ability to eliminate parasitic worms [30–32]. The avermectin family includes structural components A_1, A_2, B_1, B_2, each with major and minor subcomponents (A_{1a}, A_{1b}, A_{2a}, A_{2b}, B_{1a}, B_{1b}, B_{2a}, B_{2b}). Avermectins' broad-spectrum activity encompasses both nematodes and arthropods, classifying it as endectocides [30, 33].

1.1.1.1. Chemical structure and properties

Ivermectin (IVM), shown in Figure 2, is a semi-synthetic derivative of avermectins, a class of macrocyclic lactones with antiparasitic properties [31, 33, 34]. It is composed of a mixture of at least 80% of 22,23-dihydro-avermectin B_{1a} and no more than 20% of 22,23-dihydro-avermectin B_{1b} [31, 33, 35].

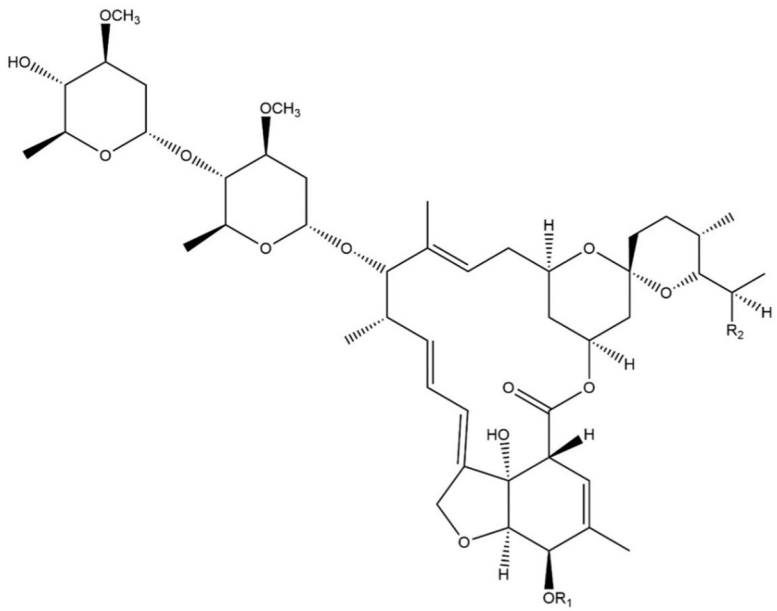


Figure 2 –Structure of avermectins and 22,23-dihydro avermectins from Gaisser et al. 2003 [34]. Avermectin A_{1a}: R_1 = –H, R_2 = $–CH_2CH_3$; Avermectin A_{1b}: R_1 = –H, R_2 = $–CH_3$. Avermectin B_{1a}: R_1 = $–CH_3$, R_2 = $–CH_2CH_3$; Avermectin B_{1b}: R_1 = –CH3, R_2 = $–CH_3$.

The molecules, having chemical formulas $C_{48}H_{74}O_{14}$ and $C_{47}H_{72}O_{14}$, have molecular weights of 875.10 and 861.07, respectively. Ivermectin is a white to yellowish-white, crystalline, non-hygroscopic powder, freely soluble in methanol and ethanol but insoluble in water (water solubility: ~4 mg/L) [36]. Like other avermectins, IVM is highly lipophilic [31].

1.2.1.1. Mechanisms of action

Ivermectin acts as a positive allosteric modulator of glutamate-gated chloride channels, which are present exclusively in invertebrates. However, this ion channels family seems to be very diverse across various parasitic nematodes, even in closely related species [32]. Eight genes, identified in nematodes, encode different subunits of the pentameric glutamate-gated chloride ion channels. These genes are referred to as: avr-14, avr-15, glc-1, glc-2, glc-3, glc-4, glc-5, and glc-6 [37].

By binding to these channels, IVM increases chloride ion permeability, resulting in hyperpolarization of neuronal and muscle cells [32, 37]. This induces flaccid paralysis and

death in parasites, either directly or through starvation [30]. The effects include the suppression of pharyngeal pumping –when the pharyngeal muscle is targeted–, reduced motility –when motor neurons are the primary focus–, inhibition of egg deposition or microfilaria release when the female reproductive tract is affected, and loss of host immunosuppression –when the excretory-secretory pore fails to release immunosuppressive substances [37].

Glutamate-gated chloride channels are not present in vertebrates, and, as such, are thought to confer the broad safety margin of IVM [32]. In mammals, IVM is safe due to its inability to cross the blood-brain barrier under normal physiological conditions [31].

1.2.1.2. Spectrum of action

As reported, ivermectin, like all avermectins, is effective against nematodes –such as intestinal ascarids and lungworms, as well as larvae of filarial worms– and arthropods such as mites, lice and ticks [30, 32, 36, 37]. It is ineffective against adult filarial worms, tapeworms and flukes. It does not work as a repellant [30]. Ivermectin is also effective in clearing human co-infections with various soil-transmitted helminths, such as *Ascaris lumbricoides* and *Strongyloides stercoralis* [32].

Widely used in veterinary medicine for the prophylaxis and treatment of parasitic diseases, IVM is often given through mass drug administration (MDA) strategies to protect entire populations of animals at-risk [32]. In human medicine, MDA has been employed to control neglected tropical diseases such as onchocerciasis, lymphatic filariasis, and malaria, with treatments typically administered once or twice yearly to achieve sustained disease reduction [9, 37]. It has also been employed for treating conditions such as scabies, head lice infestations, rosacea, strongyloidiasis, and pediculosis capitis in children [31].

Ivermectin is not classified as an effective antibacterial agent [38]. However, some reports suggest that at very high concentrations (3–24 μg/ml), it may exhibit activity against *Mycobacterium tuberculosis* [32, 37].

Numerous studies have highlighted the antiviral activity of IVM against several RNA viruses, including those causing dengue, yellow fever, Zika, Hendra, Newcastle disease, West Nile, chikungunya, Venezuelan equine encephalitis, Sindbis, avian influenza A, Semliki Forest, porcine reproductive and respiratory syndrome, and HIV type 1. Additionally, it has shown activity against DNA viruses such as equine herpesvirus type 1, pseudorabies virus, BK polyomavirus, bovine herpesvirus 1, and porcine circovirus 2 [37, 39].

Interest in IVM has recently grown due to studies suggesting its efficacy against SARS-CoV-2. However, this effect has been demonstrated only *in vitro* and at concentrations far exceeding those shown to be effective in humans [39]. Although IVM has shown potential for treating metabolic syndromes like non-alcoholic fatty liver disease, there is no evidence supporting its efficacy for such conditions at the concentrations used for antiparasitic therapy; indeed, higher concentrations would be needed to obtain these effects [37].

1.2.1.3. Authorization: formulations and registered species

Ivermectin has demonstrated high potency and effectiveness when given through various administration routes [30, 36, 37]. It is commercially available in various formulations, including oral, injection -subcutaneous and intramuscular- and topical preparations [31, 36]. Ivermectin is used either alone or in combination with other agents such as triclabendazole [40], rafoxanide [41], doramectin [42], moxidectin [43], abamectin, emamectin, eprinomectin and selamectin [44]. It is widely employed to treat internal and external parasitic infections in cattle, horses, pigs, sheep, goats, domestic pets, buffaloes, reindeer, and camels as reported in Table 1 [31, 32].

Table 1 –Veterinary drug products containing ivermectin authorized in Italy [45–47]. FPA = food-producing animals, NFPA = non-food-producing animals

Product name	Formulation	Association	Destination species
Avalon	Injectable	-	NFPA Horses
Avatar	Oral paste	-	FPA Horses
Cardotek-30	Tablets	-	Dogs
	Chewable tablets	-	Dogs
Cardotek-30 plus	Chewable tablets	Pyrantel embonate	Dogs
Chanectin	Pour on	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers
Closamectin	Pour on	Closantel sodium dihydrate	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers
Clovertin plus	Injectable	Clorsulon	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers
Ecomectin	Oral paste	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers Ovine: meat-producing, milk-producing, lactating ewes, dry ewes, pregnant ewes, lambs Swine
	Pour-on	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers
Equimax	Oral gel	Praziquantel	FPA Horses
	Tabs	Praziquantel	FPA Horses
Eqvalan	Oral paste	-	FPA Horses, NFPA Horses
Eqvalan duo	Oral paste	Praziquantel	FPA Horses
Eraquell	Oral paste	-	FPA Horses
F.mectin	Oral paste	-	Horses

Fatromectin	Pour on	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers
	Oral powder	-	Swine
Filaprev	Injectable	-	Dogs
Filarive	Tabs	Praziquantel, Pyrantel embonate	Dogs
Gabbromec	Oral premix	-	Swine
Ivertin b.o.s.	Injectable	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers Ovine: meat-producing, milk-producing, lactating ewes, dry ewes, pregnant ewes, lambs Swine
Ivomec	Injectable	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers Swine
	Pour on	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers Ovine: meat-producing, milk-producing, lactating ewes, dry ewes, pregnant ewes, lambs
	Oral premix	-	Swine
Karimectin plus	Injectable	Clorsulon	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers
Maximec	Injectable	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers Ovine: meat-producing, milk-producing, lactating ewes, dry ewes, pregnant ewes, lambs

			Swine
	Oral paste	-	FPA Horses
Maximec plus	Injectable	Clorsulon	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers
Neomectin	Oral gel	-	Horses
Nexmectin	Oral paste	-	Horses
Noromectin	Injectable	-	Bovine: meat-producing, dry cows Swine
	Drench	-	Ovine: meat-producing, milk-producing, lactating ewes, dry ewes, pregnant ewes, lambs
	Pour on	-	Bovine: meat-producing, dry cows
Noromectin praziquantel duo	Oral paste	Praziquantel	Horses
Oramec	Oral solution	-	Goats: dry female goat, kids, milk-producing goats
Otimectin	Ear gel	-	Cats
Paramectin	Injectable	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers Swine
	Pour on	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers
Tolomec	Injectable	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers Ovine: meat-producing, milk-producing, lactating ewes, dry ewes, pregnant ewes, lambs Swine
Tolomec o.s.	Oral solution	-	Goats: dry female goat, kids, milk-producing goats

			Ovine: meat-producing, milk-producing, lactating ewes, dry ewes, pregnant ewes, lambs
Tolomec plus	Injectable	Clorsulon	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers
Vectimax	Oral paste	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers Ovine: meat-producing, milk-producing, lactating ewes, dry ewes, pregnant ewes, lambs Horses Swine
	Oral premix	-	Swine
Vectimec	Injectable	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers Ovine: meat-producing, milk-producing, lactating ewes, dry ewes, pregnant ewes, lambs Swine
Virbamec	Injectable	-	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers Ovine: meat-producing, milk-producing, lactating ewes, dry ewes, pregnant ewes, lambs Swine
	Injectable	Clorsulon	Bovine: meat-producing, milk-producing, dry cows, calves, bucket calves, lactating cows, pregnant cows, heifers

Typical dosages range from 0.1 to 0.5 mg/kg body weight (b.w.), administered parenterally, topically, or orally. In farm animals, doses can vary according to the route of administration and range from 200 µg/kg b.w. in cattle, sheep and goats to 300 µg/kg b.w. in pigs [48, 49].

Due to its high lipophilicity and prolonged clearance times, which contribute to its persistence, IVM is associated with extended withdrawal periods ranging from 10 to 58 days, depending on its approved use in specific contexts (species and animal categories) [47].

1.2.2. Pharmacokinetics in mammals

Ivermectin exhibits slow absorption, wide distribution, and slow excretion predominantly through feces. Its bicompartmental pharmacokinetics varies depending on factors such as route of administration, formulation, species, body condition, age, and physiological status [31, 50, 51]. Genetic factors, including breed differences, may also influence pharmacokinetic profiles [52].

Ivermectin undergoes minimal hepatic metabolism, with most of the administered dose excreted unchanged [31, 36, 53]. Regardless of the administration route, IVM undergoes enterohepatic recirculation, and excretion primarily occurs via bile, with less than 1% eliminated in urine [6, 31, 54, 55]. In lactating animals such as dairy cows, sheep, and goats, IVM is also excreted through the mammary gland, a process linked to its high lipophilicity [50]. This lipophilicity facilitates accumulation in adipose tissues, potentially prolonging retention time (RT) in animals with higher fat content [31]. The principal active component, ivermectin B_{1a} (22,23-dihydroavermectin B_{1a}), serves as the marker residue [33, 54]. Liver and fat tissues generally exhibit the highest drug concentrations, while brain tissues display the lowest [36]. Ivermectin's high tissue distribution efficacy makes it effective against external parasites, such as ear mites and scabies: pigs treated subcutaneously have shown wide distribution to all tissues and body fluids after 24 hours post-injection and high concentrations have also been registered in skin, ears and earwax [56].

Ivermectin’s low water solubility contributes to precipitation in subcutaneous and muscular tissues, resulting in slower absorption [36]. The bioavailability of IVM increases following a

high-fat meal. The half-life elimination varies significantly across species and formulations [36, 57]. For humans, oral ethanolic solutions demonstrated approximately double the systemic availability when compared to tablets and capsules. A secondary rise in plasma levels suggests enterohepatic recycling observed between 6- and 12-hours post-administration [58].

In sheep, subcutaneous administration produces peak plasma concentrations three days post-injection, with a bioavailability of 98% [59]. In cattle, subcutaneous administration yields higher systemic bioavailability than oral treatment, while oral administration results in higher fecal concentrations, as shown in Figure 3 [60, 61]. Diet also affects excretion profiles; grain-fed cattle exhibit higher excretion peaks than pasture-fed counterparts, with prolonged excretion periods up to 6-8 days [60]. Ivermectin does not degrade in the rumen and abomasum [62, 63]. In horses, oral IVM administration results in peak fecal excretion at 2.5 days, with detectability extending up to 40 days in the ng/g range. Ninety percent clearance occurs by the fourth day after administration [43].

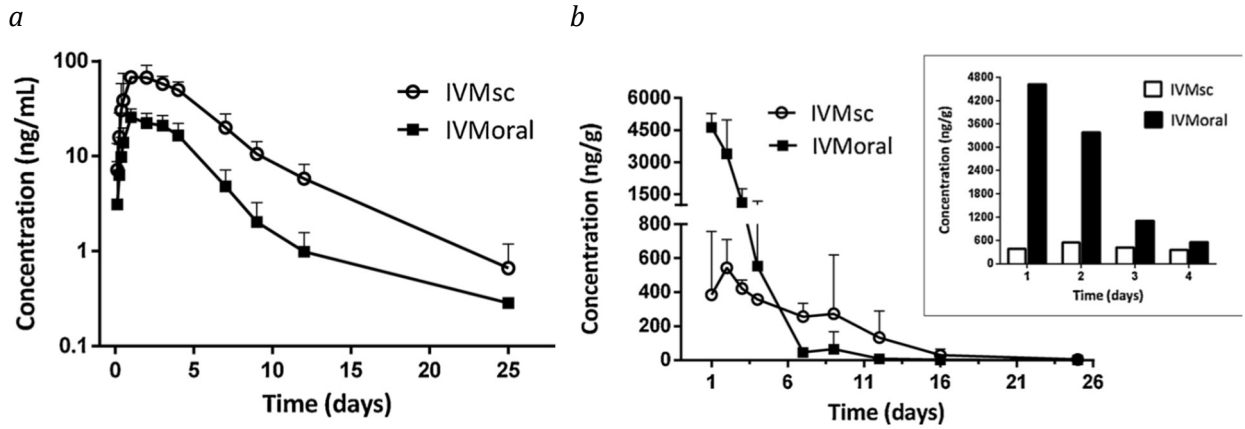


Figure 3–Mean (± SD) concentration profiles of ivermectin following subcutaneous administration (IVMsc) and oral administration (IVMoral) at a dosage of 0.2 mg/kg to parasitized calves (n = 8). a) Plasma concentration b) Fecal concentration [59].

It has been indicated that approximately 35% of IVM administered subcutaneously is excreted within 35 days [64]. Pour-on formulations can result in high fecal residues due to

self-grooming behaviors, potentially leading to elevated drug levels in untreated animals through contact and ingestion [61, 65]. This cross-contamination may induce subtherapeutic drug levels and contribute to drug resistance development [65]. Similar findings in pigs show that IVM can be detected in the tissues of untreated animals sharing enclosures with subcutaneously treated individuals [56]. Although larger fecal concentrations following oral administration of IVM may increase drug exposure to gastrointestinal worms, this does not always enhance efficacy. The potential therapeutic advantages of oral treatments must be cautiously evaluated, particularly in the context of anthelmintic resistance [66, 67].

Compared to goats, sheep, and cattle, plasma clearance of IVM is higher in pigs [36]. In pigs, earlier plasma detection compared to cattle is observed, with detectable levels persisting for up to 20 days [57]. Subcutaneous administration in pigs promotes slower absorption and prolonged bioavailability due to precipitation and adipose tissue distribution [57]. Leaner pigs exhibited earlier plasma concentration peaks and lower backfat IVM concentrations compared to fatter pigs. High variability in backfat IVM concentrations has been reported in pigs treated subcutaneously, with residues detected for up to two weeks but absent by nine weeks post-treatment [68].

After intravenous administration at 300 μg/kg in pigs, IVM remains detectable in plasma for 8-10 days. Subcutaneous administration extends elimination times compared to intravenous treatment. High individual variability of IVM backfat concentrations has been observed in pigs receiving subcutaneous treatment [56, 69]; individual differences have also been observed in the time required to reach IVM plasma concentration [69]. Body composition, including live weight and backfat thickness, as well as dietary variations, have not demonstrated significant effects on the drug's kinetic disposition in pigs [51, 69].

1.2.3. Toxicity

Ivermectin is widely regarded as a safe drug with a broad safety margin. Acute toxic effects in mammals are primarily related to central nervous system disorders, manifesting as tremors, depression, ataxia, paresis, or paralysis, depending on the species and the administered dose. Sensitivity to toxicity varies notably among species: rodents, especially mice, are highly sensitive [70], while primates, including humans, are relatively less affected. At therapeutic doses, IVM is well tolerated across all species [31, 37]. Teratogenic effects have been observed in laboratory animals only at doses toxic to the mother [35].

The toxicity of IVM in mammals is linked to a genetic anomaly that impairs the expression of P-glycoprotein (mutation in multi-drug resistance gene 1, MDR1), allowing the drug to cross the blood-brain barrier. P-glycoproteins are energy-dependent plasma membrane transporters that use adenosine triphosphate (ATP) to export xenobiotic drugs out of cells, each with a selective preference for the substances they transport. Indeed, in certain mammals with P-glycoprotein deficiencies, such as Collies, IVM can cross the blood-brain barrier, leading to neurotoxicity. This genetic anomaly is particularly common in herding dog breeds –such as Border Collies, Australian Shepherds– and their mixes, making them more susceptible to IVM toxicity [30].

Ivermectin's potential accumulation in fat tissues raises public health concerns as residues can be found in animal products like milk and meat [31]. The acceptable daily intake (ADI) represents the estimated amount of a substance in food or drinking water that can be consumed daily over a lifetime without posing a significant health risk [71]. For IVM, the ADI has been set at 0–10 µg/kg b.w. This ADI is based on a no observed adverse effect level of 0.5 mg/kg b.w. per day, determined from observations of neurological effects such as mydriasis and reduced weight gain [33, 71]. An uncertainty factor of 50 was applied to determine the

ADI, accounting for interspecies differences (factor of 5, informed by pharmacokinetic studies in dogs and humans) and intraspecies variability (factor of 10). The current ADI reflects a revision from the previous ADI of 0–1 µg/kg b.w., which has since been withdrawn [33].

1.2.4. Non-target organisms

The potential toxicity of IVM to non-target organisms has raised significant concerns [53]. Ivermectin's presence in feces has been linked to the absence of dung-degrading insects, adversely impacting pastureland ecosystems [6, 9, 27, 72].

Trans-generational effects were observed in the dung beetle *Euoniticellus intermedius*, where exposure to IVM not only impacted exposed individuals but also their progeny [73]. Trans-generational toxicity of IVM has been confirmed in *Folsomia candida* as well, affecting survival and reproduction (LC_{50}: 40 mg/kg; EC_{50}: 5 mg/kg). No avoidance behavior was observed, highlighting additional ecological concerns. Exposure across generations consistently affected survival, reproduction, and size, with F2 organisms being smaller and more numerous, while F3 organisms were larger and less abundant [74].

Gonzales-Tokman et al. demonstrated that doses of IVM in dung ranging 31.6-500 µg/kg in dung beetles reduced muscle mass, increased lipid mass at intermediate doses, altered offspring sex ratios, and increased male horn size –an important trait for mating success [75]. Lethal concentration for earthworm *Lumbricus terrestris* has been reported to be 315 µg/kg in soil [38]. Additionally, IVM concentrations of 0.502 and 2.511 mg/kg over 12 weeks resulted in 28% mortality in *Lumbricus terrestris* and a proportional decrease in motility with increasing concentrations [76]. In a field study observing spiked dung, IVM significantly reduced populations of about one-third of evaluated taxa of flies (*Diptera*) and

wasps (*Hymenoptera*), particularly small hymenopteran parasitoids, large dipteran predators, and primary coprophagous dung consumers, with reductions of 62%, 38%, and 23%, respectively. These findings underscore the vulnerability of top trophic levels due to their low population densities, larger body sizes, and susceptibility to pollutant biomagnification [77].

Soil organisms such as *Folsomia candida* and *Hypoaspis aculeifer* were shown to be affected, with LC_{50} values of 8.4 mg/kg soil's dry weight (d.w.) and 31.6 mg/kg soil d.w., respectively. Earthworms (*Eisenia fetida*) exhibited LC_{50} values of 10 mg/kg soil d.w. [78]. Although residues had limited effects on earthworms and springtails, significant impacts on the coprophilous community were observed. While dung degradation was not significantly impaired, insect activity remains only one of several factors influencing degradation. Short-term reductions in dung relocation by beetles due to IVM treatment highlighted disruptions in ecosystem functioning, including reduced species richness, abundance, and biomass, which led to long-term dung accumulation and altered soil processes [79].

Functional type 2A-RELSp nematodes have been shown to predict environmental risks associated with the bioaccumulation of IVM in seafood following sediment contamination with IVM [80]. Lethal concentration in *Daphnia magna* has been reported as low as L_{50} = 0.025 µg/kg [38]. In zebrafish embryos, IVM at 200 µg/L in water induced body axis curvature, pericardial edema, and lethality from 48 to 96 hours post-fertilization (HPF). Survival rates decreased to 76.77% at 48 HPF, 70.3% at 72 HPF, and 50.3% at 96 HPF. No mortality was observed at concentrations below 50 µg/L by 96 HPF. Significant bradycardia and reduced hatching rates were also observed at the highest concentration [81]. In mesocosm models, IVM concentrations of 1150-458 µg/kg in cattle dung were lethally toxic to *Ceriodaphnia dubia* and *Hyalella* [82]. In fish species such as *Prochilodus lineatus*, IVM

exposure in freshwater decreased swimming speed, reduced glutathione S-transferase activity, and impaired predator avoidance, potentially disrupting population dynamics [83]. Lethal concentration for rainbow trout *Oncorhynchus mykiss* has been reported at 3.0 µg/kg [38].

Ivermectin's widespread use and persistence in the environment present significant risks to non-target organisms across various ecosystems. Its impacts, ranging from direct toxicity to trans-generational effects, highlight the need for comprehensive assessments and mitigation strategies to minimize ecological harm.

1.2.5. Persistence

Veterinary medicines can persist in the soil for different durations, ranging from days to years. Their half-life is influenced by environmental factors such as temperature, pH, and the presence of manure [5, 84]. The persistence of these drugs in soil, manure, slurry, and water varies both between and within drug classes. Chauraud et al. have highlighted the need for further investigation into the environmental behavior of antiparasitic drugs in aquatic systems, a topic that remains underexplored and underestimated in the scientific literature [8].

Anthelmintics –macrocyclic lactones and IVM in particular– have been identified as a veterinary pharmaceutical of emerging concern as an environmental contaminant [6, 10, 12, 27, 85–88]. Ivermectin's predicted environmental concentrations in soil for intensively reared and pasture animals is estimated at 100 µg/kg d.w. [89]. The primary degradation mechanism for members of the avermectins family is photo-degradation. Previous studies have demonstrated that abamectin and IVM undergo rapid degradation on plant surfaces, in soil, dung, and water under light exposure. This light-driven degradation process

significantly influences the environmental persistence of these compounds, reducing their concentrations in exposed environments but potentially leaving residues in darker or shaded conditions [38].

Evidence of IVM's accumulation in the environment has been demonstrated in several studies, highlighting its persistence and the challenges associated with its degradation [38, 90, 91]. While a few studies have proposed methods for removing IVM from the environment, the practical feasibility of these approaches remains to be determined and requires further investigation. These findings emphasize the need for continued research into effective reduction strategies to address the environmental risks posed by IVM and other similar pharmaceuticals [92–95].

Ivermectin's half-life has been proven to exhibit significant variability depending on field conditions [9], as shown in Table 2. For instance, the degradation of half-life of IVM under winter conditions are more than six times longer than under summer conditions [77, 96]. Ivermectin concentrations were significantly higher in winter (4-36 mg/kg d.w.) compared to summer (2-7 mg/kg d.w.) in a research conducted in Australia [96]. Halley et al. have also reported that IVM degraded much faster in dry summer conditions (7-14 days) compared to wet winter conditions (91-217 days) in New Jersey, US [55].

Table 2 –Examples of ivermectin persistence in different matrices as reported by Bai et al. [9]

Matrix	**Concentration**	**Half-life**	**Reference**
Soil-feces mixture	Not provided	7 - 14 d	[97]
Water	1 mg in 300 mL water	6 h	[98]
Water/sediment system	1 mg in 300 mL water	127 d	[98]

Sediment	0.1, 0.2, 0.4, 0.8 and 1.6 mg/kg	Over 100 d	[99]
Milk	0.2 mg/kg b.w.	1.90 - 2.46 d	[100]
Egg	0.4 mg/kg for 5 days	1.73 d (only detected in yolk)	[101]
Liver of broiler chickens	2 mg/g of diet for 5 weeks	No residue found	[102]
Liver and breast of squabs	3.3 mg/mL of drinking water in parent pigeons for 3 days	Detected for 1 week	[103]

Furthermore, IVM degrades more quickly in sandy soil than in sandy loam soil [55, 104]. While the presence of manure or slurry in soil can accelerate the degradation of certain veterinary drugs, recent studies indicate that this effect is not universally observed [3]. The mobility of IVM in soil remains a subject of debate. Some studies suggest that IVM binds strongly to surface soil layers, rendering it largely immobile [6, 53]. However, contrasting evidence demonstrates its movement from feces to underlying soil and even to nearby plants. This mobility presents potential risks not only to soil-dwelling organisms but also to herbivorous animals that may ingest contaminated vegetation, thereby extending the negative environmental impact of IVM [105]. Accumulation of IVM in soil has also been proven at high dose rates of 1-2 mg/kg and at 10-20 mg/kg [91]. In an Australian cattle feedlot IVM (5 and 36 mg/kg d.w.) was detected in fresh manure, pad manure, harvested manure and aged manure (in the latter it reached slightly higher concentrations) [96]. Ivermectin was also found to be stable in feces after 45 days in a field study [106]. Its stability has also been confirmed in soil and feces over the course of 14 days by Bai et al. [9].

Ivermectin's dissipation time in aqueous environments has been measured over time. Its concentrations in sediments have been observed to stabilize at 20–30 ng/kg without a

discernible dissipation time[15, 105]. Acute ecological effects were observed within the first week of exposure, at nominal concentrations as low as 100 ng/L. Chronic impacts on ecosystem structure and function were evident within 97 days at nominal concentrations of 30 ng/L. Long-term effects, exceeding 229 days, were primarily observed in sediment-active organisms at nominal concentrations as low as 1000 ng/L [15].

Accumulation in adipose tissues is a concern, as residues can persist in animal products like milk and meat, raising potential public health risks. Hence, the stability of IVM under heat has been extensively studied, particularly concerning food safety. Experimental studies have examined changes in IVM concentrations after various cooking methods [107–109]. Rose et al. reported that cooking methods such as frying pig muscle and liver, microwaving cattle, pig, and salmon muscle, frying burgers, and boiling minced cattle muscle produced no significant changes beyond the analytical precision of the detection methods used [109]. Their findings indicate that IVM remains stable under typical household cooking conditions. Most of the drug residues were found in exuded juices rather than in the solid tissues. Moreover, no major metabolites or breakdown products were detected [108, 109]. Cerkvenik et al. further demonstrated IVM's resistance to pasteurization processes in ewe's milk, as well as in milk-derived products such as yogurt and cheese after subcutaneous injection of 0.2 mg IVM/kg b.w.. Their studies also identified peak IVM concentrations in raw bulk milk two days after subcutaneous administration in livestock [107]. Ivermectin's resistance to heat was confirmed in experiments involving spiked samples roasted at 190°C for 40 minutes and fried for 6 minutes, where internal sample temperatures ranged from 55 to 96°C [108]. While it has been suggested that weak acids, such as lactic acid, may hydrolyze IVM similarly to strong acids like sulfuric acid, there is currently no direct evidence supporting this in scientific literature. Notably, milk from treated animals is excluded from production to prevent contamination [107].

These findings underscore the importance of establishing maximum residue levels (MRLs) for IVM. MRLs for IVM are outlined in Table 3. According to the latest annual report on veterinary drug residues in animals and foods by the European Food Safety Agency (EFSA) in 2022, the category B2a, which includes IVM, registered 14 non-compliant results in the entire EU over 26,464 compliant results (0.05% and 99.95%, respectively). Four out of the fourteen (28.57%) were IVM incompliances: one in bovine, two in milk and one in sheep/goats category [110, 111].

Table 3 –Maximum residue levels (MRLs) for ivermectin as per the Regulation (EU) 418/2014 [112].

Marker residue	Animal species	MRLs	Target tissues	Other provisions (according to Article 14 of Regulation (EC) No 470/2009)
22, 23-Dihydro-avermectin B_{1a}	All mammalian food producing species	30 µg/kg	Muscle	"For porcine species the fat MRLs relates to "skin and fat in natural proportions". Not for use in animals from which milk is produced for human consumption"
		100 µg/kg	Fat	
		100 µg/kg	Liver	
		30 µg/kg	Kidney	

Despite its widespread use, studies on the environmental presence of IVM are limited, with only a few investigations reporting its detection in environmental matrices [10, 11, 96].

1.2.6. Referrals against its use

When pharmaceuticals are authorized and introduced to the market, producers and pharmaceutical companies are required to submit an environmental risk assessment (ERA). These assessments aim to identify potential environmental risks associated with the drug's use while ensuring the continued availability of medications to patients. ERAs rely on predicted environmental concentrations, which are influenced by the drug type, treatment characteristics, and the pathways through which residues enter the environment [113]. If

the predicted environmental concentrations exceed the threshold of 100 µg/kg d.w. in soil for intensively reared or pasture animals, additional studies on environmental fate and effects on selected non-target species are mandated during Phase II of the assessment. Similarly, in aquatic environments, if the predicted environmental concentrations surpass 100 ng/L in water compartments, further risk assessment steps are required [53]. This provides a deterministic framework to evaluate potential ecological impacts, guiding reduction strategies and regulatory decisions. However, these reports do not always reflect real-world conditions, prompting calls to enhance the regulatory framework for pharmaceutical ERAs [88, 89]. This is particularly crucial for drugs that are not metabolized or produce metabolites with activity against non-target species [13, 114, 115]. Ivermectin exemplifies such a case, as it undergoes minimal metabolism and is excreted in feces in an active form.

Ivermectin has been utilized extensively in agriculture and horticulture to protect various crops, including fruits, cotton, vegetables, and ornamental plants, showcasing their versatility and effectiveness in pest management [6, 53]. When avermectins are employed in crop protection, evidence indicates potential ecotoxicity for non-target species. In particular, insects, including pollinators such as bees, are at heightened risk [9]. The vulnerability of these species underscores the importance of careful application and the development of strategies to mitigate adverse environmental impacts while maintaining agricultural efficacy [53]. While much remains to be discovered about IVM, its continued promise lies in the development of more sustainable strategies for helminth control programs [6, 32, 53, 84].

Despite its extensive use, regulatory agencies like the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have explicitly advised against using IVM intended for animals in the treatment of COVID-19 in humans [116, 117].

Kools et al. ranked IVM fourth among European veterinary medicines posing environmental risks, particularly in pasture scenarios due to dung release [118]. Similarly, a report by Boxall et al. highlighted endectocides, including IVM, as among the most commonly used veterinary medicines with significant potential for environmental contamination [5]. Given IVM's neurotoxic, hepatotoxic, immunotoxic, and ecotoxic properties, its quantification in biological matrices is essential [31]. Recently, drug resistance to IVM has been identified, although no robust links between specific candidate genes and resistance have been established [119].

1.3. Objective of the study and research questions

This study aimed to investigate the environmental and biological implications of IVM through the following questions:

1. How can ivermectin be detected in the environment?
2. Does ivermectin pollute the surroundings of swine farms?
3. Does ivermectin affect gut microbiota in pigs?

2. Research question 1: How can ivermectin be detected in the environment?

Given the reasons outlined in the introduction, an accurate system to monitor ivermectin levels in organic substrates is of crucial importance to ensure effective environmental management and risk assessment. The present method was specifically developed to detect the marker residue—ivermectin B1a (22,23-dihydroavermectin B1a), the parent drug itself. Method development was carried out in collaboration with the Food and Feed Chemical Department of the Experimental Zooprophylactic Institute of Lombardy and Emilia-Romagna "Bruno Ubertini" under the supervision of Dr. Simonetta Menotta and Dr. Giulia Segato.

A high-performance liquid-chromatography (HPLC) method was initially developed and validated based on a reference method already employed in the Accredia-approved laboratory. This method utilizes doramectin as a process standard for the analysis of ivermectin in feed, soil, and water. The HPLC method was successfully published in March 2023 [120].

Subsequently, the pilot study revealed that higher sensitivity was required for soil sample analysis. To address this, the method was adapted to liquid chromatograph tandem mass spectrometry (LC-MS/MS), which enabled a more precise analysis of ivermectin in feces, soil, and slurry. This updated LC-MS/MS method was successfully published in October 2024 [121].

The entire process was conducted under the continuous oversight of the Italian Accreditation Body, Accredia, which ensures compliance with quality standards and certifies the reliability of laboratory results.

2.1. Development of HPLC Method

HPLC has long been employed for detecting veterinary pharmaceuticals and IVM in food and environmental samples [105, 120, 122, 123]. For example, a method involving HPLC with a fluorescent detector has been used for detecting IVM in feces after derivatization with N-methylimidazole and trifluoroacetic anhydride [52, 105, 122]. Several Authors have reported different methods for detection of ivermectin. Åsbakk et al. employed HPLC with fluorescence detection to detect ivermectin in feces from reindeer [122]. Similarly, Iglesias et al. used HPLC for the analysis of ivermectin in soil and cattle feces [105]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been widely recognized for its applicability across multiple matrices, including plasma, milk, liver, and whole blood [44, 52, 124–128]. Moreover, liquid chromatography-electrospray ionization–mass spectrometry/mass spectrometry (LC-ESI-MS/MS) has proven effective in detecting macrocyclic lactones, including ivermectin, in fish muscle [124].

In Italy, the National Reference Center for the Surveillance and Control of Feed for Animals (CReAA - Centro di Referenza Nazionale per la Sorveglianza e il Controllo degli Alimenti per gli Animali) has established a detection limit of less than 1 mg/kg in feed. This stringent threshold underscores the importance of accurate and sensitive analytical methods to ensure compliance with regulatory standards.

This research aimed to develop and validate a HPLC method equipped with a diode array detector (DAD) for the detection and quantification of IVM in feed and water intended for

animal consumption and in soil for environmental monitoring. The method targets quantitative detection of ivermectin at levels exceeding 0.5 mg/kg in solid matrices such as feed and 0.1 mg/kg in water. Doramectin was employed as the internal process standard to ensure accuracy and reproducibility during the analysis [105].

2.1.1. Equipment

The equipment used in this experiment included an ultrasound water bath (SONICA Sweep System Mod. 5300EP, SOLTEC, Milan, Italy), a homogenizer (Grindomix Mod. GM200, RETSH Grindomix, Verder Scientific, Haan, Germany), a flask shaker (Janke & Kunkel Mod. HS501 digital, IKA Labortechnik, Staufen, Germany), a centrifuge (Eppendorf Mod. 5804 R, Merck KGaA, Darmstadt, Germany), and a vacuum pump (KNF Neuberger, Mod. Laboport N820 FT.18, Milan, Italy). A water bath with a nitrogen current device (Buchi Mod. B-461 water bath, GEMINI LAB sustainable equipment, Apeldoorn, The Netherlands) was maintained at 50°C ± 5°C. Additional tools included a vortex mixer (Ika MS2 Munishaker, IKA Labortechnik, Staufen, Germany) and precision scales (Mettler Toledo Mod. XPE12025, Columbus, OH, USA; Sartorius Mod. ME235P, Sartorius, Göttingen, Germany). The two scales had a sensitivity of 10 mg and 0.01 mg.

The HPLC system was equipped with degasser, pump, autosampler, diode array detector (UV to visible spectrum), and software Agilent ChemStation Rev. A.10.02 (1757). It included a pre-column (Supelguard for reverse phase, C-18, length 2 cm, internal diameter 4.6 mm, particle diameter 5 µm, SUPELCO, Bellefonte, PA, USA) and a chromatographic column (Supelcosil for reverse phase, C-18, length 25 cm, internal diameter 4.6 mm, particle diameter 5 µm, SUPELCO, Bellefonte, PA, USA).

Additional smaller equipment included plastic adapters for reservoirs, 50 mL polypropylene tubes with pressure caps (EuroClone S.p.A., Milan, Italy), 50 mL plastic reservoirs, and a

manifold system for solid phase extraction (SPE) (SUPELCO, Bellefonte, PA, USA), pipettes with a range of 10–1000 µL (Gilson, Middleton, WI, USA), SPE columns (500 mg/6 mL C-18 ENVI, Supelco code 57064, and silica LC-Si, Supelco code 505374, SUPELCO, Bellefonte, PA, USA), 0.45 µm filters (INCOFAR, Modena, Italy), 6 mL reservoirs with 13 mm internal diameter (STRATA, Phenomenex, Torrance, CA, USA), frits (Phenomenex, Torrance, CA, USA), 10 mL plastic syringes (Rays S.p.A., Osimo, AN, Italy), and 250 mL centrifuge bottles with screw caps (Nalgene, Rochester, NY, USA).

Glassware employed included graded v-bottom and u-bottom test tubes (10–20 mL), amber test tubes with screw caps, class A or B glass pipettes, Pasteur pipettes, graduated cylinders, class A flasks, and amber vials.

2.1.2. Chemicals and reagents

All reagents used in the project had certified analytical purity. The water used was either deionized milliQ or of comparable quality. Methanol for analytical purposes, sourced from Carlo Erba Reagents (DASIT Group S.p.A., Milan, Italy), was employed during the extraction process. At the same time, neutral aluminum oxide 90 from Merck (Merck KGaA, Darmstadt, Germany) was employed in the preliminary chromatography phase. For the activation and elution of SPE columns and as components of the HPLC mobile phase, the following solvents from Carlo Erba Reagents were used: methanol for HPLC, acetonitrile for HPLC, and dichloromethane for analysis. Ivermectin with certified purity, supplied by Dr. Ehrenstorfer (LGC Labor GmbH, Augsburg, Germany), and doramectin with declared purity, obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), were used for fortified specimens and as an internal standard, respectively.

Before each experiment, a solvent mixture was prepared for dilution, comprising 56% acetonitrile, 37% methanol, and 7% water (v/v). Additionally, alumina (100 g) was activated by moisturizing with 6 mL of deionized water.

All solvents and solutions intended for use in the HPLC system were filtered through a 0.2 µm filter to ensure purity and remove particulates.

2.1.2.1. Preparation of standard and quality control samples

All standards and controls were freshly prepared before use. Stock solutions of ivermectin and doramectin were created in 10 mL flasks, considering their declared purity, at a concentration of 1000 µg/mL. These solutions were prepared using HPLC-grade methanol and stored in amber tubes in a freezer for a maximum of 12 months [129]. Intermediate solutions for each compound were prepared at a concentration of 10 µg/mL by diluting the stock solution in a solvent mixture composed of 56% acetonitrile, 37% methanol, and 7% water (v/v), using a 1:100 ratio in a 10 mL flask. Working solutions of ivermectin were then derived from the intermediate solution, with concentrations of 0.1, 0.2, 0.4, 1, and 2.5 µg/mL used as calibration points. A 0.05 µg/mL working solution was also prepared by adding 50 µL of the intermediate solutions of ivermectin and doramectin to 9.95 mL of the solvent mixture.

All prepared samples, including controls and tests, underwent identical extraction, purification, and analysis procedures to maintain consistency across the analytical process. The analytical procedure has been summarized in Figure 4.

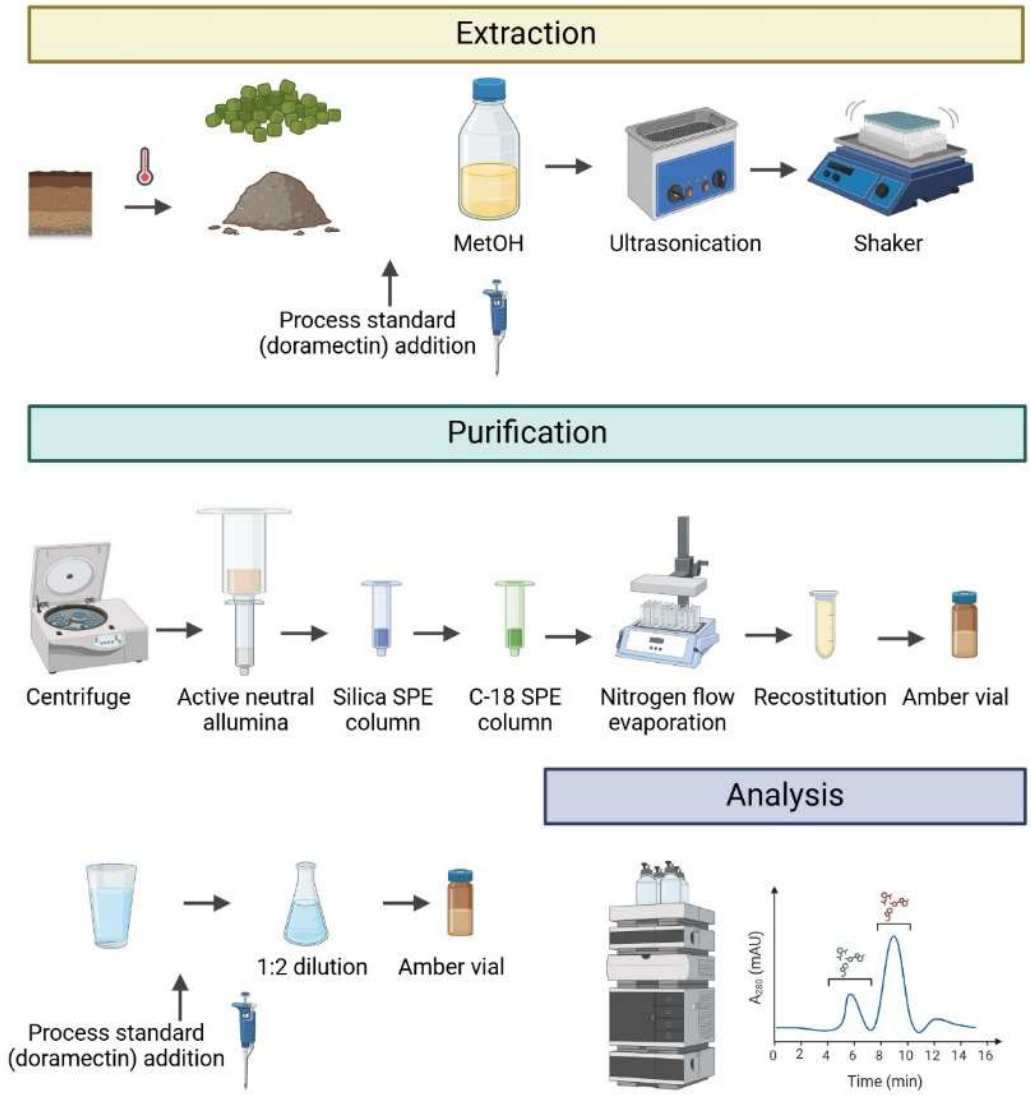


Figure 4–Workflow of the developed HPLC method.

For each analytical session, controls and fortified samples were prepared as follows:

- Water samples: a blank control and a fortified sample at 0.1 mg/L were prepared. For the fortified sample, 100 µL of the intermediate solution (10 µg/mL) was added to a 10 mL flask partially filled with deionized water, brought to volume, and subsequently diluted 1:2 with the solvent mixture.
- Solid samples (feed or soil): a blank control (negative control) was prepared using 10 g of an ivermectin-free sample. The fortified sample, at a concentration of 0.5 mg/kg, was created by adding 500 µL of the intermediate solution (10 µg/mL) to 10 g of an

ivermectin-free sample. This mixture was allowed to stand for at least 10 minutes to ensure uniform distribution.

2.1.2.2. Extraction and purification of the sample

Water samples were diluted 1:2 with a diluting solution composed of 56% acetonitrile, 37% methanol, and 7% water (v/v). To each, 500 µL of a process standard solution containing doramectin at 10 µg/mL were added. A 1.5 mL aliquot was then transferred to an amber vial.

Solid samples were extracted with methanol and purified using solid-phase extraction. The purified extracts were subsequently diluted and analyzed by HPLC.

Soil samples were completely dried in an oven at 45°C prior to processing. At least two-thirds of the total solid samples (feed or soil) were ground to a fine, homogeneous powder. Using a precision scale, 10 g of the ground sample were weighed into a conical flask. Fifty mL of methanol and 500 µL of the internal standard (doramectin, 10 µg/mL) were added. The flask was sealed, placed in an ultrasonic water bath for 20 minutes, and shaken for one hour at 700 oscillations/minute. After extraction, 40 mL of supernatant was transferred to plastic test tubes with pressure caps and centrifuged at 3000 rpm for 5 minutes. The supernatant was then passed through SPE reservoirs containing activated neutral alumina. These reservoirs were prepared by placing a frit at the bottom, followed by 5 g of activated neutral alumina, and a frit on top. Fifteen mL of the supernatant was added to the reservoir. The first 5 mL of eluate was discarded, and the remaining eluate was collected in glass tubes. SPE Columns' conditioning proceeded as follows:

- Silica SPE Columns: each column was preconditioned by percolating 5 mL of acetonitrile (HPLC grade) followed by 5 mL of dichloromethane. After each solvent, the columns were dried for 1 minute under vacuum.

- C-18 SPE Columns: these were conditioned by percolating 5 mL of dichloromethane, followed by drying under vacuum for 1 minute. Then, 5 mL of a water/acetonitrile mixture (50:50 v/v) was added, and the column was kept wet.

Using 10 mL syringes attached to the C-18 SPE columns, 2 mL of each sample and 5 mL of the water/acetonitrile mixture (50:50 v/v) were added and allowed to percolate, ensuring the column remained wet. Columns were then washed with 1 mL of the same mixture and dried under vacuum for 15 seconds. The walls of the columns were rinsed with 2 mL of the mixture, followed by drying under vacuum for 1 minute. The C-18 SPE columns were then attached to the silica SPE columns using adapters. A dichloromethane/acetonitrile mixture (9:1 v/v) was added to the C-18 SPE columns in two 5 mL aliquots, percolated under vacuum. Afterward, the C-18 columns were discarded, and ivermectin was eluted from the silica SPE columns using 5 mL of acetonitrile (HPLC grade) by gravity. An additional 2 mL of acetonitrile was added and eluted under vacuum.

The eluate was collected in conical tubes and dried under nitrogen stream in a water bath at 50°C ± 5°C. The residue was reconstituted with 2 mL of solvent mixture (acetonitrile 56%, methanol 37%, water 7%), vortexed for 1 minute, and filtered through 0.45 µm filters. Approximately 1.5 mL of the final extract was transferred to amber vials for HPLC analysis.

2.1.2.3. Analysis by HPLC

Reverse-phase liquid chromatography was employed for the analysis. The mobile phase consisted of a mixture of acetonitrile (56%), methanol (37%), and water (7%), delivered in an isocratic mode. The stationary phase included a Supelguard reverse-phase pre-column (C-18, length 2 cm, 4.6 mm internal diameter, 5 µm particle size) and a Supelcosil reverse-phase column (C-18, length 25 cm , 4.6 mm internal diameter, 5 µm particle size). Analyses were performed at 20°C. Prior to sample injections, the system was equilibrated for 30

minutes under the specified conditions. The flow rate was 1.2 mL/min, and the detection wavelength was 245 nm. For each sample, 100 µL were injected.

The procedure began with the analysis of the ivermectin standard (0.05 µg/mL), the internal standard (0.05 µg/mL), the blank, and the fortified controls. Subsequently, all samples were analyzed, with standard injections repeated every three sample runs to ensure consistency.

2.1.3. Results and discussion

The calibration curve was created by plotting the nanograms of ivermectin injected on the x-axis against the area under the chromatographic peak on the y-axis. The calibration line and instrument sensitivity were deemed acceptable when the following criteria were met:

1. The coefficient of determination (r^2) was ≥ 0.99.
2. The peak corresponding to the lowest calibration point was clearly identifiable and quantifiable.

The concentration of IVM in the samples was determined using the photometric response recorded in the chromatogram report. Examples of chromatograms from different runs are shown in Figure 5. For samples where the measured concentration exceeded the maximum range of the calibration curve, the samples were diluted appropriately to fall within the quantifiable range.

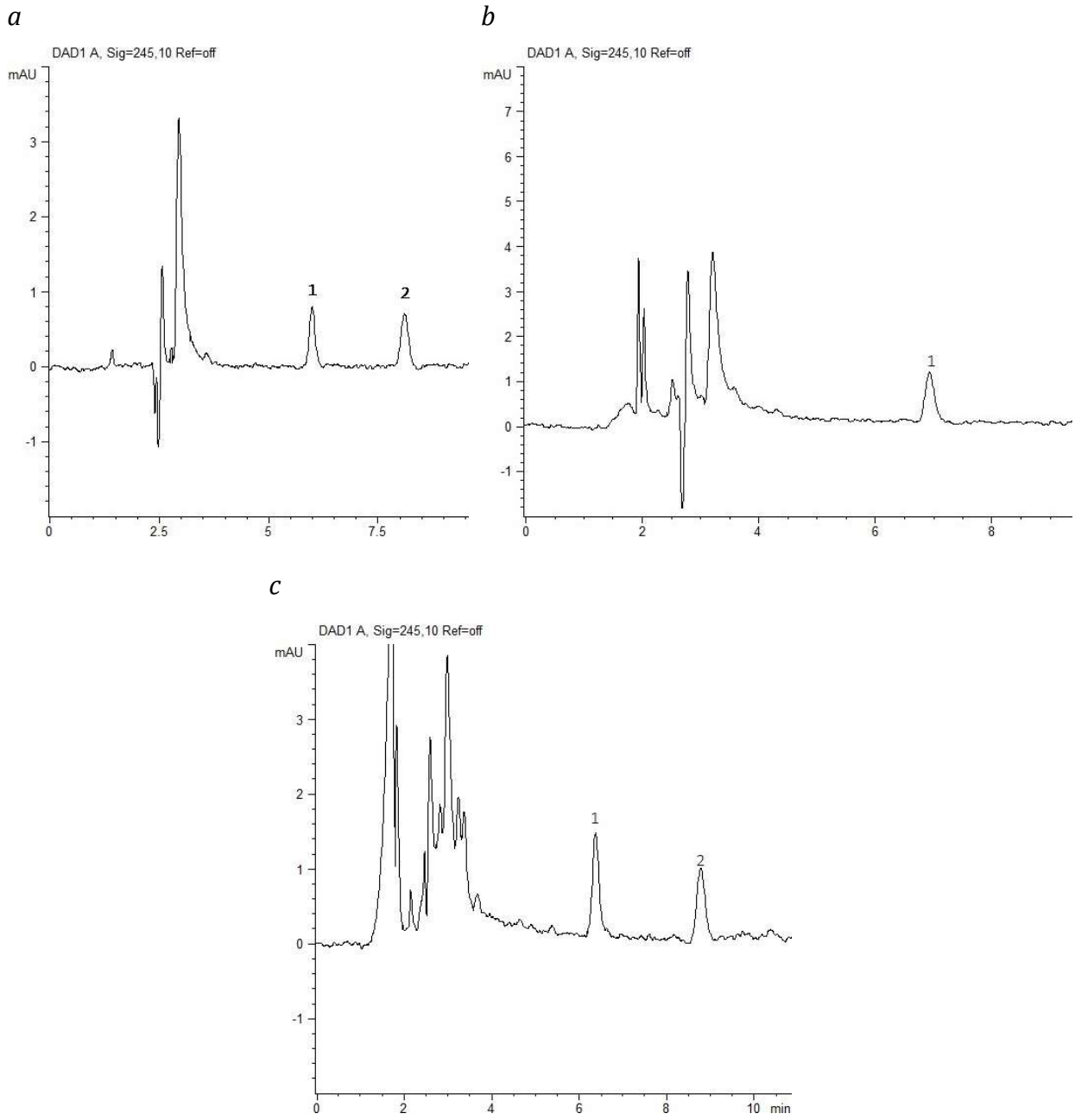


Figure 5 Part a –Chromatogram of the spiked sample, with peaks of interest annotated by their respective retention times (RT). Peak 1 corresponds to doramectin (RT 6.120), while peak 2 represents ivermectin (RT 8.100).

Figure 5 Part b –Chromatogram of a blank sample. No peak is detected at the retention time corresponding to ivermectin, while the doramectin peak is observed and labeled as peak 1 (RT 6.929).

Figure 5 Part c –Chromatogram of a positive sample, where peak 1 indicates doramectin (RT 6.372), and peak 2 corresponds to ivermectin (RT 8.582).

The concentration of ivermectin in the sample was determined using the following equation:

$$Concentration\ (mg/kg)\ =\ A/(W * 2)$$

where A is the injected amount of ivermectin (ng) as determined from the chromatogram, W is the weight of the sample (g), and the factor "2" is a constant derived from the simplification of the calculation process: $\frac{\text{ng injected x 1000 µI x 50 mL x 2 mL}}{\text{100 µL x W x 2 mL x 1000}}$.

The concentration of ivermectin added to the fortified sample was determined by interpolating the value on the calibration line.

To confirm the presence of ivermectin qualitatively, co-chromatography was performed to verify that the analyte in the suspect sample corresponded to the target compound. This process involved injecting 50 µL of a known sample extract together with 50 µL of an IVM working solution. The working solution was selected to match the approximate concentration of the analyte in the sample. A perfect overlap of the standard and sample peaks confirmed the identity of the analyte, provided the peak exhibited a symmetrical shape without splitting or distortions that could suggest the presence of interfering substances.

The area under the peak was expected to equal the combined contributions of half the standard and half the sample, reflecting the 50 µL injection volumes. Additionally, the spectrum of the sample peak was compared with that of a standard at intermediate concentration, assessing the overlap factor (match factor), which had to meet or exceed a threshold of 900/1000 to confirm the analyte's identity [130].

The quantitative confirmatory method was validated in compliance with Annex III of the EU Regulation 2017/625. The measurement uncertainty, evaluated at the validation

concentration (detection limit of 0.5 mg/kg), was expressed using the β error (false negative rate), ensuring it did not exceed 5%. This provided a statistical certainty of 1-β = 95%.

Specificity was evaluated by applying the complete testing method to 20 blank samples for both solid and liquid matrices. None of the blank samples exhibited chromatographic peaks at the analyte's RT. To assess detection capability, extracts from 20 blank samples and 20 samples spiked at 0.5 mg/kg were processed and analyzed over two separate analytical sessions. In all fortified samples, a chromatographic peak corresponding to the ivermectin RT was observed, while no peaks were detected in the blank samples. This ensured compliance with the maximum allowable error rate of 5%. The limit of detection (LOD) was determined to be 0.25 mg/kg and verified experimentally. The limit of quantification (LOQ) was established at 0.5 mg/kg and confirmed through experimental testing of fortified feed samples at the LOQ level, conducted in six independent replicates.

Accuracy was determined by evaluating the recovery rates at three concentration levels within the method's scope. Precision was assessed by calculating the standard deviation and the coefficient of variation (CV%) based on six replicate tests conducted for each concentration level. Linearity was evaluated to determine the method's applicable range (0.5–10 mg/kg). The calibration process yielded a r^2 value of 0.9999 for ivermectin, demonstrating excellent linearity. Specificity was confirmed by applying the entire test method to six blank samples. These tests verified the absence of instrumental responses at or near the analyte RT.

Uncertainty (U) was expressed as the maximum extended relative uncertainty (Ů $\bar{y}$). In this calculation, the effective degrees of freedom (v), derived using the Welch-Satterthwaite formula, the calculated coverage factor (k), and a 95% confidence level (p) were considered. The parameters used in the uncertainty assessment are summarized in Table 4.

Table 4 –Summary of uncertainty parameters.

Analyte	Validation range (mg/kg)	Extended relative uncertainty (Ů (ȳ))	Degrees of freedom (v)	Coverage factor (k)
Ivermectin	0.5-10	12.56	24	2.06

From the described uncertainty assessment, the uncertainty of the result (C) is calculated using the following equation:

$$Ů\,(\bar{y}_r) = C * Ů\,(\bar{y})/100$$

This equation considers the Ů (ȳ) and other associated parameters to provide a precise estimation of the uncertainty associated with the measured concentration of ivermectin in the sample (Ů ($\bar{y}_r$)).

2.1.4. Method validation

The method was validated for both screening and confirmatory purposes. For screening validation, the selectivity, specificity, and quantification limit were verified by performing 20 independent analyses of blank solid samples and 20 independent analyses of blank solid samples spiked at the quantification limit concentration set at 0.5 mg/kg. The same validation was conducted for drinking water, involving 20 independent analyses of blank water samples and 20 independent analyses of water samples spiked at the quantification limit concentration of 0.5 mg/L. The method demonstrated applicability to all considered matrices as well. Specifically, the applicability of the method was established for feed matrices, with a concentration range set from the limit of quantification (0.5 mg/kg) up to 10 mg/kg, in line with the ivermectin content in medicated feed.

Linearity was evaluated across the entire range of applications. For each calibration curve, five concentration levels (0.5, 1, 2, 5, 12.5 mg/kg) were selected, with each level replicated

three times. Calibration curves were constructed using the least squares regression analysis. The data were further analyzed using an ANOVA approach, which confirmed that the linear regression was highly significant ($p < 0.001$), the intercept values did not differ significantly from zero, and $r^2 > 0.999$ demonstrated excellent linearity as shown in Figure 6.

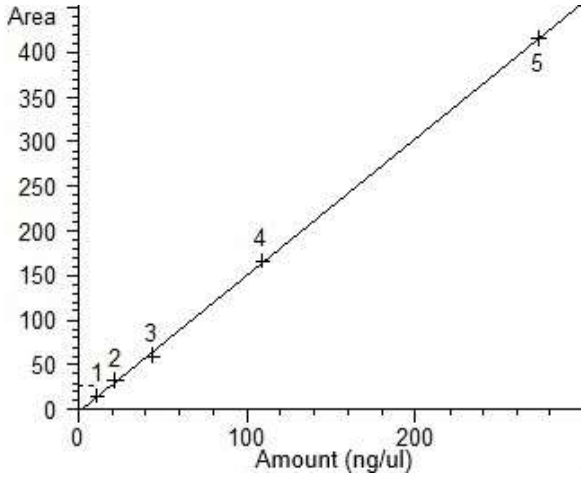


Figure 6–Calibration curve.

Selectivity and specificity were evaluated by analyzing six samples from different types of feed. No analytical substances with RTs similar to ivermectin were identified, confirming the method's specificity. The LOD was established and verified at 0.25 mg/kg, corresponding to half of the LOQ. This was achieved by analyzing six feed samples spiked at the LOD concentration. The LOQ was set at 0.5 mg/kg, in line with half the minimum quantification level concentration recommended by CReAA. This LOQ is higher compared to other validated methods that achieved greater sensitivity using liquid chromatography with different detectors or using LC-MS/MS. For example, Morbidelli et al. achieved n LOQ of 0.5–20.0 ng/mL in plasma using mass spectrometry [126], Duthaler et al. reached 1 ng/mL in dried blood spots [44], and Durden et al. detected 0.5–60 ppb in milk [127]. Methods employing fluorescence detectors, such as those by Åsbakk et al. and Iglesias et al., achieved sensitivities of 5–2000 ng/g and 1–2000 ng/g, respectively [105, 122].

Due to the lack of certified reference material for ivermectin, trueness, precision, recovery, and repeatability were assessed using spiked blank feed samples. Blank feed samples were fortified with ivermectin at three distinct concentration levels before the extraction process, with six independent replicates performed for each level. The results of these assessments, including recovery rates, standard deviations, and coefficients of variation, are detailed in Tables 5 and 6.

Table 5–Data elaboration for each concentration level.

Concentration level (mg/kg)	N° of replicas	Recovery rate (%)	Relative standard deviation	Coefficient of Variation (%)
0.5	6	91.8	2.99	3.26
2	6	98.3	1.67	1.70
10	6	96.1	3.10	3.20

Table 6–Total elaboration of 18 spiked feed samples

Total recovery rate (%)	Total relative standard deviation	Total coefficient of variation (%)
95.4	3.77	3.95

Robustness of the analytical method was assessed through ongoing quality control data. This was achieved by comparing results from quality control samples processed by different operators, under varying environmental conditions, at different times, and using different batches of standards and laboratory materials. The method demonstrated robustness, as quality control results remained stable under the planned operating conditions. Reproducibility was determined by calculating the coefficient of variation (CV%) from 40

ongoing quality control data points. The reproducibility CV% confirmed the method's reliability for consistent application.

2.1.5. Main outcomes

The HPLC method described is efficient for detecting and quantifying ivermectin in various matrices using a calibration curve and doramectin as a standard. It is validated as simple, cost-effective, precise, repeatable, and suitable for small sample sizes. Its applicability spans solid and liquid matrices, making it ideal for monitoring, environmental studies, and ecotoxicity research on ivermectin. The method also holds promises for future adaptation to other macrocyclic lactones or related compounds.

2.2. Development of LC-MS/MS method

Liquid chromatography tandem mass spectrometry provides more sensitive and specific analytical approach compared to HPLC for IVM detection and is potentially more efficient for complex biological and environmental samples [31, 124, 131–133]. LC-MS/MS has been used to investigate the pharmacokinetics of IVM in animal plasma [126, 128] and to quantify its residues in fish, bovine tissue [134], milk [44], and eggs [131]. Sample preparation for mass spectrometry analysis has involved techniques such as liquid-liquid extraction [126], solid phase extraction [127, 128], and the use of dried blood spots [127]. Numerous efforts have been made to detect IVM in soil, feces, and water at concentrations suitable for ecotoxicological studies [15, 43, 96, 98, 105, 135–137]. However, these often involve complex and time-consuming extraction and purification steps, underscoring the need for simplified and reliable LC-MS/MS-based methods for analyzing environmental samples at µg/kg concentrations [75, 138].

This research aimed to develop and validate an LC-MS/MS method for detecting and quantifying IVM in environmental samples, including feces, soil, and slurry. The extraction process was adapted from our prior HPLC-DAD detection method and optimized for LC-MS/MS to enhance sensitivity in the µg/kg range.

2.2.1. Equipment

The equipment employed included standard laboratory apparatus such as scales, micropipettes, vortex mixer, ultrasonic bath, reservoirs, vacuum pump, and centrifuge. Additional specialized equipment included a homogenizer, a manifold, and a Geno/Grinder sample shaker. For the analysis, a Waters Acquity I Class UHPLC system (Waters, USA) was used, coupled with a Waters TQS-micro mass spectrometer. The data acquisition and processing were conducted using MassLynx V4.1 software from Waters Corporation (Milford, MA, USA). The required glassware comprised 10 mL graduated glass test tubes with conical bottoms (Saniver Srl, Italy), 10 mL glass test tubes (Isolab, Italy), class A glass pipettes ranging from 1 to 20 mL (Blaubrand, Germany), Pasteur pipettes (VWR International Srl, Italy), as well as 100 mL and 50 mL graduated glass cylinders (Poulten & Graf Wertheim, Germany), class A glass flasks of 2, 5, 50, and 1000 mL (Hirschmann Laborgeräte, Germany). Consumables included spatulas, spoons, frits (Phenomenex, USA), plastic bags, 250 mL plastic centrifuge jars with screw caps (Nalgene, Rochester, NY, USA), micropipette tips (Gilson Middleton, USA), 50 mL and 15 mL polypropylene test tubes with screw caps (Biosorfa, China), C-18 SPE cartridges (500 mg/6 mL, cod. COC186500, Biocomma, China) and silica SPE cartridges (500 mg/6 mL, cod. COSIL6500, Biocomma, China).

2.2.2. Chemicals and reagents

The chemicals and reagents used were HPLC-grade methanol, acetonitrile, and formic acid, procured from Carlo Erba (Italy). Ammonium formate suitable for HPLC applications was obtained from Merck (Sigma-Aldrich, Germany). Deionized and filtered water was sourced from Millipore Corporation (Merck, Germany), and the ivermectin standard (96% purity 22,23-dihydroavermectin B1a) was supplied by LGC Labor GmbH (Augsburg, Germany).

The aqueous mobile phase (mobile phase A) consisted of a 5 mm ammonium formate buffer with 0.1% formic acid, while the organic mobile phase (mobile phase B) was prepared with 0.1% formic acid in acetonitrile. For the dilution of extracts, a solvent mixture of methanol and mobile phase A in an 80:20 v/v ratio was used.

2.2.2.1. Standard preparation

The stock solution was prepared using ivermectin powder, which was dissolved in methanol to achieve a concentration of 1 mg/mL. Intermediate and working solutions were prepared fresh prior to each analysis by diluting the stock solution. The intermediate solution had a concentration of 10 µg/mL. Standard working solutions were required for the calibration curve and the intermediate solution was diluted in methanol to final concentrations of 0.05, 0.1, 0.2, 0.4, and 1 µg/mL. The standard curve for calibration was then prepared by diluting the standard IVM solution to 1:100 into vials.

2.2.2.2. Preparation of feces samples

The workflow for fecal samples has been summarized in Figure 7.

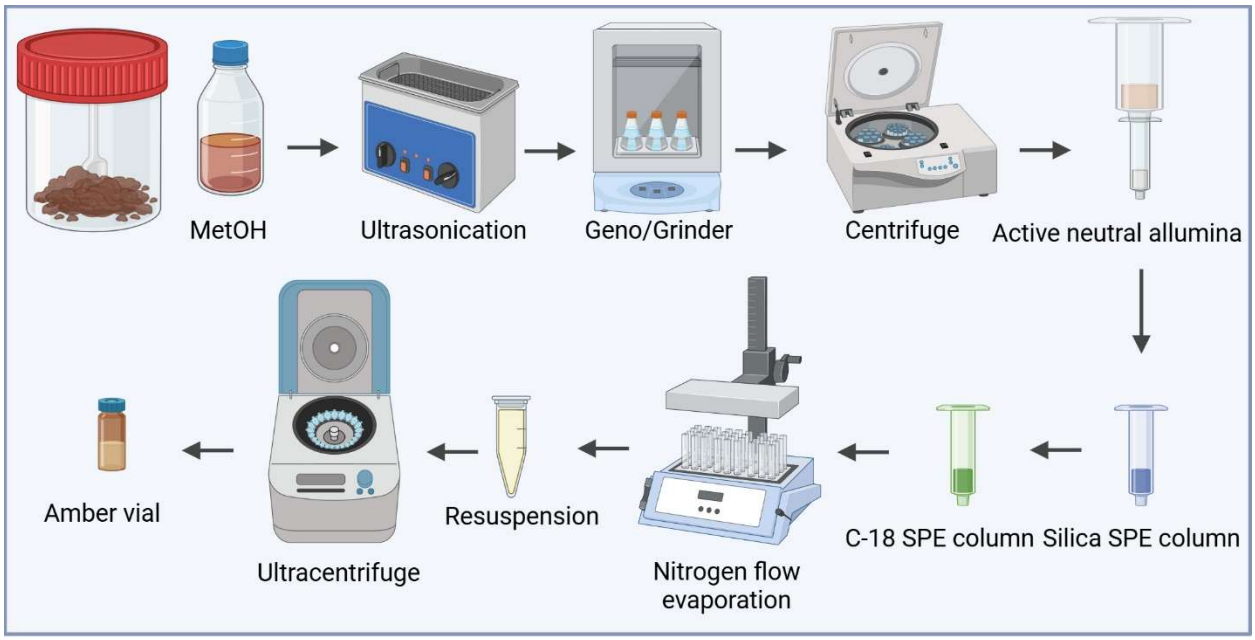


Figure 7–Workflow for IVM extraction in fecal samples for LC-MS/MS analysis.

Each analytical batch included a blank control sample and a fortified sample with 2.5 µg/L of IVM, processed under identical conditions. A 10 g portion of homogenized fecal sample was weighed into a plastic jar, followed by the addition of 50 mL of methanol. The mixture was subjected to ultrasonication for 20 minutes and shaken on a Geno/Grinder at 1500 oscillations/min for 4 minutes. After centrifugation at 1006 × g for 5 minutes, 40 mL of the supernatant was transferred into 50 mL plastic tubes. A 15 mL aliquot of the extract was loaded onto reservoirs prepared with 5 g of activated neutral alumina and frits, and the collected volume was stored in 15 mL plastic tubes.

SPE C-18 columns were conditioned with 5 mL of dichloromethane, dried under vacuum for 1 minute, and subsequently flushed with 5 mL of a 50:50 water/acetonitrile mixture (v/v). After conditioning, 10 mL of the extract and 5 mL of the water/acetonitrile mixture were loaded onto the column and allowed to percolate by gravity. The columns were then washed with 1 mL of water/acetonitrile mixture and dried under vacuum for 15 seconds. The walls were rinsed with 2 mL of the same mixture and dried again under vacuum for 1 minute. SPE

silica columns were prepared by conditioning with 5 mL each of acetonitrile and dichloromethane, with 1-minute drying steps between applications. The silica columns were placed on a manifold alongside the C-18 columns. A 9:1 dichloromethane/acetonitrile mixture (v/v) was added in two 5 mL portions to the SPE C-18 columns, with vacuum applied for 1 minute before discarding the columns.

The elution of IVM from the SPE silica column was carried out using 5 mL of acetonitrile by gravity, followed by 2 mL of acetonitrile under vacuum. The eluate was evaporated to dryness under nitrogen flow at 50°C ± 5°C, resuspended in 0.5 mL of solvent mixture, vortexed for 1 minute, and sonicated for 5 minutes. The resuspended extract was transferred into an Eppendorf tube and ultracentrifuged at 17530 × g for 10 minutes at 4°C. The supernatant was analyzed and stored at -20°C within 24 hours.

2.2.2.3. Preparation of soil samples

The workflow for soil samples has been summarized in Figure 8.

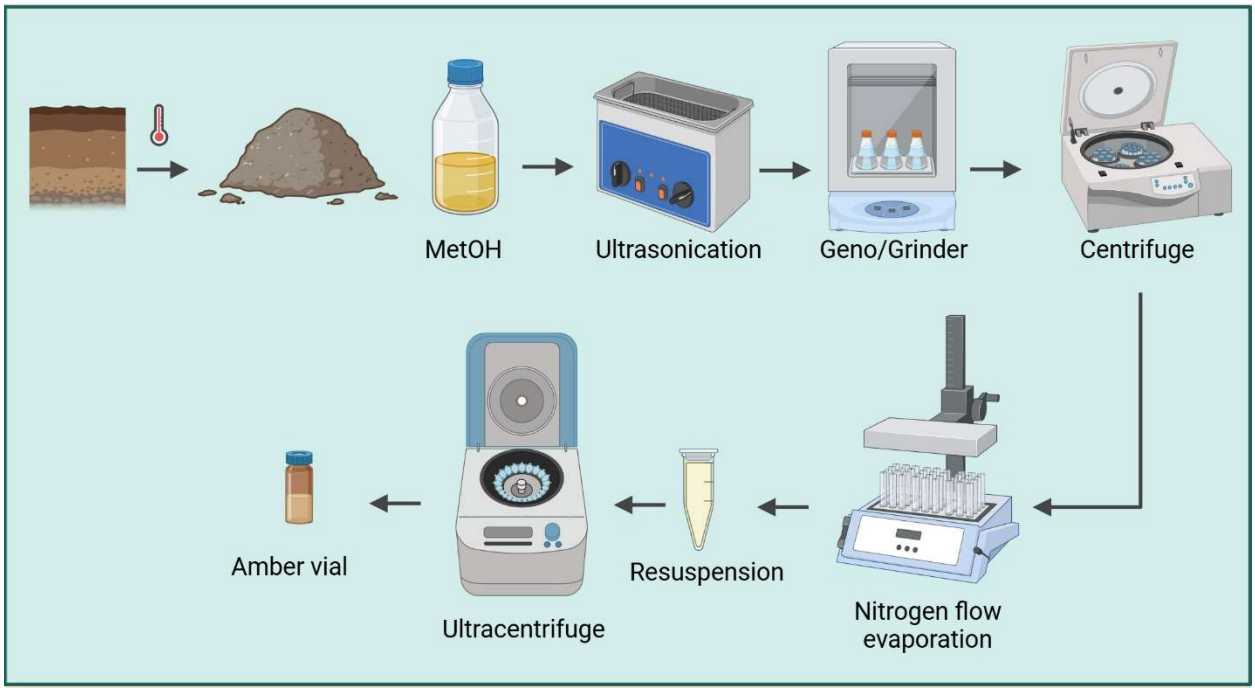


Figure 8–Workflow for IVM extraction in soil samples for LC-MS/MS analysis

Blank and fortified samples were prepared for each batch under identical conditions, with fortified samples spiked to 2.5 µg/L of IVM. Ten grams of dried soil sample were weighed into a plastic jar, and 50 mL of methanol was added. Ultrasonication was performed for 20 minutes, followed by shaking on a Geno/Grinder at 1500 oscillations/min for 4 minutes. Forty milliliters of the supernatant were collected in plastic test tubes with screw caps and centrifuged at 1006 × g for 5 minutes. Twenty milliliters of the extract were evaporated to dryness under nitrogen flow at 50°C ± 5°C. The dried extract was resuspended in 0.5 mL of solvent mixture, vortexed for 1 minute, and sonicated for 5 minutes. The solution was ultracentrifuged for 10 minutes at 17530 × g at 4°C. The resulting supernatant was transferred to a vial with an insert for analysis and stored at -20°C within 24 hours.

2.2.2.4. Preparation of slurry samples

The workflow for slurry samples has been summarized in Figure 9.

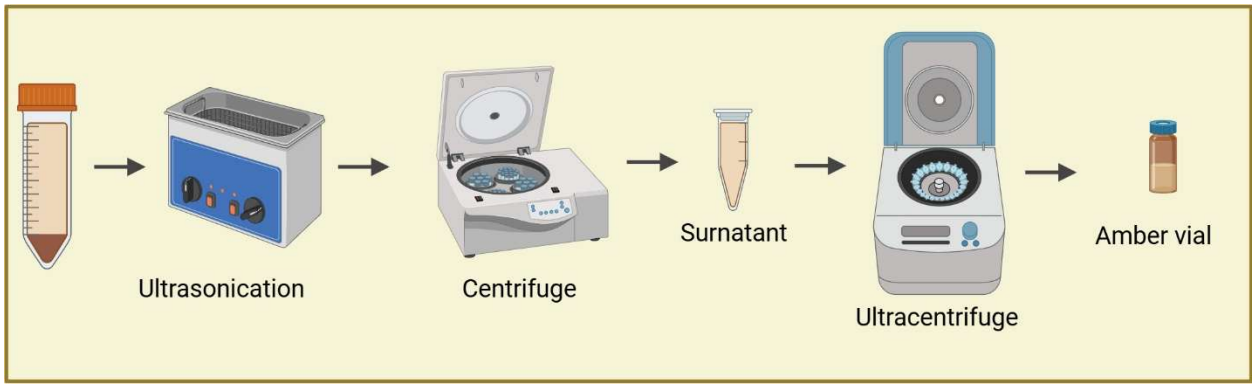


Figure 9 –Workflow for IVM extraction in slurry samples for LC-MS/MS analysis

Both blank and fortified samples were processed identically to the other samples. Ten milliliters of slurry sample were ultrasonicated for 10 minutes and centrifuged at 1006 × g for 5 minutes. A 1.5 mL aliquot was transferred into an Eppendorf tube and ultracentrifuged at 17530 × g for 10 minutes at 4°C. The supernatant was transferred to a vial for immediate analysis and stored at -20°C within 24 hours. For fortified samples, 25 µL of IVM solution

(1 µg/mL) were added to a 10 mL flask containing blank slurry, and the final volume was adjusted with blank slurry.

2.2.2.5. Analytical conditions

Chromatographic separation was performed using a Supelcosil C-18 reverse-phase column (25 cm × 4.6 mm ID, 5 µm particle size) with a Supelguard C-18 pre-column (2 cm × 4.6 mm ID, 5 µm particle size), both maintained at 40°C ± 5°C using a temperature-controlled system. A 4 µL sample injection was used, with separation achieved in a 8.5-minute run using a binary gradient system, as detailed in Table 7. The mass spectrometer parameters used for the acquisition are summarized in Table 8.

Table 7–LC-MS/MS working conditions, binary solvent usage and distribution over time.

Time (min)	Flow (mL/min)	Aqueous mobile phase (%)	Organic mobile phase (%)
0.00	0.30	98.0	2.0
0.20	0.30	98.0	2.0
1.00	0.30	5.0	95.0
5.50	0.30	5.0	95.0
5.60	0.30	98.0	2.0
8.50	0.30	98.0	2.0

Table 8–Working conditions of the mass spectrometer, ionization parameters.

Parameter	Values
Capillary (kV)	1
Source Temp (°C)	150
Desolvation Temp (°C)	400
Desolvation Gas Flow (L/Hr)	600
Cone (L/Hr)	50

In ESI+ mode, three IVM transitions were monitored: 892.4 > 145.0, 892.4 > 307.3, and 892.4 > 569.3. These parameter values were determined through optimization via the infusion of standard analyte solutions.

The IVM calibration curve was developed by plotting sample concentration on the x-axis against the area response on the y-axis. The calibration curve and instrument sensitivity were considered acceptable when r^2 was ≥ 0.999. Recovery for fortified samples was required to fall within the 80–120% range, and the signal-to-noise ratio (S/N) had to be ≥ 20 for both standard and fortified samples. If these criteria were not satisfied, the analysis was repeated.

2.2.3. Method validation

The method was validated according to the guidelines outlined in *The Fitness for Purpose of Analytical Methods* by Eurachem , which establishes the requirements for ensuring analytical methods meet international standards [139]. This guideline provides definitions and quality measures, as well as validation criteria. The validation process evaluated several parameters, including accuracy, applicability, LOD and LOQ, precision, repeatability, recovery, selectivity, linearity, and matrix effects.

In cases where a matrix effect was observed, calibration curves were made using blank matrix extracts instead of solvent mixtures. The LOD was calculated using the ADVeRSE (Analytical methoDs Validation Results Speedy Elaboration) 2.0 software package for R Studio [140, 141]. LOD calculations for each matrix were based on six samples at three concentration levels, analyzed over three days (54 samples in total). The LOQ, set at 1.50 µg/mL, was experimentally verified using the same samples.

Selectivity was assessed by analyzing 18 blank samples. Linearity was evaluated using three replicas of calibration curves within a specified concentration range. Calibration curves were considered valid if r^2 was ≥ 0.999, and the peak for the lowest concentration was quantifiable. The matrix effect was tested by comparing calibration curves prepared in solvent and blank extracts. Differences exceeding 20% indicated a matrix effect. If the curves differed by less than 20%, no significant matrix effect was present. Accuracy was evaluated through recovery testing at three concentration levels: 1.5, 250, and 500 µg/L for feces; 1.5, 5, and 15 µg/L for slurry and soil. Applicability was determined for feces, slurry, and soil based on preliminary research tracing ivermectin residues in environmental samples. Precision was assessed by calculating standard deviation and CV% from six replicates at each concentration level. Specificity was tested using the full analytical method on six samples for each matrix across three separate days. Repeatability was evaluated over three days with 18 samples. Measurement uncertainty was calculated using ADVeRSE software [140], estimating combined (u_{comb}) and expanded uncertainty (U) at three validation levels. Expanded uncertainty was determined by multiplying u_{comb} by a coverage factor of k = 2.

Six blank samples per matrix were processed to confirm no significant instrumental response at IVM retention time. For real sample quantification, concentrations were calculated through interpolation using software-generated chromatogram reports. Samples exceeding the calibration curve's highest concentration were diluted to fall within the method's measurable range.

2.2.4. Results and discussion

2.2.4.1. Sample preparation

The project’s object comprehended complex matrices which made of vegetal, mineral, and starchy components in variable proportions, influenced by the animals’ diet and the soil's

texture; soil's composition and stratification may range from sandy to loamy or clay-rich as well. Given the pronounced lipophilicity of IVM, its extraction was relatively uncomplicated in feces and soil samples. In contrast, recovery rates in sewage were notably lower due to IVM's limited solubility and affinity for aqueous environments. Recovery rates in slurry were notably lower, as IVM has limited solubility in aqueous solutions. Recovery rates for fortified samples were highly satisfactory in feces and soil, with average recoveries of 92.27 ± 12.01% and 96.19 ± 13.78%, respectively. These results confirm methanol's efficiency for IVM extraction in these matrices, as supported by previous research [120, 128], though additional solid-phase extraction and purification were necessary for feces.

In contrast, spiked slurry samples yielded lower recovery rates (33.04 ± 8.43%), representing a limitation in the validation process. Nonetheless, these findings provide a useful basis for future research. Slurry samples predominantly consist of water with variable solid fractions, and analyte loss likely occurs during the centrifugation step when the solid fraction is removed. Future efforts should explore improved recovery methods, focusing on extracting analytes from the solid components. Previous methodologies designed for water analysis handled higher concentrations, which may not adequately represent real life situations in environmental studies. Investigating particulate matter in slurry and analyzing the sediment formed during centrifugation could offer valuable insights. Enhancements to the extraction protocol could increase method applicability for environmental residue analysis at lower concentrations [9, 131].

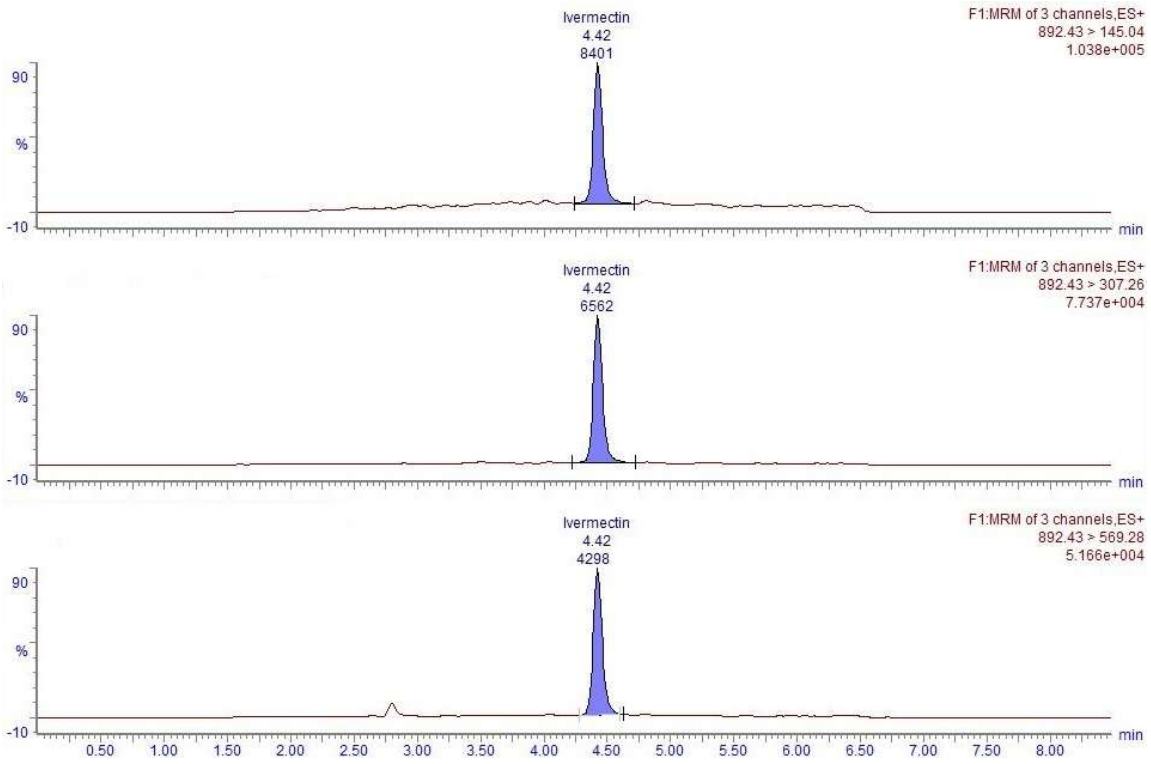
2.2.4.2. Method validation

The method's performance was validated for fecal samples across a concentration range of 1.5-500 µg/L, with no dilution effect observed even at concentrations as high as 5000 µg/L. For slurry and soil samples, validation covered a narrower range of 1.5-15 µg/L. Calibration

curves were generated by plotting the chromatographic peak areas against the analyte concentrations across five different concentration levels (0.05, 0.1, 0.2, 0.4, and 1 μg/mL).

Significant matrix effects were observed only in soil samples, with no interfering signals detected at the analyte's RT. Figure 10 shows a chromatogram for a fortified fecal sample. Each concentration level (1.5, 250, and 500 μg/L for feces; 1.5, 5, and 15 μg/L for soil and slurry) was analyzed in triplicate. A least squares regression model was applied to the data, yielding a r^2 above 0.999, confirming the method's linearity and reliability. No interferences were observed in the chromatograms during the analysis, as illustrated in Figure 10.

a



b

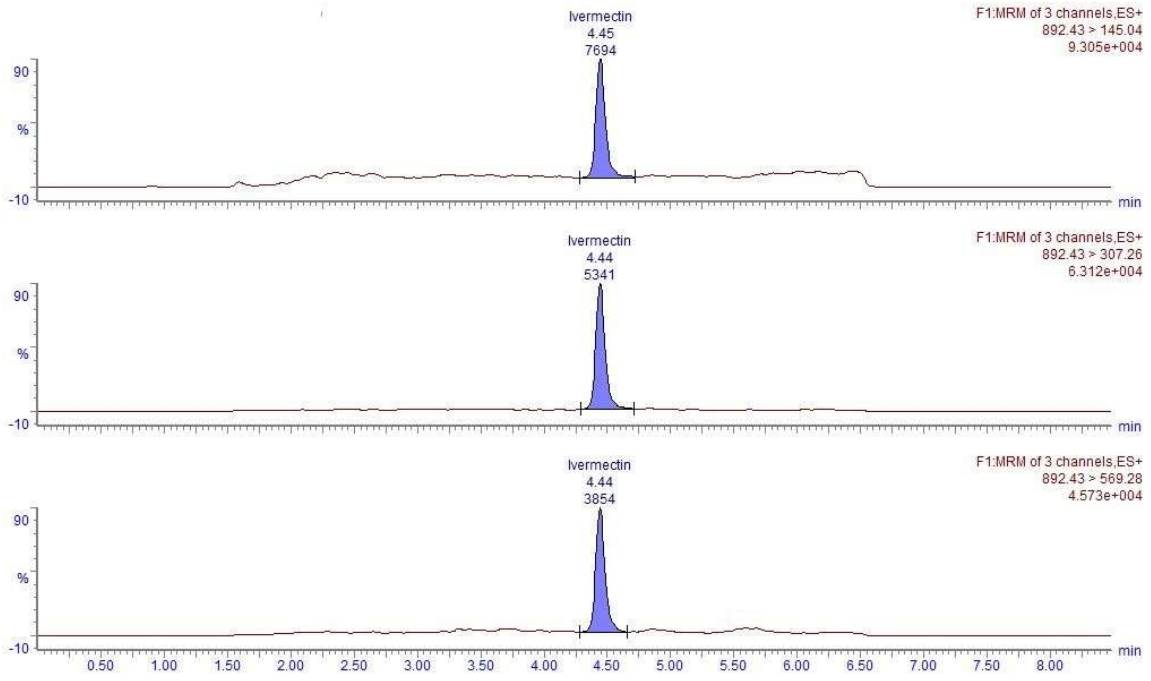
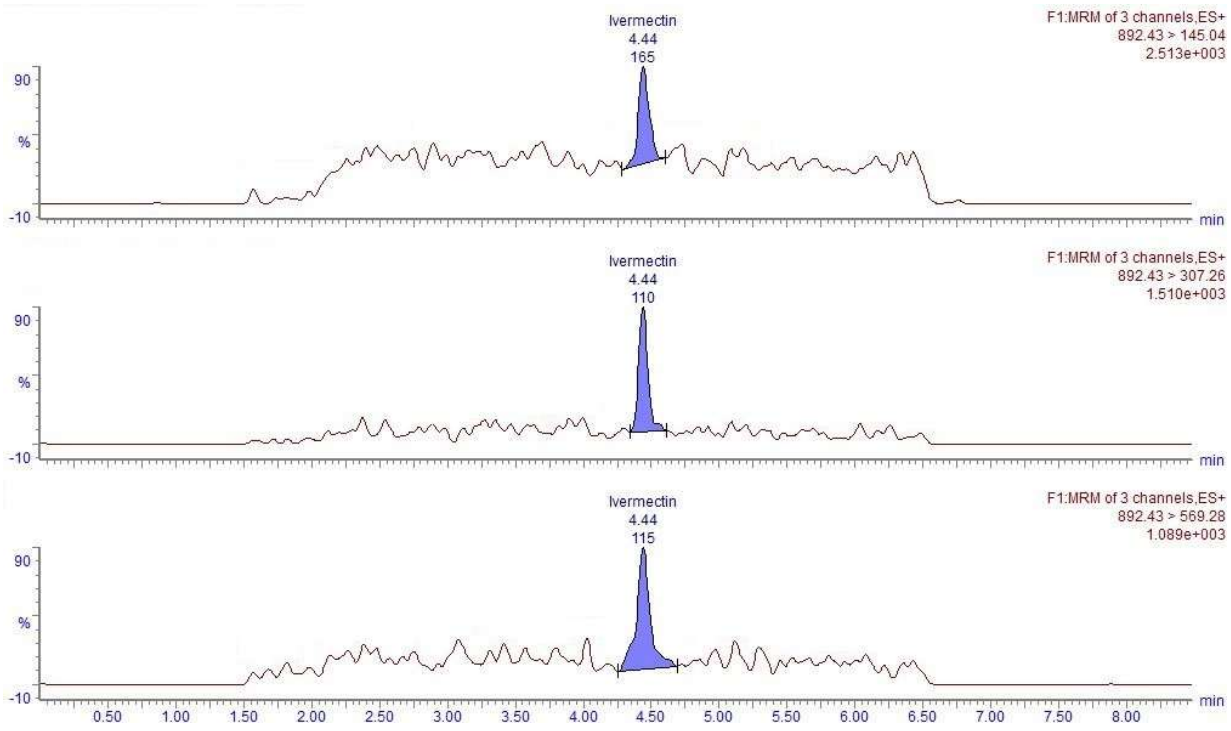


Figure 10 Part a –Chromatograms of fortified fecal sample: each chromatogram shows the three monitored IVM transitions.

Figure 10 Part b –Chromatograms of fortified soil sample: each chromatogram shows the three monitored IVM transitions.

c



d

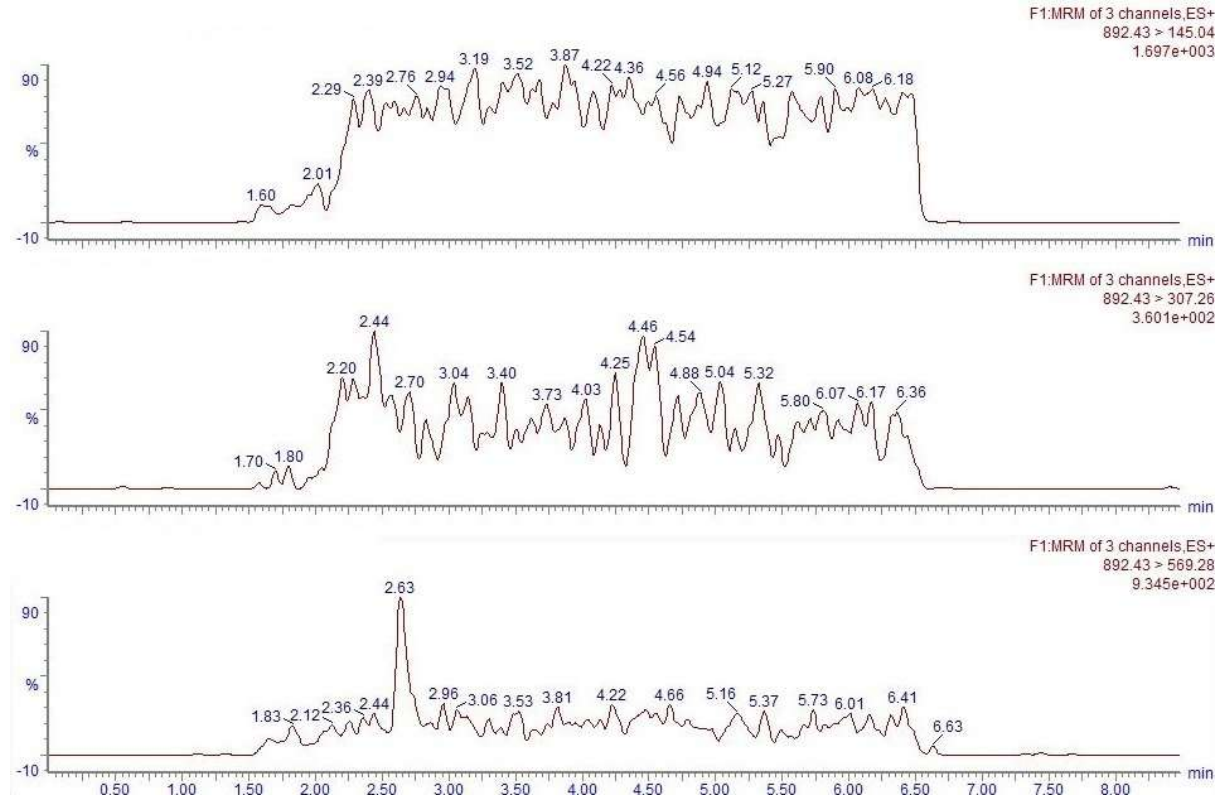


Figure 10 Part c –Chromatograms of fortified slurry sample: each chromatogram shows the three monitored IVM transitions.

Figure 10 Part d –Chromatogram of a blank sample: each chromatogram shows the three monitored IVM transitions.

The trueness of the method was tested by determining the percentage of recovery at three concentrations within the validation range. Recovery rates which fell between 80% and 120% were considered acceptable.

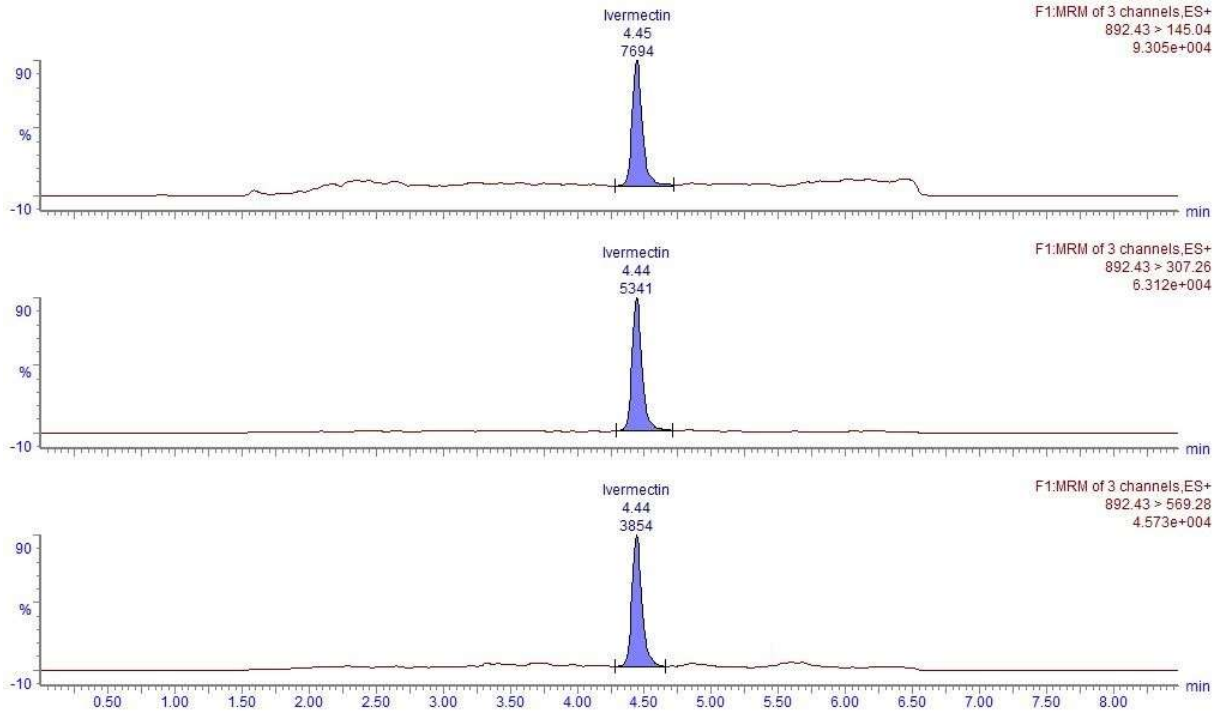
LOD and LOQ parameters are detailed in Table 9. LOD values aligned with those reported for IVM detection in other organic matrices such as plasma (0.2 ng/mL) [126], milk (0.36 ng/mL) [44], and fish muscle tissue (0.46 µg/kg) [124]. These results confirm the method's suitability for environmental studies involving complex matrices like feces and soil. Similarly, the LOQ values were comparable to those in the literature for other matrices: 1 ng/mL [52] and slightly lower 0.5 ng/mL [126] in plasma, 0.72 ng/mL in milk [44], and 1.4 µg/kg in fish muscle tissue [124].

Table 9–Validation results for the determination of ivermectin in environmental samples using LC-MS/MS.

Parameter	**Feces**	**Soil**	**Slurry**
Concentration range (µg/L)	1.5 - 50	1.5 - 15	1.5 - 15
Calibration curve	In solvent	In matrix (standards added prior to injection)	In solvent
Calibration curve (r^2)	0.999	0.991	0.996
LOD (µg/kg)	0.66	0.54	0.36
LOQ (µg/kg)	1.5	1.5	1.5
Overall recovery (%)	92.27 ± 12.01	96.19 ± 13.78	33.04 ± 8.43

The method was successfully employed to quantify IVM in fecal and soil samples in the pilot study, with one example of each shown in Figure 11.

a



b

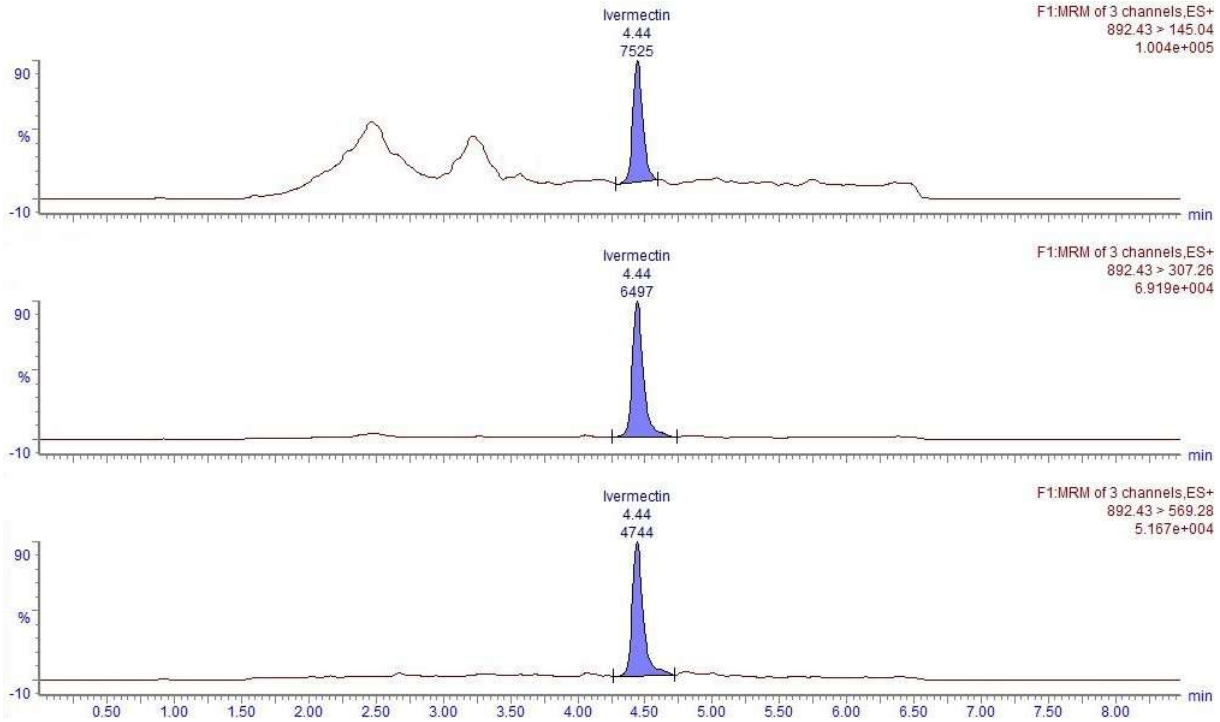


Figure 11 Part a –Chromatograms of positive samples are presented for feces: sample: each chromatogram shows the three monitored IVM transitions.

Figure 11 Part b –Chromatograms of positive samples are presented for soil: sample: each chromatogram shows the three monitored IVM transitions.

2.2.5. Main outcomes

The findings indicate that this revised method provides an efficient approach for extracting and purifying IVM from feces and soil, marking a substantial improvement over previously reported procedures. For soil samples, in particular, the extraction process is significantly quicker and more cost-effective for laboratories. Regarding feces, the method has been optimized to achieve higher magnification compared to earlier techniques involving feed. The enhanced selectivity and sensitivity obtained through mass spectrometry have resulted in considerably lower LOD and LOQ values. Utilizing LC-MS/MS, a gold-standard analytical technique, ensures exceptional accuracy and reliability, making this method highly effective for environmental and residue analysis in complex matrices. Its successful implementation underscores its suitability for such research applications. The inability to validate the analytical method for slurry and wastewater matrices at low concentrations can be primarily attributed to the high lipophilicity of ivermectin, which limits its solubility in aqueous environments. This limitation underscores the need for further methodological development to ensure robust monitoring of ivermectin residues in complex aqueous systems at concentrations in the µg/kg range, especially given their importance in assessing environmental contamination.

3. Research question 2: Does ivermectin pollute the surroundings of swine farms?

The data presented in this chapter were obtained through a collaboration with Ca' Lumaco Farm (Montetortore, 41059 Zocca (MO), Italy), which generously provided access for sampling for the pilot project as well as the control samples for the field study.

This research aimed to systematically evaluate and quantify ivermectin residues in environments associated with swine farming, a sector where such analyses remain limited. The findings contribute to the growing body of knowledge necessary for promoting sustainable livestock practices, mitigating environmental drug pollution risks and avoiding onsets of resistance.

3.1. Study design

The research focused on identifying ivermectin residue pathways in intensive swine farming systems through various environmental compartments. As depicted in Figure 12, sampling included on-farm sampling (fecal matter, slurry) and near-farm sampling (soil and wastewater). It was conducted at multiple time intervals and depths to detect ivermectin residues and evaluate their potential to exceed toxicity thresholds for non-target organisms.

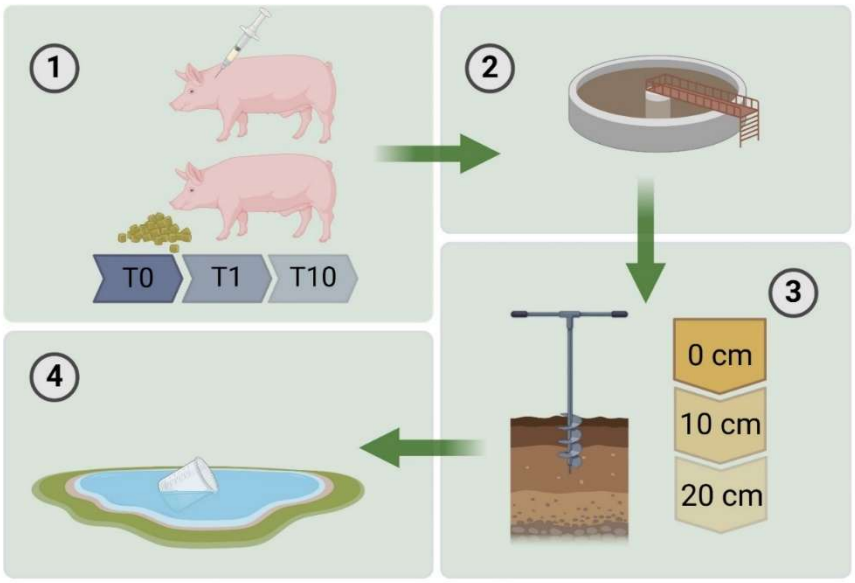


Figure 12 –Study design to trace the pathway of ivermectin residues through various environmental compartments.

3.1.1. Farm selection criteria

Emilia-Romagna region in Italy, known for its high density of livestock farms and significant pig farming activity, was chosen as the study area, as illustrated in Figure 13.

Figure 13 –Map shows the location of farms involved in the study. They are all located in Emilia-Romagna region in Italy.

The region's livestock industry, closely tied to Parma ham production, is characterized by large-scale, established operations. To ensure consistency with the study design and animal welfare, farms were selected based on strict inclusion criteria. Farms were chosen based on compliance with European and Italian regulations on animal health and welfare, presence of qualified personnel, and adherence to sound husbandry practices. The selected farms housed breeding sows with internal replacement programs and included both fattening pigs and breeding individuals on the same site, although not necessarily in the same building. Given the extended withdrawal periods associated with ivermectin, the research focused on breeding sows – as growers are usually treated with other anthelmintics, and boars are not usually present on the farms as artificial insemination is commonly used. Both farms routinely administered ivermectin for antiparasitic prophylaxis in sows. All participating farms followed intensive husbandry practices, where sows were housed individually and fed a standard commercial diet formulated for their needs. All procedures were approved by the Ethics Committee for Animal Testing of the University of Parma (protocol number: 14/CESA/2022).

Two representative intensive swine farms (Farm 1: N 44° 59' 34.978'' E 10° 1' 31.255'' and Farm 2: N 44° 47' 23.108'' E 10° 37' 38.302'') were selected. Additionally, a third farm, Ca' Lumaco (N 44° 19' 50.735'' E 11° 0' 31.451'', hereafter referred to as Farm 3), located in the province of Modena, Italy, was included in the study as a negative control. This farm specializes in the rearing of Mora Romagnola, a native pig breed, and operates using a free-range breeding system. Notably, the farm declares no use of veterinary drugs, including ivermectin, apart from mandatory vaccinations required by law. At Ca' Lumaco farm, animals which require drug treatment with non-essential pharmaceuticals are excluded from production to ensure compliance with farm's production policies. This farm is characterized by its geographical isolation from intensive production systems, minimizing potential

contamination from external pollutants. Additionally, all meat products from the farm are processed on-site, with animal slaughter also taking place within the premises. Samples collected from Farm 3 were utilized for the development of analytical methods. Furthermore, a pilot study was conducted in collaboration with the farm, although the data from this study are not included in the present thesis.

3.1.2. Animal sampling

At each farm, thirty healthy sows for each treatment group were randomly selected. Both treatments utilized Ivomec®, sourced from Boehringer Ingelheim Animal Health Italia S.p.A. Farm 1 utilized both injectable and oral ivermectin treatments, while Farm 2 exclusively employed the injectable solution. Treatments adhered to the Summary of Product Characteristics (SmPC): injectable ivermectin at 300 µg/kg b.w. as a single subcutaneous administration or oral ivermectin at 100 µg/kg b.w. for seven consecutive days.

Fecal samples were collected directly from the rectal ampoule, identified and trasnsported into sterile plastic tubes on ice before being stored at -20°C pending analysis. Sampling occurred at three time points: one hour before treatment (T0), one day post-treatment (T1, corresponding to the eighth day for oral treatment), and ten days post-treatment (T10, corresponding to the eighteenth day for oral treatment).

3.1.3. Environmental sampling

Slurry was sampled from collection and maturation tanks. The slurry was thoroughly mixed to ensure homogeneity before sampling. Ten 50 mL samples were collected per farm, and pooled when multiple tanks were present. Samples were carried on ice and stored at -20°C until analysis.

Slurry was spread on the fields in compliance with current regulations, which specify the permitted spreading periods for each region based on climate conditions and air pollution levels. In the Emilia-Romagna region, slurry spreading is allowed from October 1st to April 30th.

Soil samples were taken from ten random points in fields where, as indicated by farmers, slurry was used as fertilizer. Sampling occurred at the surface and at depths of 10 cm and 20 cm. A sanitized shovel was used for collection, ensuring no contamination between sampling points. Samples were stored in sterile plastic tubes at -20°C until analysis.

Wastewater samples were collected from drainage channels in the premises of fertilized fields, with at least five samples per farm. These were also stored at -20°C until analysis.

3.1.4. Sample Analysis

Ivermectin residues were quantified using LC-MS/MS [121]. The extraction and purification protocols were adapted from previously validated methods to accommodate different matrices (feces, soil, slurry, and wastewater). Method's LODs were 0.66 µg/kg for feces, 0.54 µg/kg for soil, 0.36 µg/kg for slurry, and 0.5 mg/kg for wastewater, while LOQ was set at 1.5 µg/kg. Blank and fortified samples (2.5 µg/L) underwent the same procedures as real samples for quality control.

3.1.5. Statistical Analysis

Data analysis was conducted using R (v4.3.2) and RStudio, employing open-source packages including car, dplyr, factoextra, FactoMineR, ggbreak [142], ggplot2, ggpubr, and readxl. Normality was assessed within individual groups (Farm, Time, Treatment, and Depth) using density plots (ggdensity function, ggplot2) and the Shapiro-Wilk test (shapiro.test function, stats package). Descriptive statistics were reported as mean ± standard deviation for

normally distributed data, and median with range for non-normal distributions. Group comparisons were performed using ANOVA with Tukey's HSD test (R stats package), applying significance thresholds of 0.05 (*), 0.01 (**), and 0.001 (***).

Post hoc power analysis was performed using G*Power v3.1.9.7, keeping a medium effect size (Cohen's d = 0.5) and standard error ($\alpha = 0.05$).

3.2. Results and discussion

A total of 495 samples were included and processed in the study, as shown in Table 10: 225 from Farm 1, 135 from Farm 2, 135 from control Farm 3.

Table 10 –Number of samples from each farm included in the study

Sample type		**Farm 1**		**Farm 2**	**Farm 3**	**Total**
Fecal	**Time**	**Treatment**				
	T0	Injection	30	30	30	120
		Oral	30			
	T1	Injection	30	30	30	120
		Oral	30			
	T10	Injection	30	30	30	120
		Oral	30			
Slurry			10	10	10	30
Soil		**Depth**				
		0 cm	10	10	10	30
		10 cm	10	10	10	30
		20 cm	10	10	10	30
Wastewater			5	5	5	15

Total	225	135	135	495

3.2.1. Feces

The sows included in this research ranged in parity from their first to sixth litters and were between 1 and 6 years of age. A total of 360 fecal samples were collected from 120 sows. A post hoc power analysis (F test – MANOVA repeated measures) revealed that the achieved power for detecting a medium effect size (d = 0.25) with the final sample size (n = 360) was approx. 100% at $\alpha = 0.05$. Residue distribution analysis showed a non-normal pattern across all groups. Fecal samples from Farm 3 confirmed the absence of ivermectin residues at all time points.

In sows treated subcutaneously with ivermectin, fecal residue levels at T0 had a median of 17.54 µg/kg, with a minimum of 0.00 µg/kg and a maximum of 68.96 µg/kg. The positivity rate was 73.33% (n = 44). At T1 the median concentration was 13.50 µg/kg, with a range from 0.00 µg/kg to 63.14 µg/kg, and a positivity rate of 80.00% (n = 48). By T10 the median concentration had increased to 69.46 µg/kg, with a minimum of 0.00 µg/kg and a maximum of 629.53 µg/kg, while the positivity rate dropped to 13.33% (n = 8).

For sows treated with oral ivermectin, no residues were detected at T0, with all samples showing concentrations of 0.00 µg/kg and a positivity rate of 0.00% (n = 0). At T1, the median concentration increased significantly to 941.73 µg/kg, ranging from 203.59 µg/kg to 5767.44 µg/kg, with a positivity rate of 100.00% (n = 30). By T10, the median concentration had decreased to 11.55 µg/kg, with a range from 0.00 µg/kg to 32.58 µg/kg, and a positivity rate of 96.67% (n = 29).

The highest median was obtained in the orally treated group at T1, while the lowest positive median was found for the same group at T10. The oral treatment regimen resulted in a notable and statistically significant elevation in ivermectin levels between T0 and T1 ($p < 0.001$) and at T10 compared to T1 ($p < 0.001$), while no discernible change was observed when comparing T0 to T10. Orally treated sows exhibited higher median ivermectin concentrations following treatment at T1 (941.73 µg/kg) than injected sows (13.50 µg/kg) as shown in Figure 14. Conversely, at T10, injected sows showed higher concentrations (69.46 µg/kg) compared to orally treated sows (11.16 µg/kg). On the other hand, injectable treatment did not show a significant change between T0 and T1, while there was a significant increase when comparing T1 to T10 ($p < 0.001$) and between T0 compared to T10 ($p < 0.001$). Significant differences in ivermectin quantities were observed between the two treatments at all time points. Notably, the oral treatment produced a peak immediately after administration, while the regimen spans several days in the case of a single administration of injectable treatment. In agreement with previous findings, oral administration typically leads to rapid excretion, with the majority of the dose being expelled within a few days [6, 53]. As suggested by previous work, this discrepancy may be attributed to the comparatively delayed distribution of IVM to the gastrointestinal tract associated with the injectable administration route [36].

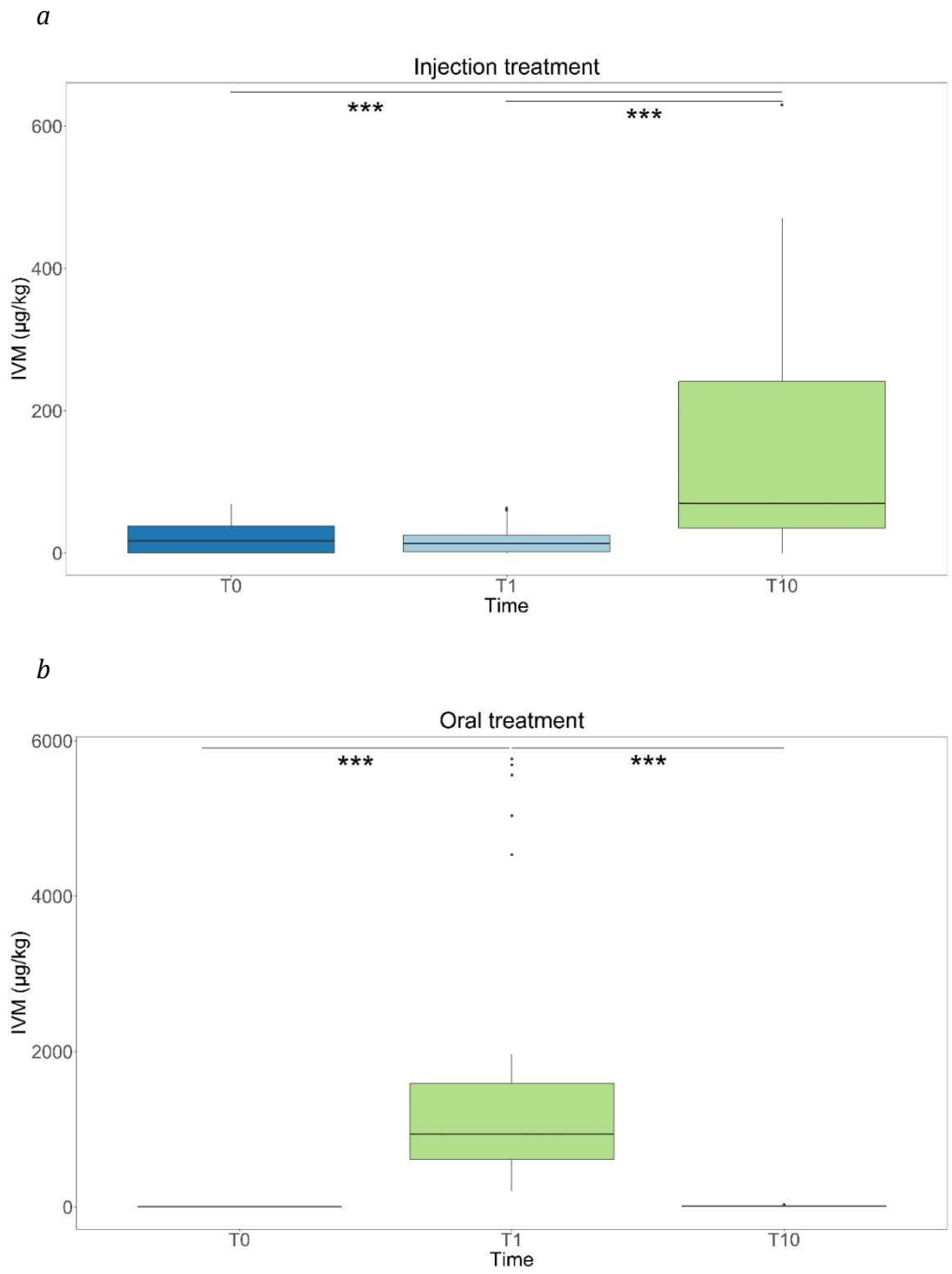


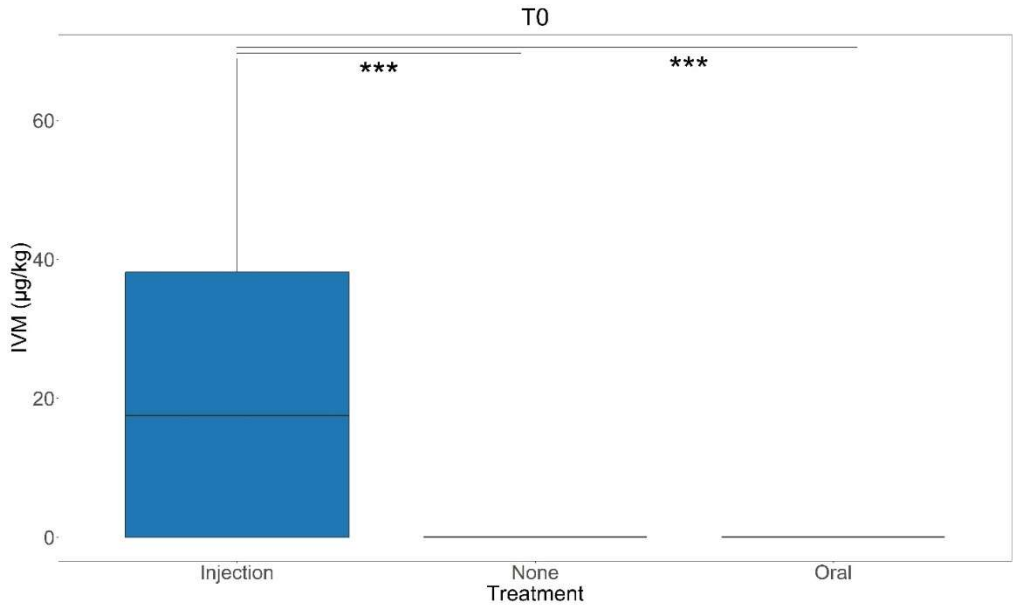
*Figure 14–Results of fecal samples compared at the three timepoints: before treatment (T0), 24 hours after treatment (T1) and 10 days after treatment (T10). The top part shows data relative to subcutaneously injected animals. The bottom part shows data on orally treated animals. Significance has been indicated as follows: '***' = 0.001, '**' = 0.01, '*' = 0.05.*

Pharmacokinetics of IVM through feces have been investigated widely in cattle and a few other species, but not in swine [43, 59, 60, 64, 65, 143]. To our knowledge, no studies have focused on quantifying the environmental IVM residues produced by intensive swine farming. Previous research reported that injectable and topical treatments are rapidly

eliminated, with peak clearance occurring within 2-7 days and a gradual elimination taking over 4-6 weeks or longer. Sustained-release formulations, however, can maintain peak levels for several weeks post-administration [6, 53]. Pharmacokinetic studies in cattle show that IVM elimination in feces reaches its highest concentration a few days after subcutaneous injection (3 days according to Herd et al. [143], 5.60 ± 3.44 days according to Fernandez et al. [64]). Fernandez et al. reported that the release of IVM in cattle feces occurs over a period of more than a month, with only 35% of the total administered IVM being excreted within the first 30 days [64]. On the other hand, oral administration of IVM in horses has shown a 90% clearance in 4 days, and IVM was detectable in feces up to 40 days post-treatment [43].

At T1, the difference in treatment was highly significant between oral and injection ($p < 0.001$) and between oral and the control group ($p < 0.001$) as shown in Figure 15. At T10 the injection group differed significantly from both the control group ($p < 0.001$) as well as the orally treated group ($p < 0.001$). Canton et al. have underlined how the subcutaneous injection in cattle guarantees a greater bioavailability while the oral treatment provokes a greater concentration of IVM in the feces [67].

a



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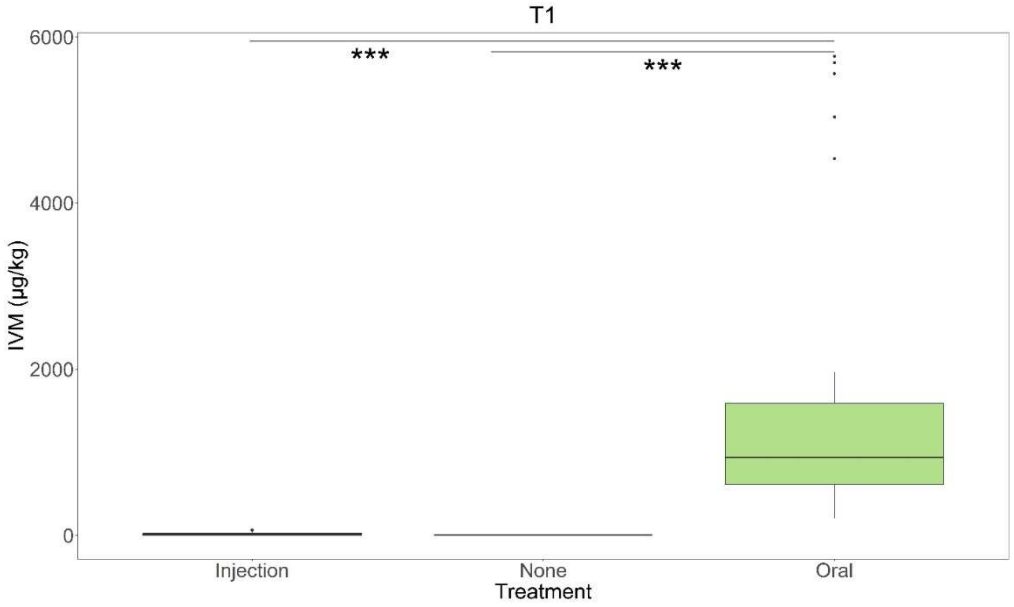


Figure 15 Part a–Results of fecal samples compared between treatments before treatment.
Figure 15 Part b–Results of fecal samples compared between treatments 24 hours after treatment

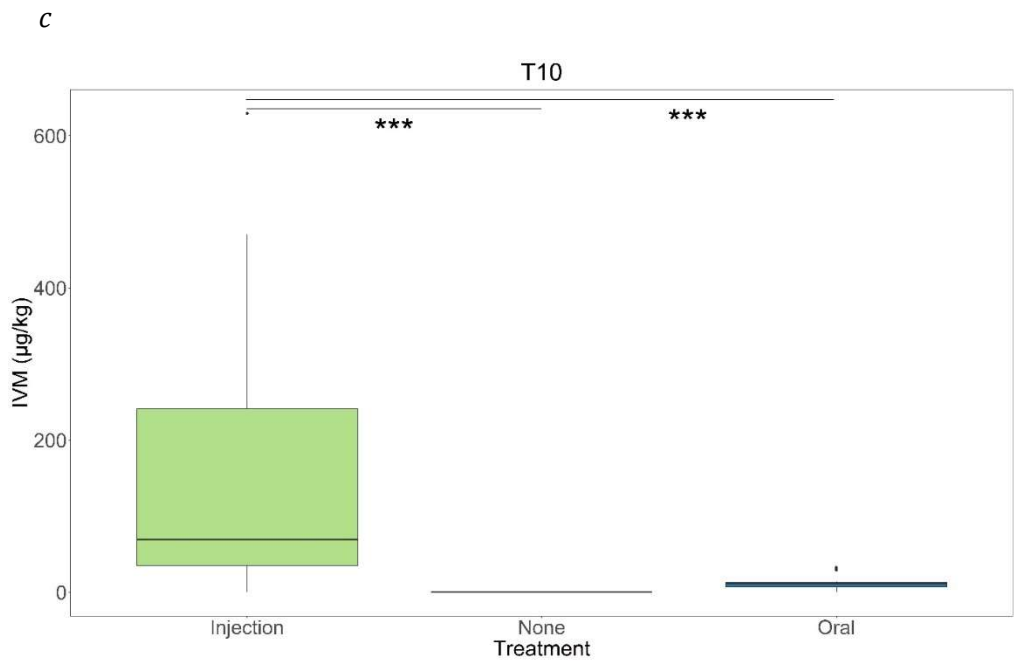


*Figure 15 Part c–Results of fecal samples compared between treatments 10 days after treatment. Significance has been indicated as follows: '***' = 0.001, '**' = 0.01, '*' = 0.05.*

Although this study took for granted the efficacy of the treatments, some Authors have reported that in cattle a high concentration in the feces does not always coincide with greater success in eliminating gastroenteric nematodes [67]. Routes of administration that result in reduced drug exposure to parasites are more likely to contribute to the development of anthelmintic resistance. Therefore, the relationship between administration methods and enhanced efficacy should be carefully evaluated, especially when managing IVM-resistant worms [67]. The potential for chemical residues to exert a lasting ecological impact is influenced by various farm management practices, including the timing and spatial distribution of chemical applications, the number of animals treated, and the selection of the active compound. Additionally, insect-specific factors such as population size, dynamics, and dispersal rates play a pivotal role in determining the overall impact [27].

Notably, several samples (n = 13) from the injection group were positive at T0 in both farms. The sows were not treated by mistake, and their management had been very rigorous. We hypothesize that other factors may have intervened in these cases. One previous research

reported positivity in body fluids and tissue samples of untreated pigs living together with treated animals and underlining the need for direct contact to result positive [56]. We believe that, in our study, animals have been positive since their last treatment, which may be due to slower clearance of the drug, which several groups have reported in the case of subcutaneous treatment [36, 59, 64]. The slower clearance could be affected by the animal's body condition scores, as IVM is proven to be highly lipophilic and could have accumulated in the fat tissue [31, 134]. Other Authors have also emphasized how the role of body fat may play a role that is still unclear in pigs [36, 69]. To better clarify these findings, further studies should investigate the possible effects of body condition scores, back fat thickness, and parity time-lapse.

Authors have also underlined how routes of administration in cattle cause reduced drug exposure and therefore may increase the risk of selecting for anthelmintic resistance [67]. Therefore, the route of administration's potential impact on efficacy, especially when dealing with IVM-resistant nematodes, should be carefully considered.

3.2.2. Soil

Thirty samples for each farm were included for analysis. A post hoc power analysis (F test – MANOVA repeated measures) revealed that the achieved power for detecting a medium effect size (d = 0.25) with the final sample size (n = 90) was approx. 76% at α = 0.05. Normality was evaluated across farm, time, and depth groups, as well as for each individual group, but none displayed a normal distribution.

At Farm 1, soil samples from the surface (0 cm depth) had a median ivermectin concentration of 3.56 µg/kg, ranging from 1.17 µg/kg to 5.43 µg/kg, with a positivity rate of 100.00% (n = 10). At 10 cm depth, the median concentration was 3.89 µg/kg, ranging from 0.00 µg/kg to 7.28 µg/kg, with a positivity rate of 90.00% (n = 9). At 20 cm depth, the median

concentration decreased to 1.41 µg/kg, ranging from 0.00 µg/kg to 98.18 µg/kg, and a positivity rate of 60.00% (n = 6).

Farm 2 presented a different distribution. Surface soil samples had a median concentration of 10.76 µg/kg, ranging from 4.12 µg/kg to 105.90 µg/kg, and a positivity rate of 100.00% (n = 10). At 10 cm depth, the median concentration dropped to 0.00 µg/kg, ranging from 0.00 µg/kg to 39.23 µg/kg, and a positivity rate of 40.00% (n = 4). At 20 cm depth, the median concentration was 2.78 µg/kg, with values ranging from 0.00 µg/kg to 19.20 µg/kg, and a positivity rate of 90.00% (n = 9).

In contrast, all soil samples from Farm 3, which reported not using antiparasitic treatments, tested negative for ivermectin residues at all depths.

As illustrated in Figure 16, no statistically significant differences were observed between depths within the same farm or among Farm 1 and Farm 2 at the same depth. This lack of significant variation could be attributed to the relatively small sample size or the limited number of farms included in the study.

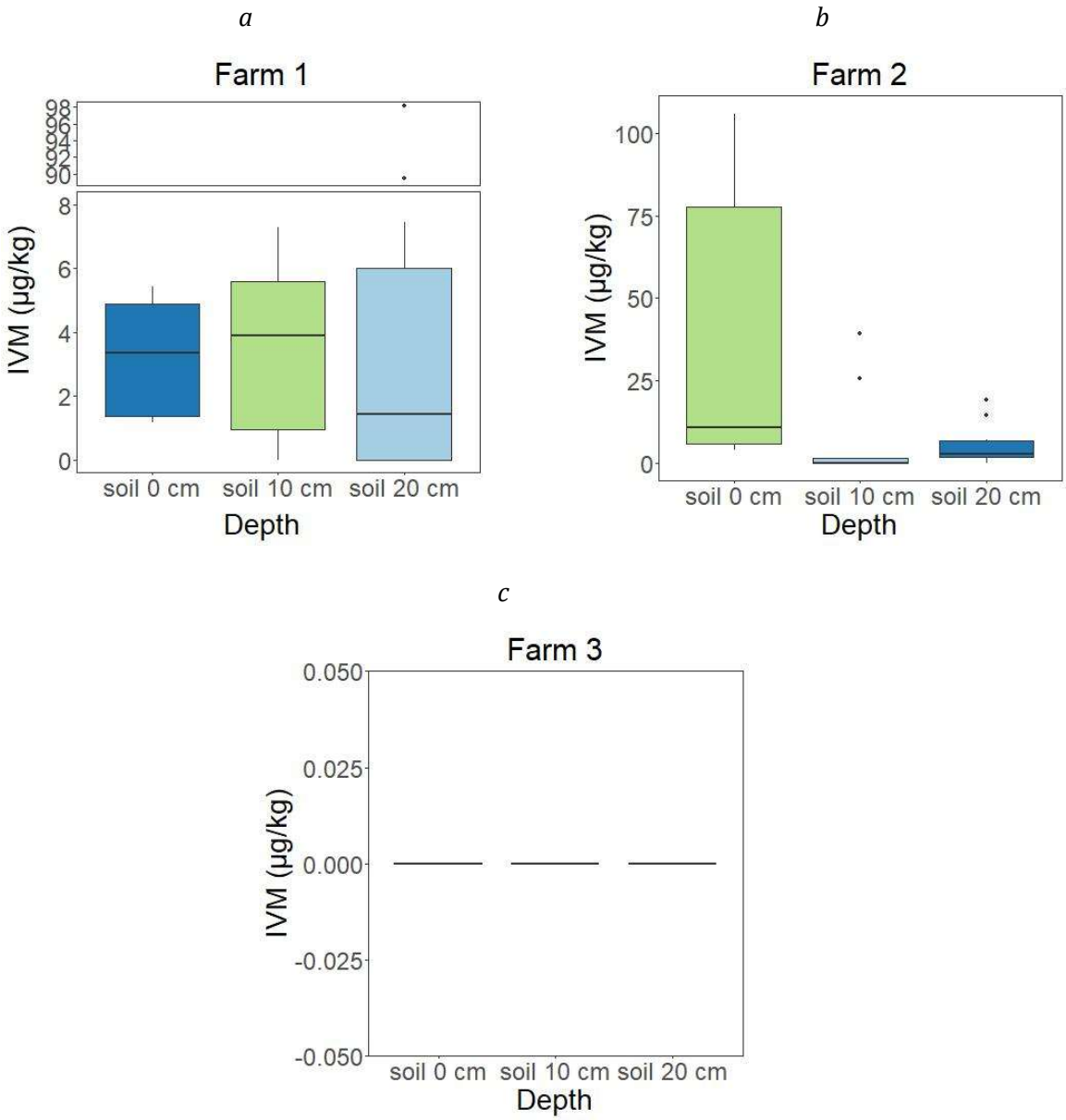


Figure 16 Part a –IVM concentration in soil samples compared at different depths in Farm 1.
Figure 16 Part b –IVM concentration in soil samples compared at different depths in Farm 2.
Figure 16 Part c –IVM concentration in soil samples compared at different depths in Farm 3.

The presented data confirms the hypothesis that the use of ivermectin in swine farms produces residues which persist in the environment. Despite the absence of significant statistical differences, the presence of ivermectin residues in soil samples, particularly at the concentrations exceeding known toxicity thresholds, raises environmental concerns. Among the samples analyzed, 47 exceeded the concentration associated with chronic toxicity in *Daphnia magna* [81], while 6 surpassed levels known to disrupt behavioral and reproductive

activities in dung beetles [75, 144]. Furthermore, 30 samples contained concentrations above 3.0 µg/kg, a level proven toxic to rainbow trout (*Oncorhynchus mykiss*) [38]. Peak concentrations detected do not reach lethal concentrations for soil dwelling species such as *Folsomia candida* (LC50 of 8.4 mg/kg soil d.w.), *Hypoaspis aculeifer* (LC50 of 31.6 mg/kg soil d.w.), and earthworm *Eisenia fetida* (LC50 of 10 mg/kg soil d.w.) [78].

Although further investigations with larger sample sizes and more diverse farm inclusion are necessary to confirm these results across broader areas, this project underscores the need for sustainable practices to mitigate environmental contamination and its potential impact on non-target organisms. Further studies could also evaluate the time-lapse between fertilization and sampling.

3.2.3. Slurry and wastewater

For slurry samples, the post hoc power analysis (F-tests - ANOVA) revealed that the achieved power for detecting the medium effect size (d = 0.25) with the final sample size (n = 360 divided into 4 groups at 3 times) was approx. 19.50% at $\alpha = 0.05$. For slurry samples, the post hoc power analysis (F-tests - ANOVA) revealed that the achieved power for detecting the medium effect size (d = 0.25) with the final sample size (n = 360 divided into 4 groups at 3 times) was approx. 10.95% at $\alpha = 0.05$.

No detectable levels of ivermectin were found in any of the analyzed slurry and wastewater samples, with concentrations falling below both the LOD and LOQ. The inability to detect ivermectin in these matrices is likely due to extreme dilution; for example fecal matter containing ivermectin residues is combined in collection tanks with waste from untreated animals. During the cleaning and collection processes, substantial volumes of water are used to wash farm surfaces and floors, further diluting any residual ivermectin in the slurry. The

aqueous nature of slurry may also hinder the analyte's stability and complicate its extraction for analysis.

Similarly, no detectable concentrations of IVM were observed in any wastewater samples, as levels remained below both LOD and LOQ. This result is likely attributable to the compound's hydrophobic nature, which promotes its precipitation rather than its dissolution in water, combined with significant dilution effects in wastewater systems. Seasonal weather patterns, such as heavy rainfall, exacerbate this dilution, particularly during periods of agricultural fertilization, which typically occur in spring and autumn when rainfall is most prevalent. Climate change could substantially influence these findings by altering precipitation patterns and intensity. Increased frequency of extreme weather events, such as heavy storms, may enhance the dilution of IVM in wastewater, further reducing detectable concentrations. Conversely, prolonged droughts could lead to reduced water volumes in waste management systems, potentially increasing the relative concentration of hydrophobic compounds like IVM. Additionally, changes in agricultural practices driven by shifting climates, such as altered fertilization schedules or increased reliance on veterinary pharmaceuticals due to changing parasite burdens, may also affect the environmental dynamics and distribution of IVM residues. These factors underscore the critical need to incorporate climate variability into future environmental monitoring and risk assessment studies. Integrating pharmacokinetic studies with environmental considerations will provide a more comprehensive understanding of the fate, persistence, and ecological impact of pharmaceutical residues under changing climatic conditions.

Previous studies have established that conventional water treatment processes are largely ineffective in removing ivermectin [8]. While traces of antiparasitic drugs, including ivermectin, have been documented in waste and tap water under specific conditions, such

findings have not been reported in settings similar to those examined in this study [8, 10, 11]. This suggests that dilution and precipitation dynamics may significantly mitigate ivermectin concentrations in liquid matrices like slurry and wastewater.

3.3. Main outcomes

This research project offers a pioneering analysis of ivermectin residues, tracing their movement from swine feces to soil and shedding light on environmental pathways. Findings reveal potential IVM accumulation in the environment, with oral and injectable treatments showing distinct residue profiles, underscoring the administration route's significance in ecological impact assessments. While no residues were detected in slurry or wastewater, the presence of IVM in soil suggests environmental persistence and raises concerns about long-term ecological effects. Given its stability and toxicity to non-target species, further research is needed to understand IVM's fate, distribution, and ecological impact, guiding mitigation strategies and promoting sustainable livestock practices.

4. Research question 3: Does ivermectin affect gut microbiota in pigs?

The data presented in this chapter were obtained through collaboration with the One Health Antimicrobial Resistance group at the Department of Veterinary and Animal Science of the University of Copenhagen, under the guidance of Prof. Luca Guardabassi.

The gut microbiota plays an important role in the health, growth, and overall performance of commercial pigs [145]. It significantly influences feed efficiency in livestock, with specific bacterial taxa associated with high and low feed conversion and in animals' growth rates [146–148]. It is well-established that drugs interact with the gut microbiota through various mechanisms, often leading to diverse outcomes [149–151]. Although knowledge of ivermectin's minimal inhibitory concentrations (MIC) against individual bacterial strains in vitro is limited [152], its activity within the gastrointestinal tract against endoparasites suggests a potential influence on microbiota composition. Other antiparasitic drugs, such as moxidectin and praziquantel, have been shown to modestly but significantly reduce gut microbiota diversity [153]. Previous studies have investigated the impact of oral ivermectin administration on gut microbiota across various species, including significant effects in some cases [154–159]. In contrast, topical formulations appear to have minimal impact on gut microbiota leaving it unaffected, as observed in Australian sea lion pups [160]. No research is available to date documenting ivermectin effects on gut microbiota in pigs, where this drug is commonly used. One paper suggested that ivermectin may not induce dysbiosis or cause adverse effects in healthy human individuals [161], but the specific effects on swine gut microbiota remain unexplored.

4.1. Study design

This field study aimed to assess ivermectin's impact on sows' gut microbiota while comparing the effects of two administration methods—oral and subcutaneous injection—through a longitudinal analysis of treatment outcomes.

Sampling included 45 multiparous sows of a commercial hybrid breed. The sows were housed on two indoor pig farms in Italy, Farm A and Farm B, that raise pigs for Prosciutto di Parma production. Sows were randomly selected from each herd, individually housed, and identified with ear tags. Each animal received an ivermectin treatment (Ivomec®; Boehringer Ingelheim Animal Health Italia S.p.A.) administered either subcutaneously or orally, following the product's summary of characteristics (SmPC).

Both injectable and oral administrations were tested at Farm B, while only injectable treatment was used at Farm A. Sampling occurred at three timepoints, as illustrated in Figure 17: one hour before treatment (T0), 24 hours after treatment (T1), and ten days after treatment (T10). IVM content in all samples was quantified using LC-MS/MS [121]. All procedures were approved by the University of Parma's Ethics Committee for Animal Testing (protocol number: 14/CESA/2022).

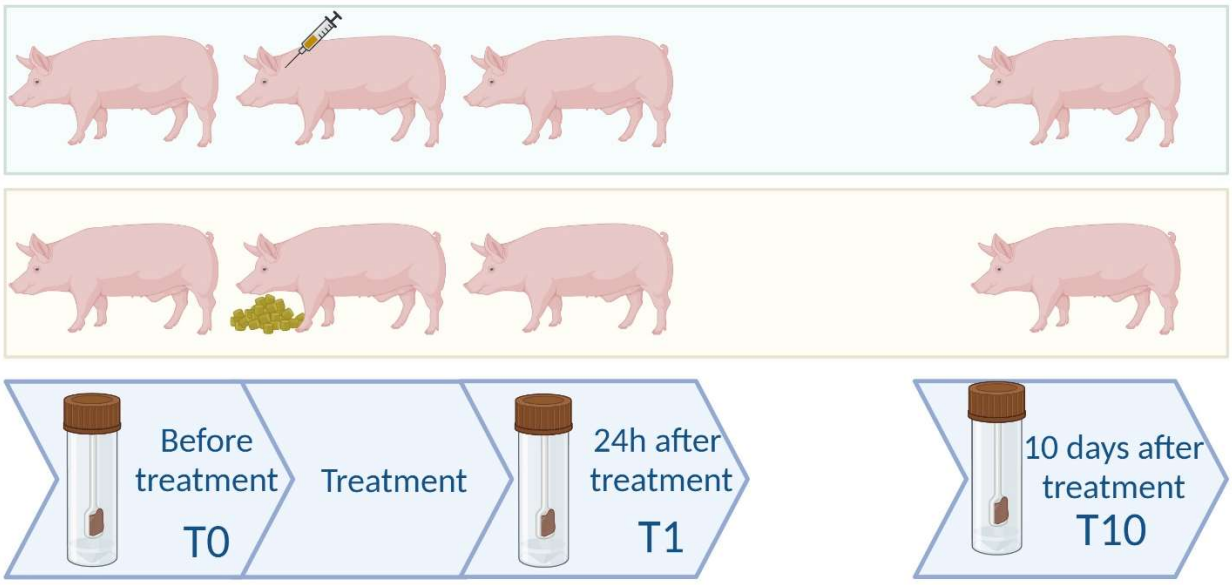


Figure 17–Study design for evaluating change in gut microbiota of sows after ivermectin treatment.

4.2. Materials and methods

Fecal samples were collected aseptically from the rectal ampulla manually of through rectal swabs, transported on ice to the laboratory, and stored at -80°C until analysis.

4.2.1. DNA Extraction and Quantification

Total DNA was extracted using Qiagen kits QIAamp UCP Pathogen Mini Kit and Pathogen Lysis Tubes S. Sterile swabs and saline solutions were used as negative controls during DNA extraction. DNA quantification was performed using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and DNA concentration was further evaluated using the Qubit quantification system (Life Technologies, Grand Island, NY, USA). Quantitative PCR targeting the 16S rRNA gene was conducted using the LightCycler 96 System (Roche Life Science, Basel, Switzerland). The Quick-16S NGS Library Prep Kit (Zymo Research, Orange, CA, USA) was used to amplify the V3-V4 region of the 16S rRNA gene through the LightCycler 96 System (Roche Life Science, Basel, Switzerland). Reactions were performed in 20 µL volumes with primers 338F (ACTCCTACGGGAGGCAG) and 530R

(GTATTACCGCGGCTGCTG). Cycles were performed as follows: 2 min at 95°C for initial denaturation, 40 cycles of 20 s at 95°C and 60 s at 61°C for amplification and 60 to 95°C for melt curve analysis.

4.2.2. Library Preparation and Sequencing

Partial 16S rRNA gene sequences (V3-V4 region) were amplified using the Quick-16S NGS Library Prep Kit (Zymo Research, Orange, CA, USA) with amplification carried out on the LightCycler 96 System. Libraries were prepared using ZymoBIOMICS DNase/RNase-Free Water and purified with Select-a-Size MagBead (Zymo Research, Orange, CA, USA). DNA concentrations of the libraries were assessed using the Qubit quantification system. Negative controls and a mock microbial community (ZymoBIOMICS Microbial Community DNA Standard, Zymo Research, Orange, CA, USA) were included to ensure quality and validate sequencing. Sequencing was performed on an Illumina MiSeq platform (2 × 300 bp paired-end reads) using the MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA) in accordance with the manufacturer's instructions.

4.2.3. Bioinformatics Analysis

Sequencing data of 16S rRNA gene were processed using the DADA2 v1.3 and Biostrings v2.70.2 packages in R v4.3.2 and taxonomy assigned with the Silva database v138.1. Contaminants were removed using negative controls and the decontam package. Reads matching mitochondria, chloroplasts, or non-bacterial sequences were excluded. Taxa were normalized 16S data using the cumulative sum scaling method. Differentially abundant taxa were evaluated using DESeq2 library in R Studio.

Alpha diversity metrics, including Shannon indices to evaluate bacterial diversity, were calculated using the MicrobiotaProcess and vegan R packages, with differences assessed

using the Wilcoxon test. Beta diversity was analyzed using the Bray–Curtis distance metric and visualized through principal coordinates analysis (PCoA) in the phyloseq R package. Differentially abundant taxa were evaluated using the DESeq2 package, with results corrected for multiple comparisons using the Benjamini-Hochberg method. The obtained data were interpreted considering the results of the residues research project.

4.3. Results and discussion

A total of 85 sows were included in the study; 5 sows were excluded due to incomplete sampling across the required time points, as these animals were either reformed, moved, or slaughtered. The distribution of sows by treatment and farm groups is shown in Table 11.

Table 11 –Number of sows for which it was possible to complete sampling three times.

	Oral treatment	**Injection treatment**	**Total**
Farm A	N/A	42	42
Farm B	20	23	43
Total	20	65	85

4.3.1. Microbiome composition

The microbiota composition of each analyzed group at the three evaluated time points is presented in Table 12. The table includes taxa with relative abundances exceeding 5.00% in at least one group. The five most abundant phyla found were, in decreasing order: Firmicutes (76.12%), Bacteroidota (15.01%), Actinobacteriota (3.19%), Proteobacteria (2.82%), Spirochaetota (1.10%).

Table 12 –Relative abundances (%) of found taxa exceeding 5% for each group considered at the three time points. Farm B has been divided in two groups based on treatment received: injection and oral, respectively.

	Farm A			Farm B (injection)			Farm B (oral)		
Taxa	**T0**	**T1**	**T10**	**T0**	**T1**	**T10**	**T0**	**T1**	**T10**
Phylum									
Actinobacteriota	2.12	2.15	1.45	5.85	3.08	1.66	2.60	5.69	5.55
Bacteroidota	16.19	18.75	9.80	14.12	7.12	15.58	16.89	17.73	18.01
Firmicutes	79.51	75.05	86.57	75.57	85.84	77.35	73.87	62.57	61.52
Proteobacteria	0.38	0.79	0.37	1.42	2.31	1.71	3.79	9.20	11.28
Family									
Christensenellaceae	2.22	3.32	5.94	6.95	5.41	17.60	7.82	7.00	3.72
Clostridiaceae	6.79	5.88	6.31	5.41	5.73	5.15	2.95	3.73	4.06
Erysipelotrichaceae	2.67	2.42	4.21	4.75	4.76	5.66	1.81	3.01	2.42
Family XI	0.01	0.55	0.97	3.23	0.06	0.23	1.01	6.46	6.30
Lachnospiraceae	10.76	8.93	7.68	5.63	8.50	8.35	5.99	4.25	4.35
Lactobacillaceae	17.62	15.68	19.33	14.81	25.37	7.26	8.17	3.86	7.49
Moraxellaceae	0.01	0.01	0.00	0.14	1.06	0.00	2.92	5.72	7.11
Oscillospiraceae	12.09	12.40	15.69	11.79	13.17	13.36	17.93	7.63	6.46
Peptostreptococcaceae	3.91	4.17	8.72	8.30	8.70	6.43	3.28	4.61	4.45
Planococcaceae	0.00	0.00	0.02	1.47	1.85	0.01	7.89	7.39	7.68
Prevotellaceae	7.66	9.10	3.78	5.81	2.61	5.57	7.15	9.23	6.72
Streptococcaceae	5.48	4.71	0.10	0.39	0.04	0.12	0.06	0.76	0.73
Genus									

Acinetobacter	0.01	0.01	0.01	0.23	1.56	0.00	5.11	5.63	11.42
Anaerococcus	0.01	0.36	1.09	3.29	0.06	0.10	1.07	4.94	6.50
Christensenellaceae R-7 group	3.65	6.75	10.66	11.91	8.69	27.39	14.48	11.85	6.04
Clostridium sensu stricto 1	11.60	12.29	12.25	10.07	9.68	8.75	5.87	6.71	6.83
Corynebacterium	0.97	1.27	0.26	5.08	0.31	0.37	2.84	6.96	6.11
Kurthia	0.00	0.00	0.02	2.11	2.00	0.01	14.25	10.57	8.59
Lactobacillus	28.02	30.23	35.84	24.96	40.33	11.77	14.90	6.55	11.65
NK4A214 group	5.73	6.71	8.68	7.51	8.20	6.74	6.35	3.99	3.18
Phascolarctobacterium	2.38	5.62	2.98	4.40	1.62	2.68	4.65	3.86	3.21
Prevotella	2.08	4.06	1.69	3.17	0.95	1.50	1.82	5.80	4.36
Prevotellaceae NK3B31 group	2.86	5.36	2.01	3.53	1.28	4.09	5.58	5.38	3.43
Streptococcus	9.43	9.93	0.20	0.74	0.07	0.20	0.13	1.30	1.23
Terrisporobacter	5.15	6.63	11.75	10.87	10.06	6.82	4.71	6.36	5.29
Turicibacter	2.23	2.94	6.73	6.68	6.38	8.13	1.54	3.31	2.58
UCG-002	5.46	6.66	11.81	5.75	3.93	6.94	18.72	3.59	2.33
UCG-005	6.56	8.50	6.41	5.69	8.02	5.79	7.39	3.96	3.69
un_f__Muribaculaceae	9.47	8.24	4.81	5.64	4.29	3.76	7.24	3.22	2.52
un_f__Ruminococcaceae	1.98	2.55	5.30	1.84	1.89	1.42	2.55	0.86	0.80
un_f__[Eubacterium] coprostanoligenes group	7.05	7.32	9.25	3.10	4.60	6.23	8.55	3.37	2.41

The observed families that composed the sample with a relative abundance greater than 5.00% were Lactobacillaceae (14.19%), Oscillospiraceae (12.38%), Lachnospiraceae (7.54%), Prevotellaceae (6.55%), Christensenellaceae (6.03%), Peptostreptococcaceae (5.80%) and Clostridiaceae (5.37%). The most abundant genera overall were Lactobacillus (13.13%) Christensenellaceae R-7 group (5.60%), Clostridium sensu stricto 1 (5.33%), Terrisporobacter (4.09%), UCG-002 (3.76%), NK4A214 group (3.48%) and UCG-005 (3.44%). Figure 18 shows genera distribution in all samples according to farm, type of treatment, and time of sampling.

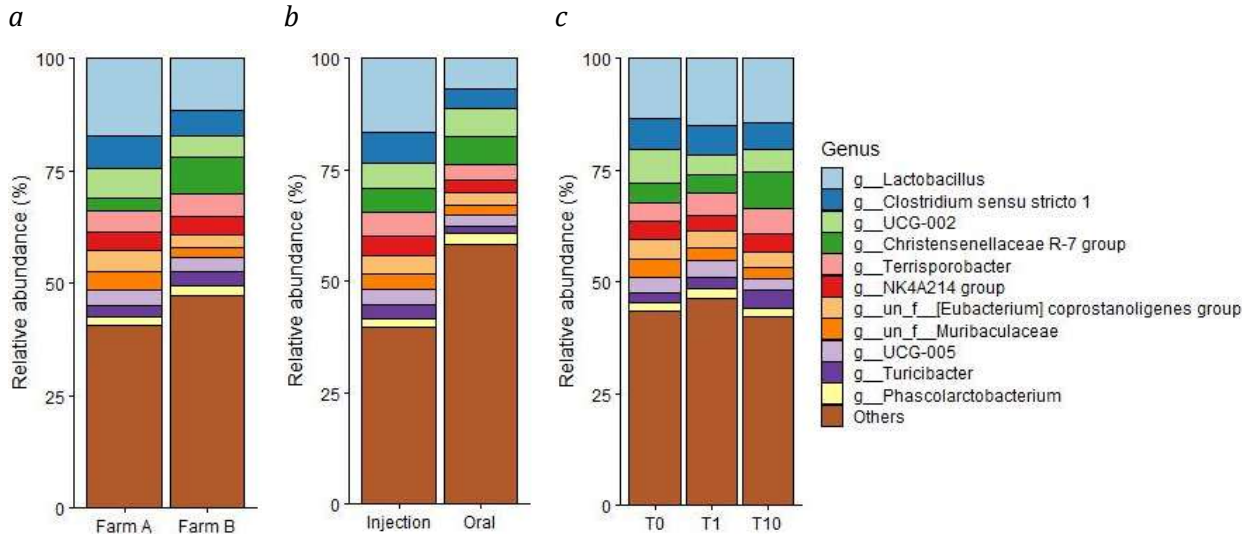


Figure 18 Part a–Relative abundances of genus in all samples divided by farm of origin.
Figure 18 Part b–Relative abundances of genus in all samples divided by treatment.
Figure 19 Part c–Relative abundances of genus in all samples divided by time.

Samples from Farm A were mostly composed of Firmicutes (81.90%), Bacteroidota (14.06%) and Actinobacteriota (2.00%) at phylum level. Lactobacillaceae (21.31%), Oscillospiraceae (13.79%), Clostridiaceae (7.55%), Lachnospiraceae (6.98%) and Peptostreptococcaceae (6.58%) were the most abundant families, making up more than 50% of the total. Lactobacillus (19.99%) and Clostridium sensu stricto 1 (7.55%), Terrisporobacter (4.83%), UCG-002 (4.68%), Streptococcus (4.53%), unidentified Muribaculaceae (4.40%) and NK4A214 group (4.27%) were the most relatively abundant

genera exceeding 4.00% and composing together more than half of all genera as shown in Figure 19.

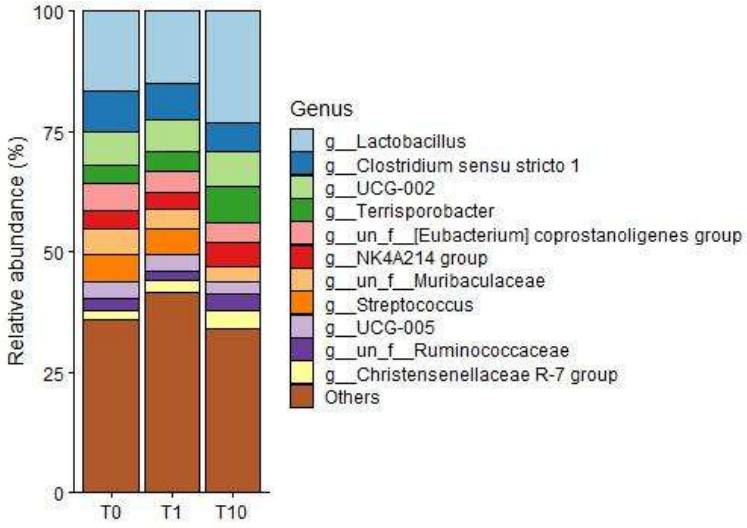


Figure 20–Relative abundances of genus in samples from Farm A divided by time.

Samples from Farm B were mostly composed of Firmicutes (77.54%), Bacteroidota (11.92%), Actinobacteriota (4.47%), Proteobacteria (3.54%) at phylum level. Lactobacillaceae (14.39%), Oscillospiraceae (12.86%), Christensenellaceae (8.65%), Peptostreptococcaceae (7.94%), Clostridiaceae (5.73%), Prevotellaceae (5.15%) were the most abundant families, exceeding 5.00% and making up more than 50%. Lactobacillus (13.52%), Christensenellaceae R-7 group (7.97%), Clostridium sensu stricto 1 (5.73%), Terrisporobacter (5.52%), UCG-002 (4.52%), NK4A214 group (4.17%), Kurthia (4.06%), Turicibacter (3.60%), UCG-005 (2.86%) were the most relatively abundant genera composing more than 50% of all genera as shown in Figure 20.

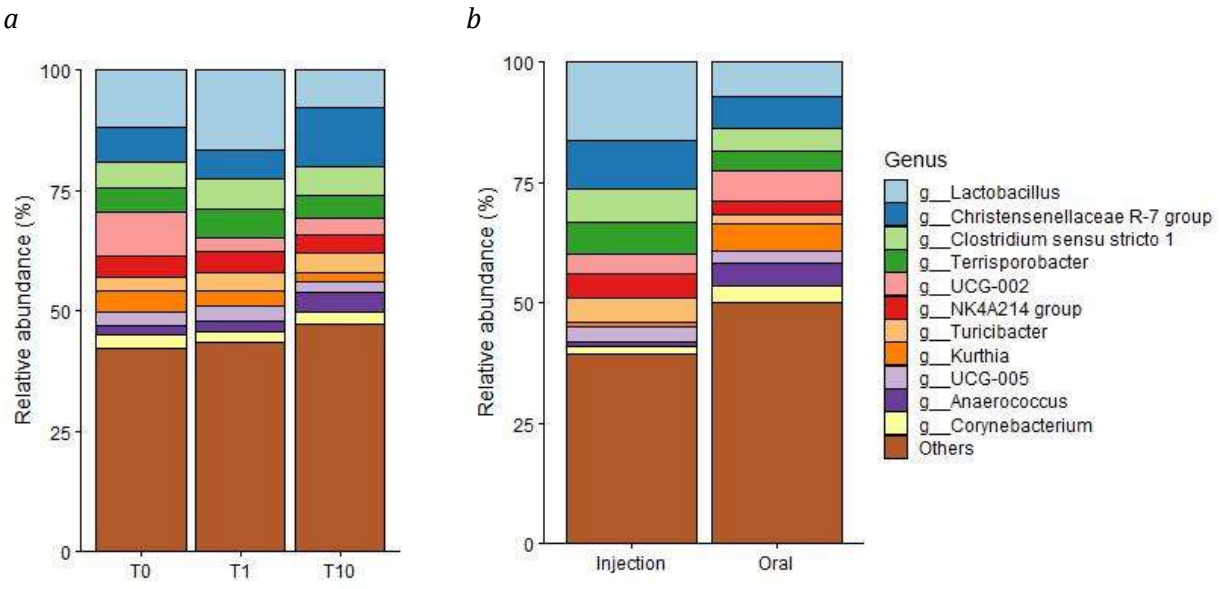


Figure 21 Part a–Relative abundances of genus in samples from Farm B divided by time.
Figure 20 Part b–Relative abundances of genus in samples from Farm B divided by treatment.

Phyla making up for more than 98% of microbiota of orally treated sows were Firmicutes (73.90%), Bacteroidota (12.50%), Proteobacteria (5.65%), Actinobacteriota (5.17%), Campylobacterota (1.23%). Families exceeding 5.00% of relative abundance in composition were Oscillospiraceae (12.54%), Lactobacillaceae (9.22%), Planococcaceae (8.97%), Christensenellaceae (7.02%), Prevotellaceae (6.30%), Peptostreptococcaceae (6.03%), Family XI (5.26%) and Clostridiaceae (5.06%). Lactobacillus (8.71%), Kurthia (8.68%), Christensenellaceae R-7 group (6.49%), UCG-002 (6.02%), Clostridium sensu stricto 1 (5.06%), Terrisporobacter (4.53%), Corynebacterium (3.90%), NK4A214 group (3.32%), Anaerococcus (3.07%), Phascolarctobacterium (2.97%) were the main genera composing more than 50% of relative abundance as shown in Figure 21.

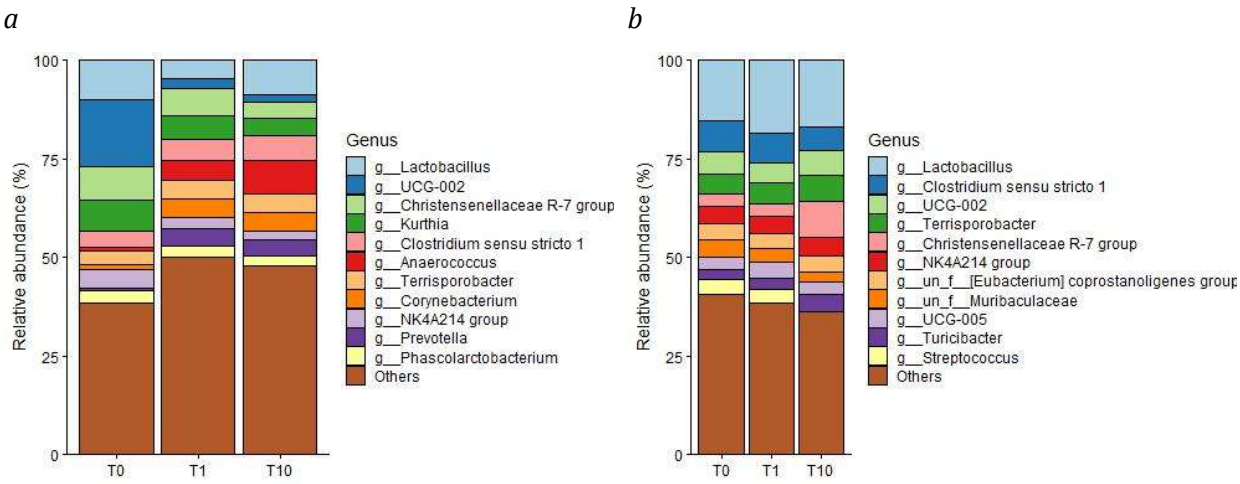


Figure 22 Part a –Relative abundances of genus in samples of orally treated sows at the three timepoints.
Figure 21 Part b–Relative abundances of genus in samples of injected sows at the three timepoints.

Phyla making up for more than 97.45% of microbiota composition of injection treated sows were Firmicutes (82.22%), Bacteroidota (12.54%) and Actinobacteriota (2.69%). Families exceeding 5% of relative abundance in composition were Lactobacillaceae (19.98%), Oscillospiraceae (13.80%), Peptostreptococcaceae (7.69%), Clostridiaceae (7.02%), Lachnospiraceae (6.67%), Christensenellaceae (5.80%) and Prevotellaceae (5.38%). Lactobacillus (18.73%), Clostridium sensu stricto 1 (7.02%), Terrisporobacter (5.43%), Christensenellaceae R-7 group (5.35%), NK4A214 group (4.50%), UCG-002 (4.25%), unidentified Muribaculaceae (3.68%) and UCG-005 (3.48%) were the main genera composing more than 50% of relative abundance as shown in Figure 21.

In samples from T0, the only phyla exceeding 1.00% of relative abundance were Firmicutes (81.01%), Bacteroidota (13.83%) and Actinobacteriota (3.56%). In the same samples almost 60% of microbiota were made of Lactobacillaceae (19.22%), Oscillospiraceae (14.74%), Clostridiaceae (7.12%), Peptostreptococcaceae (7.02%), Prevotellaceae (5.91%), Lachnospiraceae (5.41%) families. As shown in Figure 22, the most abundant genera exceeding 3% were Lactobacillus (17.87%), Clostridium sensu stricto 1 (7.12%), UCG-002 (5.85%), Terrisporobacter (5.17%), unidentified Muribaculaceae (4.81%), NK4A214 group

(4.46%), Christensenellaceae R-7 group (3.96%), Streptococcus (3.35%), unidentified [Eubacterium] coprostanoligenes group (3.07%).

In samples from T1, the only phyla exceeding 1.00% of the relative abundance were Firmicutes (80.04%), Bacteroidota (12.80%), Actinobacteriota (3.15%), Proteobacteria (1.48%). In the same samples, almost 60% of the microbiota was made of Lactobacillaceae (20.63%), (Oscillospiraceae (12.65%), Peptostreptococcaceae (7.55%), Clostridiaceae (7.03%), Prevotellaceae (5.75%) and Lachnospiraceae (5.37%) families. As shown in Figure 22, the most abundant genera exceeding 3.00% were Lactobacillus (19.29%), Clostridium sensu stricto 1 (7.03%), Terrisporobacter (5.52%), NK4A214 group (4.46%), Christensenellaceae R-7 group (4.07%), UCG-005 (3.40%), UCG-002 (3.35%), unidentified Muribaculaceae (3.29%) and Streptococcus (3.29%).

In samples from T10, the only phyla exceeding 1% of relative abundance were Firmicutes (85.74%), Bacteroidota (8.57%), Actinobacteriota (2.26%), Proteobacteria (1.43%). In the same samples almost 60% of microbiota was made of Lactobacillaceae (17.84%), Oscillospiraceae (14.64%), Christensenellaceae (10.05%), Peptostreptococcaceae (9.92%), Clostridiaceae (7.29%) families. As shown in Figure 22, most abundant genera exceeding 3% were Lactobacillus (17.26%), Christensenellaceae R-7 group (9.21%), Clostridium sensu stricto 1 (7.29%), Terrisporobacter (6.90%), UCG-002 (5.88%), NK4A214 group (4.91%), Turicibacter (4.89%) and Romboutsia (3.02%).

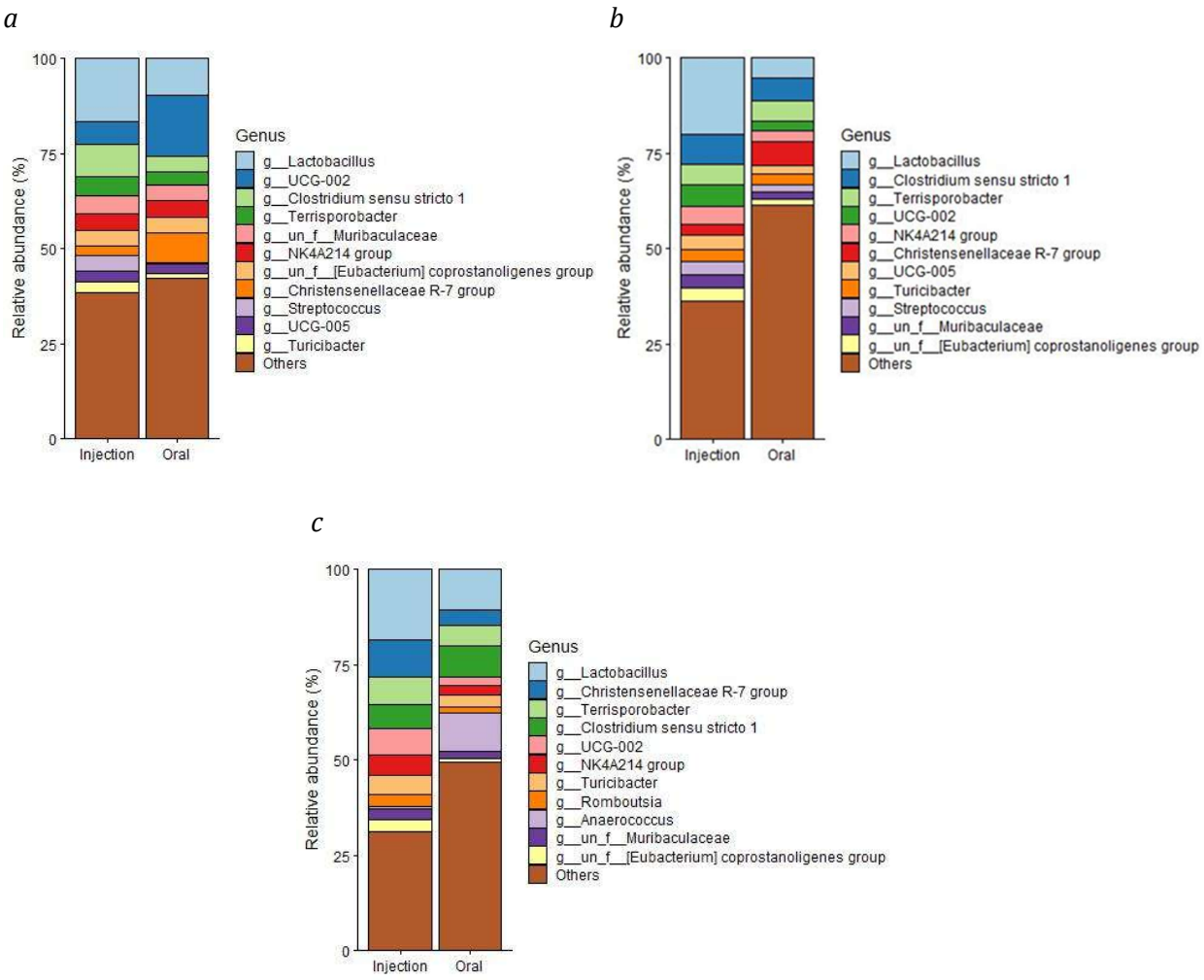


Figure 23 Part a–Relative abundances of genus in samples at T0 by treatment.
Figure 22 Part b–Relative abundances of genus in samples at T1 by treatment.
Figure 22 Part c–Relative abundances of genus in samples at T10 by treatment.

4.3.2. Alpha diversity

The data shows change in gut microbiota diversity and compositions, thus confirming an antimicrobial activity, conversely from what has been reported by Halley et al. [38]. A statistically significant difference in alpha diversity, based on the Shannon index, was observed within Farm A across time points, with significant changes detected between T1 and T10 ($p < 0.0001$) and between T0 and T10 ($p < 0.01$). For Farm B, no significant differences in alpha diversity were observed across all sows when comparing treatments and timepoints, although differences emerged within treatment subgroups. Specifically,

sows in Farm B receiving oral treatment did not show any significant difference at the considered times, while sows in the injection group showed significant changes between T1 and T0 ($p < 0.01$) and T1 and T10 ($p < 0.001$). The Shannon index distributions for these comparisons are shown in Figure 23.

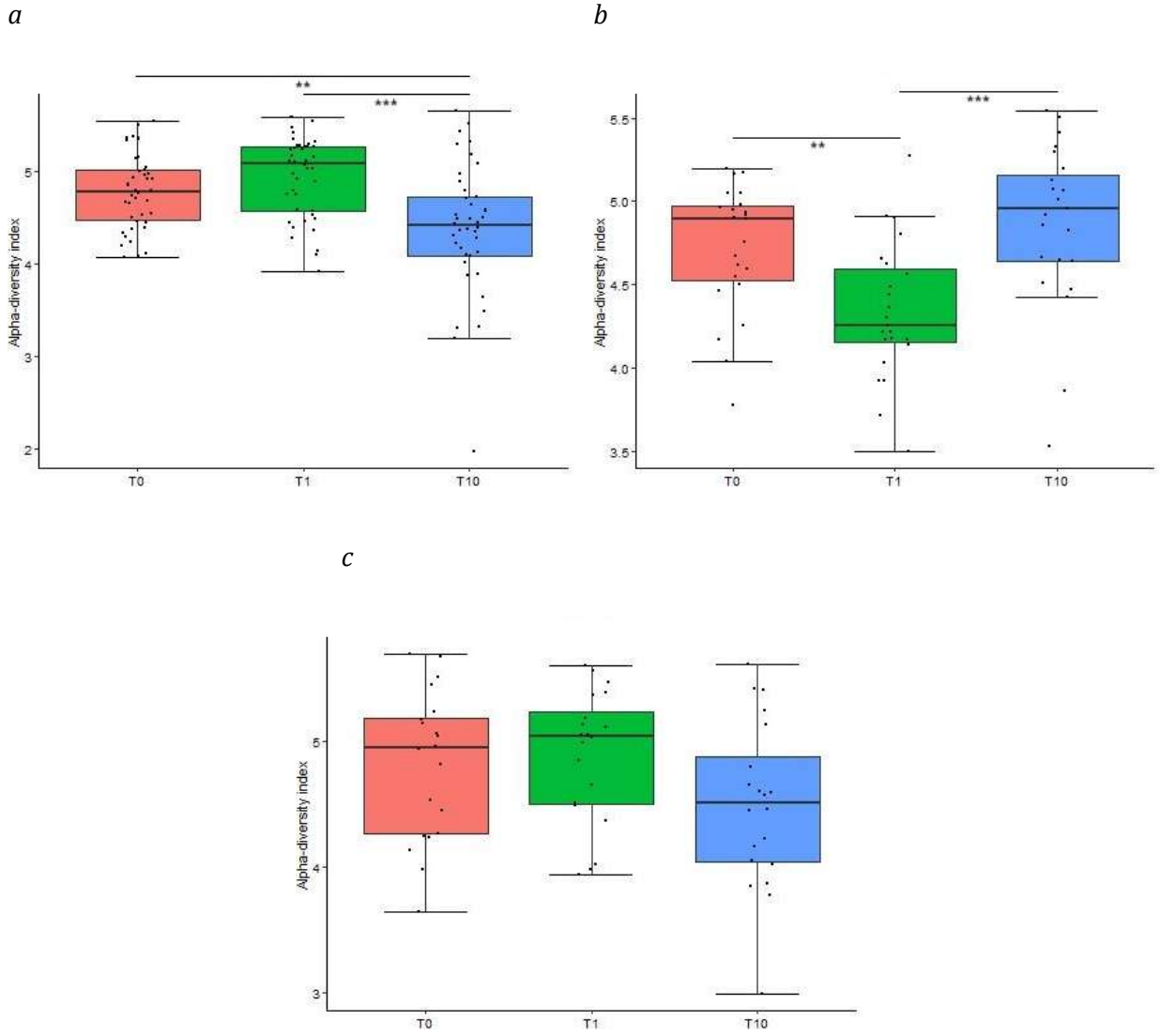
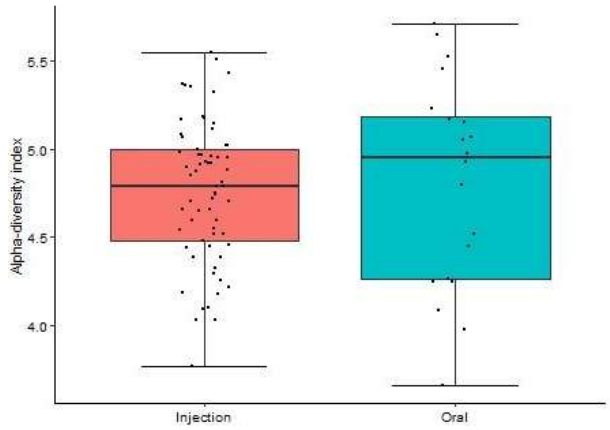


Figure 24 Part a –Alpha diversity according to Shannon index of sows from Farm A.
Figure 23 Part b –Alpha diversity according to Shannon index of sows from Farm B (injection treatment).
Figure 23 Part c –Alpha diversity according to Shannon index of sows from Farm B (oral treatment).

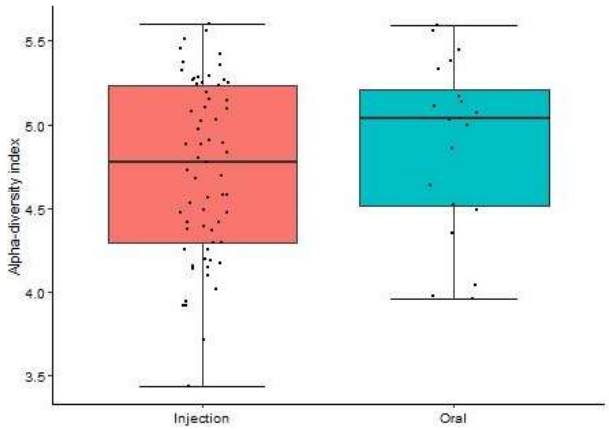
No statistically significant difference was observed between the two injection groups, from Farm A and Farm B respectively. While injection and oral treatment caused a difference in

alpha diversity within the respective groups at the three timepoints, no difference was detected among the two groups at the three points as shown in Figure 24.

a



b



c

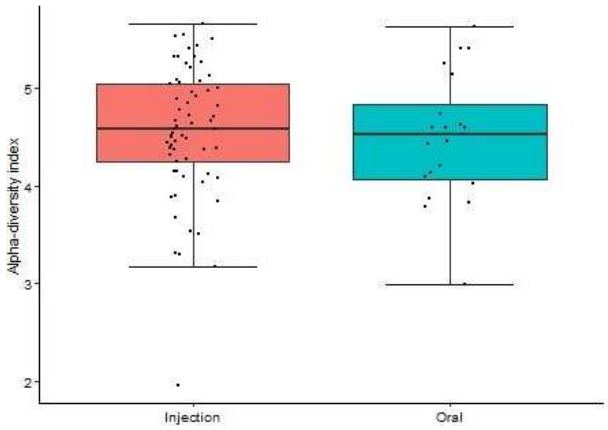


Figure 25 Part a –Distribution of Shannon index among the three timepoints when comparing treatment groups at T0.

Figure 24 Part b –Distribution of Shannon index among the three timepoints when comparing treatment groups at T1.

Figure 24 Part c26 –Distribution of Shannon index among the three timepoints when comparing treatment groups at T10.

These findings suggest that although the mode of ivermectin administration (oral or injection) influenced alpha diversity changes within each treatment group over time, it did not lead to a statistically significant difference between the two treatment groups when assessed at each time point. Therefore, it appears that the administration route did not

differentially impact the two groups' microbial diversity profiles, at least in terms of Shannon diversity index. This consistency between groups suggests that other factors, possibly related to farm-specific practices or environmental influences, might also play a role in shaping alpha diversity.

4.3.3. Beta diversity

Beta diversity analysis, based on Bray-Curtis distances, revealed significant clustering of samples from Farm A across the three timepoints. Notable differences were observed between T0 and T1 ($p < 0.05$), T0 and T10 ($p < 0.01$), and T1 and T10 ($p < 0.01$), thus confirming IVM activity on microbiota. Similarly, in Farm B, beta diversity analysis indicated distinct clustering patterns over time, with significant differences detected between T10 and T0 ($p < 0.01$), T10 and T1 ($p < 0.05$), and T0 and T1 ($p < 0.05$). A pronounced effect was observed when comparing the oral and injection treatment groups ($p = 0.001$) in Farm B. These findings suggest that temporal shifts in microbiome composition occurred within both farms; however, the route of ivermectin administration (oral vs. injection) introduced additional variability in Farm B. The marked differences between treatment groups in Farm B may be attributed, as mentioned earlier in the text, to variations in the pharmacokinetics of the drug and its localized effects on the microbiome, which are influenced by the method of administration.

Beta diversity, illustrated through principal coordinate analysis, is shown in Figure 25. The impact of ivermectin treatment on gut microbiota at Farm B, showed significant differences were observed between T0 and both T1 and T10, while no significant changes were evident between T1 and T10. This temporal shift in microbiota composition following treatment indicates a distinct influence of the administration method. In animals receiving ivermectin via injection, alterations in gut microbiota persisted over time, likely due to the prolonged

presence of ivermectin residues. In contrast, animals treated orally exhibited transient microbiota changes, consistent with the shorter persistence of ivermectin residues associated with this administration route. These results underscore the role of administration method in shaping the dynamics of gut microbiota following ivermectin treatment.

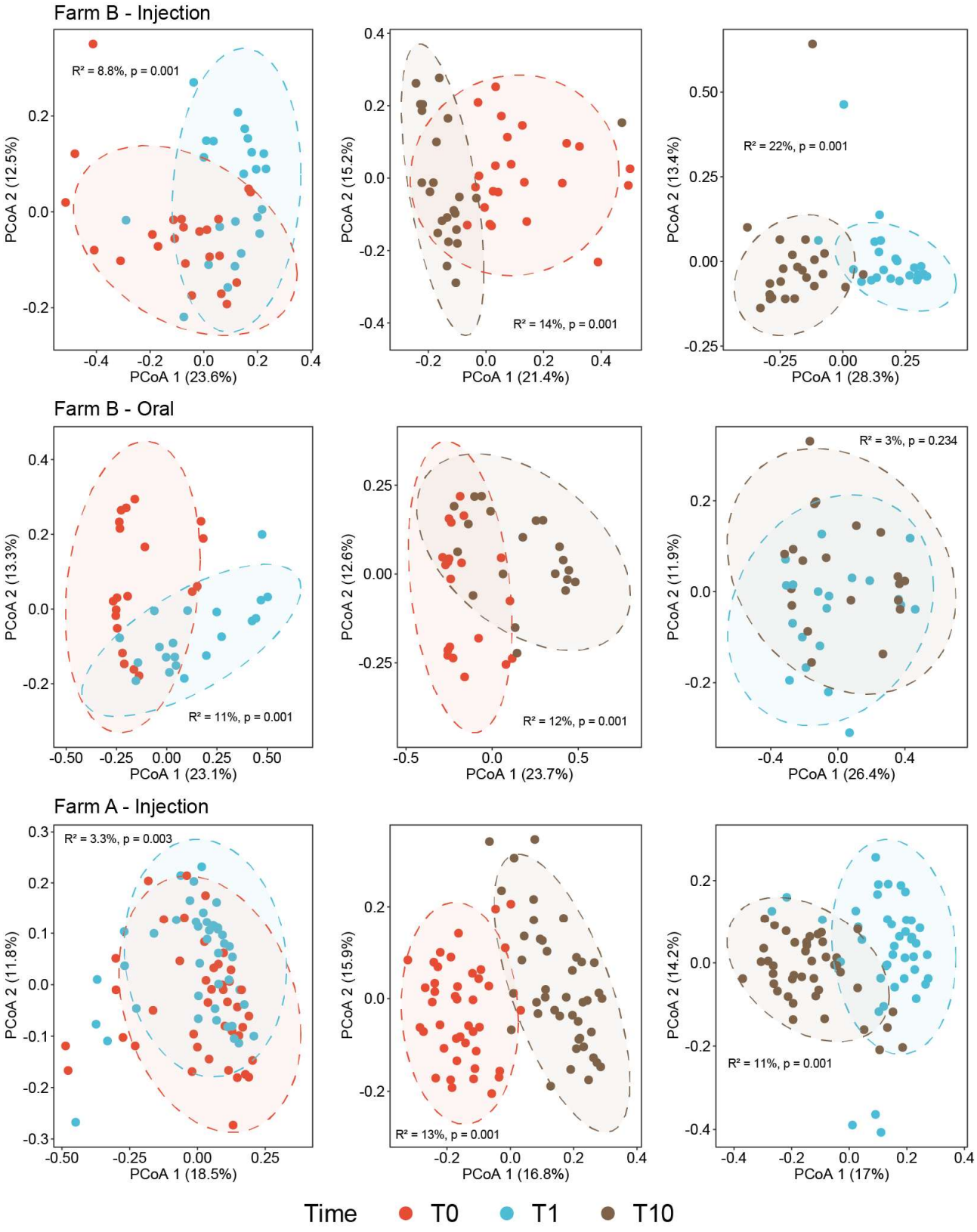


Figure 27–Beta diversity of each timepoint comparison.

4.3.4. Differential analysis

Taxa significantly influenced by time within the two treatment groups, as determined by differential analysis, are summarized in Table 13. For clarity and conciseness, only the phylum and family levels are presented.

*Table 13 –Trends in differentially abundant taxa over time in the two treatment groups. Only statistically significant changes are included. The "Trend" column indicates whether the abundance of each taxon increased (↑) or decreased (↓). Statistical significance levels are denoted as follows: ''***" = p < 0.001, "**" = p < 0.01, and ''*" = p < 0.05.*

	Injection	
Taxa	**Trend**	**Significance**
	Phylum	
Actinobacteriota	↓	***
Bacteroidota	↓	**
Desulfobacterota	↑	***
Firmicutes	↓	***
Spirochaetota	↓	***
un_k__Bacteria	↓	***
Verrucomicrobiota	↑	***
	Family	
Actinomycetaceae	↓	**
Akkermansiaceae	↑	***
Bifidobacteriaceae	↓	**
Butyricicoccaceae	↓	***
Christensenellaceae	↑	***
Clostridiaceae	↓	***
Corynebacteriaceae	↓	***
Desulfovibrionaceae	↑	***

Eggerthellaceae	↓	*
F082	↑	**
Muribaculaceae	↓	***
Planococcaceae	↓	***
Porphyromonadaceae	↓	*
Prevotellaceae	↓	**
Rikenellaceae	↓	**
Selenomonadaceae	↓	***
Spirochaetaceae	↓	**
Streptococcaceae	↓	***
T34	↓	***
un_k__Bacteria	↓	***
Veillonellaceae	↓	***

Oral		
Taxa	**Trend**	**Significance**
	Phylum	
Actinobacteriota	↑	**
Bacteroidota	↓	*
Campylobacterota	↑	***
Firmicutes	↓	*
Fusobacteriota	↑	***
Proteobacteria	↑	*
	Family	
[Eubacterium] coprostanoligenes group	↓	***
Actinomycetaceae	↑	**
Aerococcaceae	↑	***

Akkermansiaceae	↓	*
Bacteroidaceae	↑	**
Campylobacteraceae	↑	***
Christensenellaceae	↓	***
Clostridiaceae	↑	***
Enterobacteriaceae	↑	***
Erysipelotrichaceae	↓	***
Family XI	↑	***
Fusobacteriaceae	↑	***
Hungateiclostridiaceae	↑	***
Lachnospiraceae	↓	***
Moraxellaceae	↑	*
Muribaculaceae	↓	***
Oscillospiraceae	↓	***
p-2534-18B5 gut group	↓	***
Peptostreptococcaceae	↓	***
Porphyromonadaceae	↑	***
Prevotellaceae	↓	*
Ruminococcaceae	↓	***
Staphylococcaceae	↑	*
Streptococcaceae	↑	***
UCG-010	↓	***

Differential analysis of relatively abundant taxa of injection treated sows at the phylum level showed that Actinobacteriota, Desulfobacterota, Firmicutes, Spirochaetota, un_k__Bacteria, and Verrucomicrobiota were the most affected by treatment over time (p<0.0001) even if

with different trends. Verrucomicrobiota, specifically Proteobacteria and Tenericutes, have been reported to increase after IVM administration [155]. Additionally, the phyla Bacteroidota (p<0.01) demonstrated significant changes over time. This confirms findings from Belo et al. in mice where there was a decrease in Bacteroidetes and Firmicutes following repeated treatments with IVM [155]. At the family level, Akkermansiaceae, Butyricicoccaceae, Christensenellaceae, Clostridiaceae, Corynebacteriaceae, Desulfovibrionaceae, Muribaculaceae, Planococcaceae, Selenomonadaceae, Streptococcaceae, T34, un_k__Bacteria and Veillonellaceae showed the most significant alterations across the three timepoints (p<0.001).

Differential analysis of relatively abundant taxa of orally treated sows at the phylum level showed that Campylobacterota and Fusobacteriota were the most affected by treatment over time (p<0.0001). Additionally, the phyla Actinobacteriota (p<0.01), Bacteroidota (p<0.05), Firmicutes (p<0.05) and Proteobacteria (p<0.05) demonstrated significant changes over time, the latter confirms what has been seen in research on mice [155]. At the family level, [Eubacterium] coprostanoligenes group, Aerococcaceae, Campylobacteraceae, Christensenellaceae, Clostridiaceae, Enterobacteriaceae, Erysipelotrichaceae, Family XI, Fusobacteriaceae, Hungateiclostridiaceae, Lachnospiraceae, Muribaculaceae, Oscillospiraceae, p-2534-18B5 gut group, Peptostreptococcaceae, Porphyromonadaceae, Ruminococcaceae, Streptococcaceae and UCG-010 showed the most significant alterations across the three timepoints (p<0.001).

An increase in Desulfobacterota and Verrucomicrobiota and a decrease in Spirochaetota were observed exclusively in the injected group (p<0.001), while the oral group was characterized by an increase in Campylobacterota (p<0.001), Fusobacteriota (p<0.001) and Proteobacteria (p<0.05). At family level, Actinomycetaceae, Akkermansiaceae,

Christensenellaceae, Clostridiaceae, Porphyromonadaceae, Streptococcaceae showed opposite trends over time in the two treatment groups. Muribaculaceae (p<0.001) and Prevotellaceae (injection group p<0.01, oral group p<0.05) families decreased in both groups.

In the orally treated group Clostridium sensu stricto 1 increased significantly (p<0.001) over time after IVM administration as reported in a study conducted in horses, while the opposite trend was observed in the injected group (p<0.001). The same study reported an effect on the Eubacterium genus, which was not confirmed by our results [154]. Bacteroidota phylum was also negatively affected by IVM treatment in both groups (injection group p<0.01, oral group p<0.05), which confirms previous research results [154, 155]. Hu et al. also reported a significant decrease in Prevotella species, which has been confirmed in our study by a significant decrease of the Prevotellaceae in both groups (injection group p<0.01, oral group p<0.05) [154].

Firmicutes were negatively affected by IVM treatments in both groups (injection group p<0.001, oral group p<0.05), confirming data from an *in vivo* study conducted in mice [155]. Conversely from what has been observed in mice, Proteobacteria increased over time (oral group p<0.05), also no difference was observed in Tenericutes [155]. An increase in Verrucomicrobia phylum was observed in the injection group (p<0.001) as reported previously [155]. To the best of our knowledge, no comparable studies have been published to date in swine, precluding direct comparisons with existing literature.

4.4. Main outcomes

The data indicates that ivermectin (IVM) influences the composition of gut microbiota, supporting its classification as a compound with antimicrobial properties. These findings

underscore the need for further studies to characterize its antimicrobial effects in greater detail. No clinical signs attributable to IVM treatment were observed, and all sows involved in the study remained healthy throughout. Changes in the gut microbiota composition were induced by IVM in both orally and injection treated animals. These changes correlated with the presence of ivermectin residues in a dose-dependent manner, persisting as long as the residues remained detectable.

5. Study limitations

This study encountered a few limitations that influenced its scope and findings. One of the primary challenges was the extraction of ivermectin from water-based samples, such as slurry and wastewater. The water-based nature likely caused the precipitation of IVM, which significantly hindered its extraction. This methodological obstacle limits the capacity to accurately quantify and analyze IVM in such matrices.

Another notable limitation was the sample size for environmental samples, which, although not negligible, could have been larger to provide more robust data and enhance the statistical power of the results. However, the number of samples was primarily restricted by logistical challenges associated with the Swine Fever epidemics in Italy. The outbreaks imposed severe limitations on the transportation of swine-derived materials and necessitated multiple visits to each farm for the collection of substantial amounts of material. These efforts were further complicated by the need to adhere to stringent biosecurity measures and by the understandable reluctance of farmers to grant access to researchers. The combination of these factors significantly impacted the sample collection process. Future research endeavors should aim to include a larger number of companies to increase the representativeness of the data and provide a more comprehensive understanding of the phenomena studied across the entire swine district.

In the context of the microbiota project, additional challenges were encountered that may have influenced the findings. One major limitation was the absence of an on-farm control group that did not receive treatment. This restriction limited the ability to compare treated and untreated groups directly within the same environmental context. Furthermore, obtaining consistent fecal samples from some sows proved impossible, necessitating the use of two sampling methods (rectal ampoule collection and swabs). While this alternative

method was viable, it introduced variability in sample types, which might have influenced the results. Despite the relatively large sample size compared to other *in vivo* studies on swine, an even larger cohort would have strengthened the robustness of the data and improved the statistical power. Once again, the Swine Fever outbreaks in Italy also posed significant logistical and operational challenges for the microbiota research project with similar repercussions.

Overall, the research provides valuable insights and addressing these limitations in future research will be essential to enhance the reliability and generalizability of the findings. Expanding the sample size, ensuring the inclusion of appropriate control groups, and incorporating a greater number of farms and companies will be pivotal in overcoming the constraints faced in this study.

6. Conclusions

This PhD thesis has explored the detection, environmental impact, and biological consequences of ivermectin use in swine farms, emphasizing its significance within a One Health framework. By combining robust analytical methods, environmental sampling, and biological assessments, this research addresses critical gaps in understanding the implications of veterinary pharmaceutical residues. The findings provide a comprehensive overview of ivermectin's presence in swine farm environments, its potential for contamination, and its effects on swine gut microbiota. These results underline the interconnectedness of animal health, environmental integrity, and sustainable farming practices. This project could serve as a foundation and example for future research, bridging pharmacokinetic investigations with environmental frameworks to enhance understanding of the interactions between pharmaceutical use and its ecological implications.

The first research question focused on the detection of IVM in environmental matrices. A novel HPLC-based analytical method was developed, enabling the precise quantification of ivermectin residues in both solid and liquid matrices. Employing doramectin as a process standard and validated through stringent calibration and precision tests, the method proved to be cost-effective, reliable, and adaptable to various contexts. Its suitability for monitoring ivermectin in feces, slurry, and soil establishes it as a critical tool for environmental and ecotoxicological studies. Furthermore, its potential application to other macrocyclic lactones or structurally related molecules opens to broader environmental monitoring. The validated HPLC method represents a significant contribution to the field, offering researchers and regulatory agencies a powerful means of tracing ivermectin and assessing its ecological impact. The further development following Eurachem guidelines using LC-MS/MS for environmental matrices revealed IVM's presence in feces and soil, with an LOQ of 1.5 µg/kg,

underscoring the sensitivity and applicability of the method. A matrix effect was evident in the soil matrix, thus requiring making calibration curves using blank soil extracts instead of solvents. This research emphasizes the necessity of integrating a One Health approach, which considers the overlapping domains of animal health, environmental sustainability, and human well-being. The development and application of this analytical framework provide essential tools for mitigating environmental contamination and supporting sustainable livestock practices.

Addressing the second research question, the study demonstrated that ivermectin residues can pollute the surroundings of swine farms, particularly through soil contamination. This research marks a pivotal step in understanding IVM's environmental pathways, as to our knowledge it is the first research to systematically track IVM residues from swine feces to soil. The findings highlight a potential risk to non-target organisms, aligning with concerns about the environmental stability and bioactivity of macrocyclic lactones. While IVM was not detected in slurry or wastewater, its accumulation in soil suggests a persistent environmental pathway that warrants closer scrutiny. These results also suggest that the administration route significantly influences residue profiles, which in turn could have implications for both the treated animals and the surrounding environment. The presence of ivermectin residues in soil raises additional concerns about its environmental persistence and potential indirect effects on non-target organisms. Moreover, the marked differences in residue profiles between oral and injectable administration methods underscore the need to consider the administration route when assessing environmental impacts. Chemical residues have the potential to exert a lasting ecological impact, thus there is a critical need for further research into the broader ecological and biological consequences of ivermectin use, especially concerning its long-term effects on environmental microbiomes and soil health.

Finally, in response to the third research question, the work provided evidence of ivermectin's effects on the gut microbiota of swine, with this study representing the first to document such impacts in sows. The data demonstrates that ivermectin treatment alters the gut microbiota composition of treated sows, though the specific mechanisms and implications of these changes warrant further exploration. No clinical signs attributable to IVM treatment were observed, and all the sows involved remained healthy throughout the project. The findings revealed that ivermectin treatments –whether administered orally or via injection– alter the microbial composition of sows' gastrointestinal tracts, thus confirming the thesis of IVM being a compound with antimicrobial properties. These alterations were dose-dependent and directly correlated with the presence of detectable ivermectin residues, persisting for the duration of residue presence. This finding highlights a critical intersection between animal health and pharmaceutical use, suggesting that IVM's influence extends beyond its antiparasitic role to potentially affect broader aspects of animal physiology and productivity. Understanding these interactions will be crucial for optimizing therapeutic strategies and safeguarding the health and performance of treated livestock.

This thesis, through a multidisciplinary approach, faced the multifaceted impact of ivermectin use in swine farming, from methodological advancements in residue detection to its tangible environmental and biological consequences. The findings stress the importance of adopting sustainable practices and developing comprehensive strategies to mitigate the environmental footprint of veterinary pharmaceuticals. Through its integrated approach, we believe that this research could contribute valuable knowledge to the pursuit of sustainable agriculture, bridging the gaps between animal health, environmental conservation, and public health.

7. Future developments

The potential environmental impacts of ivermectin warrant further exploration through targeted studies addressing its presence and behavior in diverse contexts. One promising avenue involves investigating urban environments, particularly parks and recreational areas frequented by pets, where the widespread use of IVM-based treatments for heartworm and gastrointestinal parasites may lead to environmental dispersion of residues. Similarly, studies in non-anthropized environments, such as forests, untreated fields, and natural parks located near agricultural or livestock-treated zones, could provide insights into the extent of contamination in pristine ecosystems and the mechanisms driving IVM spread into these areas. A key objective for future work will be to enhance the extraction process from liquid matrices, which would enable comprehensive environmental analyses by providing more accurate and reliable data across all sample types.

Future research should expand beyond swine to include other livestock species, explore different soil types, and delve deeper into the organic components of slurry. By broadening the scope of analysis, we can gain a more accurate picture of ivermectin's ecological footprint and refine guidelines for its use in animal husbandry, ultimately safeguarding both human and environmental health. Research on farms should also aim to understand the potential infiltration of IVM residues or their degradation products into deeper groundwater layers, particularly below the reach of agricultural plow blades. Additionally, the role of insects as potential vectors for the environmental transport of IVM and its metabolites merits investigation to elucidate ecological pathways and interactions. Furthermore, given that IVM is a natural product of *Streptomyces avermitilis*, studies could explore the presence of this bacterium in untreated soils to determine whether it contributes to baseline levels of IVM in the environment.

By addressing these topics, future research will help clarify the complex interactions and impacts of IVM in various ecosystems, providing essential knowledge for mitigating environmental risks and ensuring the sustainable use of veterinary pharmaceuticals.

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