REVIEW / ARTÍCULO DE REVISIÓN

Antifungal activity of metabolites from *Trichoderma spp.* against *Fusarium oxysporum*

González M.F[1,2,3]*, Galarza L[1,2], Valdez L.L[3], Quizhpe G.M[3]

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[1] Escuela Superior Politécnica del Litoral, ESPOL, Biotechnology Research Center (CIBE), Campus Gustavo Galindo, Guayaquil, Ecuador.
[2] Escuela Superior Politécnica del Litoral, ESPOL, Faculty of Life Sciences, Campus Gustavo Galindo, Guayaquil, Ecuador.
[3] Universidad de Guayaquil, Facultad de Ingeniería Química, Facultad de Ciencias Química Cdla, Guayaquil, Ecuador.
Corresponding author: marfgonz@espol.edu.ec

Abstract: The *Trichoderma* genus is well known as one of the most valuable biological control agents against several phytopathogens used in different plant species. Managing phytopathogenic fungi using the Trichoderma genus through various associated antifungal mechanisms is a sustainable and eco-friendly strategy that reduces the harmful presence of pathogens in soil, roots and aerial parts of plants. However, using biocontrol agents combined with chemical pesticides has evidenced further potential to reduce pathogen growth and benefit plant development. A better characterization of active metabolites secreted by *Trichoderma* and their mechanisms of action is necessary to improve its use as a biocontrol agent. This review summarizes current evidence on *Trichoderma spp.*, used as a biocontrol against *Fusarium oxysporum*, the active secondary metabolites secreted by the former fungi, and the effect of three widely used agrochemicals to control the latter, namely Mancozeb, Chlorothalonil, and Propiconazole. A total of 155 studies were selected and used to extract information that was analyzed, resulting in more than 590 identified secondary metabolites. Fifty-four percent of these have at least one biological function. Results highlight the potential of *T. harzianum* and *T. reesei* as biological control agents to control *Fusarium oxysporum*. The antifungal activity of *T. Espirale* is associated with enzymatic reactions. Additional findings show that management of diseases caused by *F. oxysporum* can be combined by using Trichoderma as biological control and agrochemicals to reach: (1) higher access to the different plant tissues; (2) higher degradation of the cell wall; and (3) and activation of oxidative metabolism of Trichoderma.

Key words: *Trichoderma*, secondary metabolites, fungicide, mycoparasitism, biocontrol, *Fusarium oxysporum*.

Introduction

Fusarium oxysporum is one the most economically important phytopathogens when referring to agriculturally important crops such as bananas and other crops[1]. The fungus infects the host plant through the roots or stems, causing wilt, blight, rot, and cancer of many plant species leading to significant yield loss in economically important crops such as banana[2], onion[3], tomato[4], chili[5], watermelon[6], cabbage[7], ginger[8], chickpea[9], soybean[10,11], eggplant plants[12], as well as ornamental plants like *Chrysanthemum spp.*, *Dianthus spp.*, *Gerbera spp.*, *Gladiolus spp.* and *Lilium spp.*[1]. No curative control method is currently available against this pathogen[1]. Current approaches to control *F. oxysporum* infestation are based on prophylactic measures and cultural practices[1] like keeping tools, soils and substrates in good sanitary conditions, planting resistant or tolerant genotypes, paying particular attention to crop monitoring, appropriate management of irrigation and crop rotation[1].

Chemical fungicides have also been essential in managing *Fusarium oxysporum* wilt for decades. However, these chemical control agents often become ineffective since pathogens may develop resistance, and the chemicals may adversely affect soil fertility and accumulate in the crop[13]. Since chemical pesticides are not selective, they can influence many beneficial non-target biotas and potentially harm the farmer's health[14-16]. Due to the harmful effects of these products, different policies that limit pesticide use have been implemented in several nations in the world[1,17].

Alternatively, biocontrol agents aim to regulate the growth of the pathogen with less harm to the plant and farmers. In this line, mycopesticides are exciting products because they use several mechanisms of action that reduce plant disease caused by phytopathogenic fungi[1,13,18]. Biopesticides are often less toxic than chemical products and decompose quickly. This can avoid pollution problems, resistance and residue concerns. Biopesticides generally affect only the target pest and closely related organisms, thereby protecting other organisms living in the same environment. The commercial evolution of the biopesticide market is promising to be a potential tool for pathogen control with a current annual growth rate of 14.1%[13].

Trichoderma spp. comprises more than 200 validly described species distributed in soils worldwide and across various habitats and are considered a valuable resource for structurally novel natural products with diverse bioactivities, including biological control of phytopathogens[19]. In the interest of obtaining more effective methods of pathogen control, plant growth-promoting rhizosphere microorganisms have been used as a consortium or in combination with chemical pesticides by our group and other authors[20-23]. Bioassays have revealed great potential to improve current methods of managing antifungal treatments to plant culti-

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vars. Yet, summarized evidence about active metabolites and the mechanism of actions of both biocontrol agents and chemical pesticides is needed to better use this possibility.

In this work, we reviewed the use of *Trichoderma spp*. as a biocontrol agent and the secondary metabolites and enzymes that have been characterized as active molecules. As additional findings, we also summarized the antifungal activity of commercial fungicides Mancozeb, Chlorothalonil, and Propiconazole, which are often used to control plant diseases caused by F. oxysporum.

Narrative Findings on *Trichoderma spp*

Trichoderma is a genus that belongs to the family *Hypocreaceae* and comprises many different fungi strains found in most diverse ecosystems[19]. *Trichoderma* strains proliferate and have a characteristic morphology, white and cottons at the beginning, then developing into yellowish green to deep green compact tufts. *Trichoderma* strains are characteristically branched[24]. The distinctive species categorized in the genus of *Trichoderma* are hard to differentiate morphologically. Based on morphology, *Trichoderma* strains have been classified into five sections: *Saturnisporum, Pachybasium, Longibrahiatum, Trichoderma* and *Hypocreanum*[25]. New genetic tools and physiological activity are used to determine the different functional groups within *Trichoderma spp*. Thus, current identification methods of *Trichoderma* strains include morphological and molecular characterization.

Many *Trichoderma* fungi act as biocontrol agents of phytopathogens and plant growth promoters[26]. They can also stimulate plant defense mechanisms against insect pests and be efficient soil bioremediation agents[27]. *Trichoderma spp*. can also be used in waste/organic materials decomposition and polluted area detoxification[28]. Some examples have emerged as human pathogens, for example *T. longibrachiatum*. Consequently, while the studies on effective biocontrol fungal are ongoing, further research to avoid the risk for humans, plants, and other organisms contributed by *Trichoderma spp.* also need to be accomplished.

The Mechanisms of biological control by *Trichoderma spp*

Biological control by *Trichoderma spp.* is based on the activation of indirect and direct mechanisms. Direct and indirect mechanisms can act synergistically and depending on species and strain[29]. The indirect mechanisms are competition for space and nutrients, growth promotion, and systemic resistance induction. The mechanisms by which *Trichoderma* induces systemic resistance in plants vary depending on plant species, *Trichoderma* species, pathogen species, abiotic stress conditions, and culture methods. It has been shown that *Trichoderma* colonization of plant rhizosphere may simultaneously activate both systemic acquired resistance and induced systemic resistance mechanisms of the plant. *Trichoderma* is also known to induce the resistance of plants towards diseases by root architecture alteration during the interaction with pathogens[30].

Direct mechanisms are mycoparasitism and the production of active metabolites and lytic enzymes[3,31]. Mycoparasitism, the ability to parasitize on fungi, is a unique characteristic of *Trichoderma* since they can parasitize even taxonomically close species[19]. The antifungal activity of *Trichoderma* against phytopathogenic fungi is attributed to the combined action of secondary metabolites (SMs) and hydrolytic enzymes i.e., cellulases, proteases, chitinases, and xylanases[3,32,33]. About 500,000 secondary metabolites have been described; of these, 15.600 (47 %) are of fungal origin.

Characterization of genes involved in fungal–fungal interactions has indicated that are mainly those involved in signal transduction, fungal cell wall degradation, and production of secondary antifungal metabolites (SMs)[19].

Secondary metabolites and enzimes produced by *Trichoderma spp*

SMs are not essential for normal growth but are synthetized for specific environmental conditions. SMs can be either volatile or non-volatile organic compounds. Volatile SMs diffuse over a distance through systems in the soil affecting the physiology of competitor organisms[34-36]. Non-volatile SMs exert their activity through direct interactions between *Trichoderma* species and their antagonists[35].

Our search for current evidence on SMs secreted by *Trichoderma spp*. Or enzymes resulted in annotating 590 unique compounds listed in the sup[A5] plementary Table S1. It includes many structural classes like pyrones, butenolides, steroids, peptaibols and terpenoids[19]. Fifty-four percent of all SMs or enzymes retrieved in our search have at least one biological effect associated, described in Table S1. Even though this list of biological activities should not be considered exhaustive, it allows appreciation of the incredibly broad range of biological activities of *Trichoderma* SMs i.e, antifungal, antibacterial, antitumor, DPPH-radical-scavenging, positive effect on plant growth and development, among others (Table S1). Further investigation is required using isolated compounds to obtain a comprehensive understanding of all effects at different for the different combinations.

The list of SMs shown in supplementary Table S1 is consistent with previous papers that emphasize that the quality and the number of volatile compounds produced are variable for each strain of *Trichoderma*. As example of the diversity in SMs produced by different *Trichoderma* species. A total of 115 SMs were reported for *T. reesei, T. harzianum* and *T. spirale* (Table S1). SM or enzymes identified for *T. reesei, T. harzianum* and *T. spirale* are indicated in Table 1, 2 and 3, respectively, which could be potentially used to control *Fusarium oxysporum*, due to their antifungal activity.

This result should not be understood as only *T. harzianum* secretes all these compounds. Genes encoding for proteins responsible for synthesizing these SMs are usually not expressed constitutively but due to interactions with the pathogen in the plant rhizosphere[4,45]. For example, the SM trichosetin, presumably secreted by *T. harzianum*, has only been identified in dual culture of *T. harzianum* and calli of *Catharathus roseus* but not in single cultures$_{31}$. However, the vast diversity of SMs isolated and characterized from *T. harzianum* indicates the great potential value of this fungus as a biocontrol agent against phytopathogenic fungi.

In vitro and *in vivo* assays have shown *T. harzianum* isolates with higher inhibitory activity against *F. oxysporum* (F3) than other *Trichoderma* species[46]. Biocontrol potential of *T. harzianum* against *Fusarium Oxysporum* has been demonstrated *in vitro* e *in vivo* against *F. oxysporum* in Poplar[47], ginger[8], cucumber[29,48], lettuce[49], white yam[50], chili[51], tomato and cucumber[52]. Nonetheless, *T. reesei* is one of the top fungal species used in industrial biotechnology and is used safely for decades in enzyme production. In contrast to *T. harzianum*, *T. reesei* is considered to have a limited production of mycotoxins[53]. Table 2 lists all SMS associated with antifungal activity.

Despite the literature did not specifically listed the SMs

Reference	Secondary Metabolite (SMs)
37	1,8-dihydroxy-3-methylanthraquinone
	1-hydroxy-3-methylanthraquinone
	1-hydroxy-3-methylanthraquinone
	6-methyl-1,3,8-trihydroxyanthraquinone
	6-methyl-1,3,8-trihydroxyanthraquinone
38	6-pentyl-α-pyrone
37	8-dihydroxy-3-methylanthraquinone
38	Farzianopyridone
39	Glutaryl-CoA
31–33,37,40–43	Harzianic acid
31–33,37	Harzianolide
37	Hydroxylphenylethanol
39	N-Undecanoylglycine
13	Pachybasin
39	Psoromic acid
32,38	Stigmasterol
31,32,37	T39butenolide
43	Trichocaranes A – D
44	Trichodermaol
37	Trichodermin
37	Trichokindin II*

Table 1. SMs of *Trichoderma harzianum* with antigungal activity.

Reference	Secondary Metabolite (SMs) or Enzime *
37	Harzialactone A
54	Ilicicolin H
37,55	Oxosorbicillinol
56	Trichodermin
57	*Xylanase
58	α-aminobutyric acid
59,60	α-aminoisobutyric acid

Table 2. SMs or enzymes of *Trichoderma reesei* with antifungal activity.

of *T. spirale* associated with the antifungal activity, it was worth to list the asociated enzimes *Trichoderma spirale* (Table 3). This list is short but shows the potential use of *T. spirale* [A9] in the control of pathogenic fungi.

In this work, we reviewed the use of *Trichoderma spp.* as biocontrol agents, the secondary metabolites characterized as active molecules and the enzymes found in this bibliographic review which present antifungal characteristics that act directly on the phytopathogenic fungi. The following graph highlights the following strains of *Trichoderma spp.*, *T. reesei, T. harzianum* and *T. spirale* with the most critical metabolites and enzymes.

Additional findings on the use of *Trichoderma spp.* and 3 synthetic pesticides[A10]

The low input cost and higher crop productivity of applying biological control agents (or biopesticides) are the economic benefits observed when compared to synthetic pesticides[63]. Thus, the use of *Trichoderma* is regarded as a sustainable approach not only ecologically but also from an economic perspective.

However, using microbial-based products as biocontrols or biostimulants has some disadvantages compared to their chemical counterparts. Microbial products have a limited shelf life and require special conditions for conser-

Reference	Enzymes associated with antifungal activity on *Trichoderma Spirale*
61	Chitinase
62	Endochitinase
37	Trichodemic acid
61	β-1,3-Glucanase

Table 3. Enzymes associated with antifungal activity of *Trichoderma spirale*.

vation to maintain viability and efficacy[44]. Also, they have constraints due to dependency on the crop, geographical, and meteorological regimes and pathogens[3,63].

One interesting approach that has emerged to cope with the advantages and limitations of the different methods to control crop infestation with *F. oxysporum* is the simultaneous application of *Trichoderma* as biological control with chemical pesticides and other biological control agents. For example, a combined treatment with *T. polysporum* LCB50 and irrigation with liquid compost applied resulted in a strong synergistic effect in controlling melon wilt and a 100% increase in the productivity of commercial fruit[64].

Recent results from our group have shown a synergistic effect using *T. reesei* and Mancozeb, inhibiting the mycelial growth of *F. oxysporum* (F1)20. Also, a synergic activity was obtained in vitro assays using *T. ressei* combined with Chlorothalonil or Propiconazole (unpublished data). However, the molecular basis of these agents' biological activity that results in an increased capacity to inhibit *F. oxysporum* infection is unknown.

Chlorothalonil (tetrachloroisophthalonitrile) and Mancozeb (manganese ethylene bis (dithiocarbamate) (polymeric) complex with zinc salt) are multisite enzyme inhibitors that act as protective broad spectrum fungicides[65]. Both are non-systemic, preventive fungicides that form a protectant barrier at the surface of the plant against the germination of spores and inhibit pathogen development[65,66]. Propiconazole (((2RS 4RS;2RS,4SR)-1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-ylmethyl]-1H-1,2,4-triazole)) belongs to a group of systemic fungicides that destabilize the cell membrane integrity and affects ergosterol biosynthesis through the inhibition of C14-demethylation[65]. Systemic fungicides are adsorbed into the leafs and translocated via the xylem, thus protecting the plant and controlling the circulating pathogens[65].

The co-inoculation strategy is possible as long as the fungus used as biopesticide tolerates the minimum concentration of the chemical fungicide required. This latter does not interfere with its development but contributes to increased control over the phytopathogenic specie. In this scenario, as proposed by Pelàez-Alvarez *et al.*(2016)[23], the presence of the chemical pesticide retards the growth of the phytopathogen, providing an advantage in the competition for space and nutrients in favor of the biopesticide. Chemical sensing of the competing fungus would induce the secretion of an arsenal of SMs that may act in both senses, facilitating the activity of the chemical fungicide and stimulating the plant's defense system[29,67]. Hence, the movement of chemical fungicides takes place from the upper parts of the plant and, in the case of those of systemic activity, disseminates to lower parts.

F. oxysporum fungus enters through the roots and disseminates throughout the plant using the vascular system. In contrast to chemical fungicides, *Trichoderma* fungi are part of the rhizosphere and generally grow on plant root surfaces and therefore control root diseases in particular[68]. Consequently, using *Trichoderma* as a biological control agent will provide an effective first barrier at the site of infection that will be complemented by the activity from top to bottom of chemical pesticides.

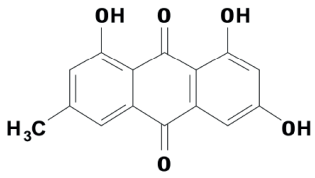
Previous work on *Trichoderma spp.* used as biocontrol agents have shown that cell wall degrading enzyme secreted by fungi i.e chitinase, cellulase, protease, and β-(1-3) glucanase and peptaibols are produced concurrently during biocontrol and interact synergistically as antifungal agents[69]. The proposed mechanism for such an effect is based on the fact that enzymes degrade the cell wall of host fungal pathogens. This activity directly inhibits the growth of the pathogen, at the same time, facilitates the access of peptaibols to the cellular membrane. Peptaibols. are small peptides of 15-20 residues characterized by non-standard amino acids in their sequences, with a special propensity for aminoisobutyric acid. The antimicrobial activity of peptaibols is related to their capacity to form pores in lipid membranes[70].

The same synergistic effect has been described for the activity of cell wall degrading enzymes and other SMs targeting specific target molecules in inhibiting *F. oxysporum* by *T. asperellum*[29]. Table 4 summarizes SMs identified as present in extracts with potent antifungal activity against *F. oxysporum*; or assayed from purified preparations and with proven inhibitory activity against this phytopathogen. Yet, important differences have been reported in the activity of cell wall degrading enzymes for *T. asperellum* and *T. harzianum*[29]. Thus, this mechanism could not be similarly effective for all *Trichoderma species*.

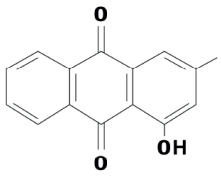
A similar synergistic activity could explain the outcome observed by co-inoculating *Trichoderma* species with biocontrol capacity against *F. oxysporum* and Mancozeb, Chlorothalonil and Propiconazole 20. It is reasonable to expect a similar effect on facilitating the penetration of these chemical fungicides.

Systemic effects resulting from *Trichoderma* interaction with the plant would also contribute to the observed synergistic effect when used with chemical pesticides. Reactive oxygen species scavenging enzymes have been found significantly increased in plants treated with *Trichoderma T-soybean, T. longibrachiatum* and *T. harzianum*, thus improving plant resistance to oxidative stress[3,11,72]. Exposure of plants to pesticides has evidenced that most of these chemicals lead to the development of oxidative stress[17]. Also, root colonization by *Trichoderma* has been found to result in intensified levels of defense-related, including β-peroxidases

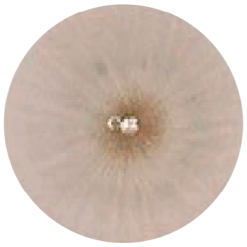
Trichoderma harzianum

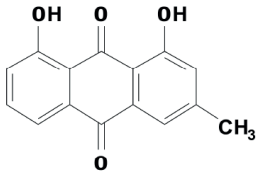


6-methyl-1,3,8-trihydroxyanthraquinone



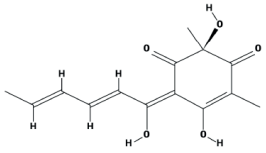
1-hydroxy-3-methylanthraquinone



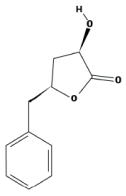


1,8-dihydroxy-3-methylanthraquinone

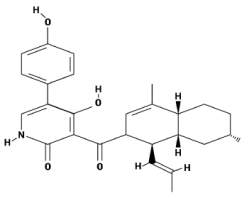
Trichoderma reesei



Oxosorbicillinol

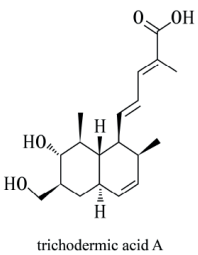


Harzialactone A

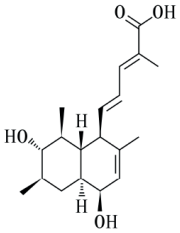


Ilicicolin H

Trichoderma spirale



trichodermic acid A



trichodermic acid B

Figure 1. Secondary metabolites and enzymes associated with antifungal activity that stand out in *T. reesei, T. harzianum* and *T. spirale* strains identified in the literature review on using *Trichoderma spp.* as biocontrol agents against *Fusarium Oxysporum.*

Metabolite	Trichoderma isolate	Experiment	Reference
N-à-(tert-Butoxycarbonyl)-L-Valine*	*T. asperellum* CCTCC-RW14	*In vitro* and *in vivo* (greenhouse)	29
6-Dimethylamino-4-keto hexanoic acid*			
1,3-Dioxolane,2-(3-bromo-5,5,5-trichloro-2,2-dimethylpentyl)*			
1-Aminocyclopentanecarboxylic acid, N-ethoxycarbonyl-, heptyl ester*			
2-[2-[2-Methoxyethoxy]ethoxy-1,3-dioxalane*			
1,6-diphenylhexane-1,3,4,6-tetrone*			
2-Octenoic acid*			
Methylmalonic acid*			
Milbemycin B*			
5-demethoxy-5-one-6,28-anhydro-25-ethyl-4-methyl-13-chloro-oxime*			
Koninginin G	*T. aureoviride*		71,38
Cremenolide	*T. cremeum*	*In vitro*	71,38
6-pentyl-α-pyrone	*T. Harzianum, T. koningii and*		71,38
trichodermin	*T. Harzianum*	*In vitro*	71
farzianopyridone		*In vitro* and *in vivo*	38
8-dihydroxy-3-methylanthraquinone			
1,8-dihydroxy-3-methylanthraquinone			
1-hydroxy-3-methylanthraquinone			
6-methyl-1,3,8-trihydroxyanthraquinone			
1-hydroxy-3-methylanthraquinone			
6-methyl-1,3,8-trihydroxyanthraquinone			
Stigmasterol			
Koninginin E	*T. koningii*	*In vitro*	71,38
trichokonin VI		*In vitro*	38
trichokoninVII			
trichokonin VIII			
Koninginin A	*T. koningiopsis*	*In vitro*	38
Koninginin B			
Koninginin D	*T. koningiopsis*, *T.harzianum, T. koningii, T. aureoviride*	*In vitro*	71,38,67,31
Koninginin F	*T. koningii, koningiopsis*	*In vitro*	71,38

*, denote compounds identified in extracts with antifungal activity. CCTCC-RW14 is represented

Table 4. Metabolites identified in extracts, or purified, with antifungal activity against *Fusarium oxysporum*.

and hydroxide lyase of lipoxygenase-pathway of the plant[73]. Moreover, it has been evidenced that *T. harzianum* alleviates oxidative stress by minimizing reactive oxygen species accumulation during *F. oxysporum* infection[52]. Thus, the contribution to activating a systemic response to oxidative stress in plants could be another level of cooperative action between chemical and biological control agents to control the attack of this phytopathogenic fungus.

Conclusions

Deleterious effects caused by *F. oxysporum* on plant species cause significant economic losses in agriculture at domestic and industrial levels. This review presents an organized narrative of information starting with the antifungal mechanism of *Trichoderma*, listing the SMs and enzymes involved in these mechanisms and finally the potential synergy of 3 synthetic pesticides for a better control of *F. oxysporum*. Our findings suggest that there is a need to develop more effective and ecologically friendly methods of controlling *F. oxysporum*, compared to the current control methods. Both chemical and biological control agents have individually played important roles protecting crops for millenniums. Also, both have advantages and disadvantages in their use. Thus, recent approaches have proposed the simultaneous use of chemical and biological pesticides and obtained promising results evidencing a synergistic activity controlling *F. oxysporum* infestation at *in vitro* and *in vivo* experiments. A better understanding of modes of action and cooperative effects of these two types of fungicide agents should let make better use of them in co-inoculation programs. The review of current knowledge on modes of action of *Trichoderma* in the control of *F. oxysporum* infection

as well as the chemical fungicides Mancozeb, Chlorothalonil and Propiconazole confirms that their inhibitory activities may be compensatory and may lead to synergistic effects.

Supplementary Materials

Supplementary Table 1 (S1) is available under request.

Author Contributions

GMF, GL, VL and QG made substantial contributions conception and design, or acquisition of data, or analysis and interpretation of data. GMF and GL contributed drafting the article or revising it critically for important intellectual content. GMF and GL revised the final version of the manuscript before publication. GMF and GL ensures that any part of the work was appropriately investigated and resolved.

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Institutional Review Board Statement

Ethical review and approval were waived for this study because it does not involve humans or animals.

Data Availability Statement

Data is available fully in open access.

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Conflicts of Interest

The authors declare no conflict of interest.

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