**Original Research Article**

**Glucose-induced Production of Secondary Metabolites by *Lasiodiplodia theobromae***

**ABSTRACT**

Microbial chemodiversity of bioactive secondary metabolites remains largely unexplored for use as new antimicrobial agents. Current advances employed to exploit this plethora of bioactive compounds from microbes are focused on the stimulation of physiological processes that induce the expression of specific enzymes (gene clusters) that eventually regulate the production of secondary metabolites. In this work, we studied the impact of glucose optimized medium in the induction of specific-gene clusters within the genome of *Lasiodiplodiatheobromae.* High-performance Liquid Chromatographic analysis was used to monitor and identify the metabolites in each crude extract. Agar well diffusion was used to assay the antimicrobial activities of each crude extract against selected isolates of vancomycin and oxacillin resistant-*Staphylococcus* *aureus,* Multidrug resistant-*Pseudomonas aeruginosa*, *Candida albicans*, and *Dermatophytes*. The induced secondary metabolites identified included Protocatechuic acid scopularide. None of these compounds were hitherto produced by the uninduced (wild type) *L. theobromae* strain when cultured under normal laboratory conditions. The resultant extracts demonstrated good microbial inhibitory activities and MIC which ranged between 2 to 8 mm and 31 to 500 µg / mL respectively. The broad-spectrum activities may be due to the presence of the glucose-induced structurally diverse secondary metabolites produced by *L. theobromae*.

**Keywords:** modulation, induction, gene clusters, bioactive secondary metabolites, *Lasiodiplodia theobromae*, antimicrobial activity, new chemical entity.

1. **INTRODUCTION**

Microorganisms especially endophytic fungi have remained a consistent producer of known and novel bioactive compounds required for combating new infectious diseases especially those caused by multi-drug resistant organisms such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, and Vancomycin-resistant *Staphylococcus aureus* (Jyotsna *et al*., 2018). However, there is a noticeable reduction in the discovery of new molecules from microbes attributed to the continuous cultivation of these microbes under normal (standard) laboratory conditions (Reen *et al*., 2015; Jyotsna *et al.,* 2018; Stroe *et al*., 2020). However, they have constantly produced novel bioactive compounds having diverse industrial applications (Wu and Chappell, 2008, Newman and Cragg, 2012). Endophytic fungi have shown vast chemical diversity (Okezie *et al*., 2022; Okezie *et al*., 2021; Okezie *et al*., 2017; Okezie *et al*., 2015) that involved already discovered and biosynthetic potentials for yet-unidentified compounds (Jyotsna *et al*., 2018; Reen *et al*., 2015). Current discoveries made that identified silent biosynthetic gene clusters (BGCs) through fungal genome analysis, is a proof that the biosynthetic capacities of this family of microorganisms for novel molecules have not been fully harnessed (Reen *et al*., 2015). Therefore, have become the primary focus of several advanced screening programs (new screening strategies) that regulate the biosynthesis of novel molecules (Jyotsna *et al*., 2018; Deepika and Satyamoorthy, 2015; 2015; Newman and Cragg, 2012). Moreover, fungi genome mining has provided natural products researchers with useful data on the existence of biosynthetic gene clusters (Blunt *et al.,* 2017; Kusari and Spiteller, 2011) at specific genomic locations which have been linked with their metabolic capacities. Also, this has prompted the Identification of key regulatory regions of these biosynthetic gene clusters (BGCs) within their genomes (Deepika and Satyamoorthy, 2015) that are inducible and responsible for the production of novel bioactive molecules. Furthermore, this development has upheld the inexhaustive nature of microbial (fungal endophytes) metabolic wealth.

The discovery of silent BGCs has been accompanied by advancements in screening techniques, development and applications of various tools that predict important putative genes (Bergman *et al.,* 2007; Khaldi *et al.,* 2010; Medema *et al*., 2011; Priebe *et al*., 2011) encoding specific enzymes with novel biosynthetic pathways. However, these genes are inactive and remain silent under normal laboratory conditions thus the natural products for which they encode remain unknown (Soltani, 2016). Nevertheless, studies have revealed the positive influence of specific cultivation-based approaches such as external cues (stress) e.g. temperature; nutrient regimes; pH, or light (Uv-radiation, electromagnetic), and co-cultivation approaches on specific regulatory signaling proteins responsible for stimulating the activation (induction) of silent gene clusters that encode for important secondary metabolites (Reen *et al*., 2015). These have helped in reversing redundancy in novel natural product discovery characterized by the re-isolation of known compounds. In particular, exposure of fungus to UV radiation or light has been exploited as an inducer for the production of new bioactive compounds (Kramer *et al*., 2015; Kramer *et al.,* 2014; Zhang *et al*., 2016). Also, studies show that the communication (interaction) that occurs between two microbes in a co-cultivation experiment such as two fungi or a fungus with a bacterium induces the production of new chemical entities (Yu *et al*., 2016; Eze *et al*., 2021). Furthermore, the incorporation of cobalt ion in the cultivation of an *Aspergillus sp*. induced the production of aspergstressin (Ding *et al*., 2016); benzylpyridin-4-one, and terpenoids (Blunt *et al.,* 2017) by this fungus. Therefore, understanding the regulatory mechanisms at the molecular level and the cultivation conditions capable of stimulating the biosynthetic pathways will have a positive impact on the generation of novel secondary metabolites.

               In our previous work, we isolated orthosporin a phytotoxic compound produced by *Lasiodiplodia theobromae* (previously known as a disease-causing fungus), isolated from the Healthy leaf of *Musa paradisiacal* (Okezie *et al*., 2022). Also, we observed several compounds have been detected and/or isolated from the fermentation product of several *Lasiodiplodia sp*, (Li *et al*., 2016; Salvatore *et al*., 2020) thus providing an insight into a possible large chemo-diversity associated with this family of fungus and the presence of silent gene clusters. We have subsequently reported the antimicrobial, antioxidant, and reverse transcriptase potentials of *L. theobromae* crude extract (Okezie *et al*., 2022).

Also, based on the identification of BGCs in endophytic fungi genome coding for diverse arrays of bioactive compounds (Reen *et al*., 2015), in addition to our preliminary data on the metabolic profiles of *L. theobromae* as well as available data on the secondary metabolites produced by species of *Lasiodiplodia*(Blunt *et al.,* 2017; Li *et al.,* 2016; Salvatore *et al*., 2020),*L.*theobromae was considered an ideal and reliable candidate for induction of novel chemical entities.

In the present study, we reported the expression of an uncharacterized gene cluster encoding a possible novel secondary metabolite in *L. theobromae* that was induced by the impact of two different cultivation-based approaches.

1. **MATERIALS AND METHODS**
	1. **MICROORGANISM USED**

The endophytic fungus*Lasiodiplodia theobromae*, was previously isolated from healthy leaves of *Musa paradisiaca* and identified by combining the molecular, macroscopic, and microscopic details (Okezie *et al.,* 2022). *L. theobromae* was maintained on malt extract agar (MEA) and sub-cultured routinely.

* + 1. **MODULATION STUDIES**

The biosynthetic genes of the endophytic fungus*Lasiodiplodia theobromae*, was modulated adopting media (glucose) optimization techniques.

* + 1. **MODULATION BY MEDIA OPTIMIZATION**

To modulate the biosynthetic gene clusters present in *L. theobromae*, pure culture of*L. theobromae*  were fermented on rice medium enriched with 10% glucose at 27°C for 21 days insterile rice medium under stationary condition (Figure 1).

The effect of a carbon source (10 % glucose) on the induction of the biosynthesis of bioactive metabolite by *L. theobromae* was assessed, “OSMAC (one strain many compounds) approach”. Here, the composition of the fermentation medium comprised of a mixture of (100 g of rice + 200 mL of water + 10 g of glucose). This was incubated at 27°C for 21 days under stationary conditions.

* 1. **EXTRACTION AND CHROMATOGRAPHIC ANALYSES OF INDUCED SECONDARY METABOLITES**

Upon completion of the fermentation process, 500 mL of ethyl acetate was transferred into each of the fermentation flasks, homogenized, filtered, and concentrated at 50°C with a rotary evaporator (Stuart, USA) Okezie *et al*., (2022). Assessment of possible induction of silent gene clusters and biosynthesis of cryptic secondary metabolites by Clr8 was analyzed using high-performance liquid chromatography system diode-array detection (HPLC-DAD) system and the resulting chromatograms (metabolic profiles) of each extract was compared with that of the unmodulated (Clr8) strain. Also, the impact of the different modulation approaches on the bioactivity of each modulated extract was assessed by subjecting to selected microbial inhibitory evaluations.

* 1. **HPLC ANALYSIS**

In other to monitor the effect of substrate modification using glucose glucose on the induction of novel secondary metabolites by the endophytic fungus, “HPLC analytical technique using a Dionex P580A LPG HPLC system coupled to a photodiode Array Detector (UVD340S, DionexSoftron GmbH, Germering, Germany) was used. Detection was at 235, 254, 280, and 340 nm. The separation column (125 × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent” Okezie *et al*., (2022).

* 1. **BIOACTIVITY**
		1. **TEST ORGANISMS**

The test organisms are Bacteria: Oxacillin and Vancomycin resistant-*Staphylococcus aureus* and Multidrug-*Pseudomonas aeruginosa* (resistant to at least one antibiotic from the beta lactams, aminoglycoside, quinolones and tertracycline); Fungi (*Candida albicans and Dermatophyte spp*).

* + 1. **ANTIMICROBIAL ASSAY**
		2. **AGAR WELL DIFFUSION ASSAY**

Each of the modulated fungal extract was subjected to antimicrobial evaluation following the same protocol described by Okezie *et al*., (2017). Each crude extract was reconstituted in dimethyl sulfoxide (DMSO) to obtain varying concentrations of 1, 0.5, 0.25, 0.13 and 0.06 mg/mL. Sterilized cork borer (8 mm) was used to bore wells/holes in the Mueller-Hinton agar and Sabouraud dextrose agar plates previously inoculated with the various test organisms. Using a micropipette, 80 µl of each extract concentration was applied to their respective wells. Zone of inhibition was measured after an incubation period of 24, 48, and 168 h for *bacteria*(*VOR-Staph aureus; MDR-Pseudomonas*), *Candida albicans*, and *Dermatophytes* respectively.

* 1. **STATISTICAL ANALYSIS**

The results were expressed as mean ± standard deviation. Statistical analysis was carried out using a one-way analysis of variance (ANOVA) and SPSS (version 20) statistical program. The measures were done in triplicate (n = 3). A confidence level of 95% was used.

1. **RESULTS & DISCUSSION**
	* 1. **EFFECTS OF MEDIA OPTIMIZATION ON INDUCTION OF SECONDARY METABOLITES PRODUCTION IN *L. THEOBROMAE***

The HPLC chromatogram shows the presence of Protocatechuic acid, citreoisocoumarinol, and scopularide A detected in the fermentation product of *L. theobromae* previously grown on glucose optimized medium. Comparisons between the compounds detected in the crude extracts of the unmodulated *L. theobromae* and *L. theobroame* modulated using glucose optimized medium is an indication that glucose may have induced the biosynthesis of some secondary metabolites hitherto not detected in the crude extract of L. theobromae. This result also provides evidence of the effect observed when there is a slight shift from the normal laboratory cultivation conditions to favorable growth conditions which also influence the production of secondary metabolites through the activation of silent genes.

**W**



**18**

**4**

**12**

Graph 1-HPLC chromatogram analysis monitored at UV/Vis wavelength of 235 nm



**Z**

**Y**

**X**

**Figure 1: HPLC chromatogram (W) of medium engineered-modulated *L. theobromae* crude extract (OSMAC approach) indicating Protocatechuic acid, Citreoisocoumarinol, and Scopularide Peaks; and their UV spectra Protocatechuic acid (X); Citreoisocoumarinol (Y), and Scopularide A (Z).**

**Table 1: Table showing the Bioactive Compounds detected in the extracts of *L. theobromae* pre- and post-induction**

|  |  |  |  |
| --- | --- | --- | --- |
| **Extract** | **Compounds**  | **Reported Biological Activities**  | **References**  |
| Extract of uniduced*L. theobromae* (Crude extract) | Orthosporin | Antimicrobial, antioxidant, reverse transcriptase, cytotoxicity | Okezie *et al*., 2022 |
| Extract of *L. theobromae* induced with Glucose-opt-medium | Protocatechuic acid  | AntimicrobialAntimicrobial, Antioxidant  | Xican *et al*., 2013  |
| Extract of *L. theobromae* induced with Glucose-opt-medium | Scopularide | Anticancer  | Mie *et al*., 2015 |

Summarized compounds identified in the extracts of uninduced (wild type) and induced strains of *L. theobromae* by HPLC analysis

**Table 2: Anti-Candida evaluation of extracts of modulated *L. theobromae***

|  |
| --- |
| **Ext -Glu / Conc. (µg/mL) / IZD (mm)** |
| **Test Orgs.** | **500** | **250** | **125** | **63** | **31** | **Mico. 50 µg** |
| *C. a 1* | 5± 0 | 4± 0 | 3± 0 | 3± 0 | 3± 0.7 | 6 |
| *C. a 2* | 6± 0 | 5± 0 | 5± 0 | 4± 0 | 0± 0 | 12 |
| *C. a 3* | 4± 0 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 10 |
| *C. g 1* | 5± 0.7 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 7 |
| *C. g 2* | 5± 0.7 | 5± 0.7 | 4± 0 | 4± 0 | 2± 0 | 4 |
| *C. tropicalis* | 6± 0.7 | 4± 0 | 3± 0 | 0± 0 | 0± 0 | 7 |
| *C. krusei* | 7± 0 | 6± 0 | 5± 0.7 | 4± 0.7 | 0± 0 | 8 |

Key: C.a: *Candida albicans*; Ext-Glu: extract of glucose-optimized medium; Mico: Miconazole 50µg (positive control)

**Table 3: Anti-Dermatophytic evaluation of extracts of modulated *L. theobromae***

|  |
| --- |
| Ext -Glu / Conc. (µg/mL) / IZD (mm) |
| **Test Orgs.** | **500** | **250** | **125** | **63** | **31** | **Mico. 50 µg** |
| *T1* | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 5 |
| T2 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 8 |
| T3 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 10 |
| T4 | 8± 0 | 5± 0 | 4± 0 | 0± 0 | 0± 0 | 6 |
| T5 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 0 |

Key: T: *Tinea species* 1-5; Ext-Glu: extract of glucose-optimized medium; Mico: Miconazole 50µg (positive control)

**Table 4: Antibacterial evaluation of extracts of modulated *L. theobromae* against some VOR-*Staph aureus* isolates**

|  |
| --- |
| **Ext -Glu / Conc. (µg/mL) / IZD (mm)** |
| **Test Orgs.** | **500** | **250** | **125** | **63** | **31** | Cipro. 5 µg |
| *VOR-S. a 1* | 5± 0 | 3± 0 | 2± 0 | 0± 0 | 0± 0 | 0 |
| *VOR-S. a 2* | 3± 0 | 2± 0 | 0± 0 | 0± 0 | 0± 0 | 0 |
| *VOR-S. a 3* | 4± 0 | 3± 0 | 0± 0 | 0± 0 | 0± 0 | 6 |
| *VOR-S. a 4* | 6± 0 | 4± 0 | 0± 0 | 0± 0 | 0± 0 | 8 |
| *VOR-S. a 5* | 2± 0 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 15 |
| *VOR-S. a 6* | 2± 0 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 4 |
| *VOR-S. a 7* | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 17 |

Key: VOR-*Staph aureus: Vancomycin & Oxacillin-resistant Staph aureus*:Ext-Glu: extract of glucose-optimized medium; Cipro: ciprofloxacin 5 µg (positive control)

**Table 5: Antibacterial evaluation of extracts of modulated *L. theobromae* against some MDR- *Pseudomonas aeruginosa* isolates**

|  |
| --- |
| **Ext -Glu / Conc. (µg/mL) / IZD (mm)** |
| **Test Orgs.** | **500** | **250** | **125** | **63** | **31** | **Cip. 5 µg** |
| *MDR-P1* | 4± 0 | 3± 0 | 2± 0 | 0± 0 | 0± 0 | 8 |
| *MDR-P2* | 4± 0 | 4± 0 | 3± 0 | 3± 0.7 | 3± 0.7 | 7 |
| *MDR-P4* | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 8 |
| *MDR-P5* | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 0± 0 | 0 |
| *MDR-P7* | 4± 0 | 4± 0 | 3± 0.5 | 0± 0 | 0± 0 | 6 |

Key: *MDR-Pseudomonas aeruginosa*: Ext-Glu: extract of glucose-optimized medium; Cipro: ciprofloxacin 5 µg (positive control)

Fungi utilize at least one of the several sugars (carbon sources) necessary for metabolism. Glucose a sugar is utilized by fungi as a source of carbon more than any other source because of their ability to metabolize it completely. Previously, we observed a low production of important bioactive secondary metabolites by *L. theobromae* when cultured under normal laboratory cultivation conditions (Okezie *et al*., 2022), hence the addition of glucose to provide a growth condition optimum for the fungus.

Several sugars such as sucrose, fructose, and glucose are sources of carbon necessary for the biosynthesis of important secondary metabolites by fungi (Sanchez *et al.,* 2010; Ramos and Said, 2011). Also, small alterations in the composition of culture mediums have been observed to influence both the amount and type of secondary metabolites produced by a fungus (Bode *et al*., 2002; Bracarense and Takahashi, 2014). In this study, the incorporation of glucose (an additional source of carbon) in the fermentation medium, was found to induce the production of protocatechuic acid, citreoisocoumarinol, and scopularide (Figure 1, Table 1). Glucose-induced production of secondary metabolites is connected to specific signaling proteins such as CreA (carbon signaling) triggered by certain physiological processes believed to be linked to the regulation of metabolic pathways responsible for the secondary metabolites (Deepika and Satyamoorthy, 2015). This is evident by the type and number of secondary metabolites produced by *L. theobromae*grown on glucose optimized mediumand detected in the fermentation product by HPLC-DAD analysis (Figure. 1; Table. 1). Similarly, incorporation of glucose in the growth medium of *Aspergillus parasiticus*, induced the production of higher amounts of kojic acid (Bracarense and Takahashi, 2014).

The induction of the biosynthesis of protocatechuic acid, citreoisocoumarinol, and scopularide by *L. theobromae* may have been stimulated by the activation of specific genes that encode them, induced by the incorporation of glucose in the medium. Glucose is metabolized by specific enzymes such as nonribosomal peptide synthetase (NRPS1), polyketide synthase (PKS2), etc. necessary for the biosynthesis of both polypeptide and polyketide compounds (Deepika and Satyamoorthy, 2015). Therefore, changes in the growth medium (i.e. addition of glucose) triggered the induction of silent genes that code for protocatechuic acid, scopularide biosynthesis, and up-regulation of citreoisocoumarinol. Similarly, studies conducted by Reen *et al*., (2015) and Sanchez, *et al*., (2010) highlighted the enhancement of secondary metabolite production by microorganisms cultured on glucose-optimized media. Our findings, therefore, provide additional information on nutrient-mediated induction of silent gene clusters and the production of different compounds by a single strain of microorganism (i.e. OSMAC approach).

At concentration of 31 to 500 µg/mL, the ethylacetate crude extracts of *L. theobromae* cultured on glucose-optimized medium exhibited good antimicrobial activities against the resistant isolates with inhibition zone diameters and minimal inhibitory concentrations (MIC) that ranged between 3 – 6 mm; 31 – 500 µg/mL against the *Candida species;* 34 – 8 mm; 125 – 500 µg/mL against the *dermatophyte species*; 2 – 6 mm; 125 – 500 µg/mL against *VOR-Staphylococcus aureus;* 2 – 4 mm; 31 – 250 µg/mL against *MDR-Pseudomonas aeruginosa* (Table 2-5). The activity demonstrated by this extract against the resistant *Staphylococcus aureus* isolates was observed to be the best (Table 5).

From the results of the antimicrobial activities recorded in this work, it is evident that the crude extract produced by *L. theobromae* cultured on a glucose-optimized medium demonstrated varying antimicrobial potentials. This may be due to the difference in the secondary metabolites present and their diverse mechanisms of inhibiting microorganisms since the compounds differ structurally and thus exhibit structure-related activity.

Structural differences in secondary metabolites are likely to affect the overall performance of the extracts tested in this work, leading to differences in the sensitivity between test organisms (Gram-positive, Gram-negative bacteria, mold, and yeast). For instance, the morphological differences between the bacteria where Gram-negative bacteria have an outer polysaccharide membrane that carries the structural lipopolysaccharide components that make the cell wall impermeable to lipophilic solutes (compounds). This explains a higher sensitivity to the extracts by *Pseudomonas aeruginosa* Gram-negative bacteria than *S. aureus* a Gram-positive. The observed activity is due to the presence of more hydrophilic compounds such as phenolic compounds capable of permeating the cell wall of these organisms.

Also, the hydrophobic nature of some endophytic fungal secondary metabolites (EFSMs) allows them to accumulate and perturb the structure and function of lipid cell membrane, disturbing biological function, and causing the failure of chemiosmotic control, thus rendering the membrane more permeable (Ahmed *et al*., 2018).

Furthermore, the observed antimicrobial activities shown by the extract may be attributed to the presence of these bioactive compounds protocatechuic acid, citreoisocoumarinol, and scopularide detected in the crude extract.

Also, studies on the antibacterial activities of some secondary metabolites have implicated Protocatechuic acid (Semaming *et al*., 2015), citreoisocoumarinol (Akpotu*et al*., 2017), and scopularide A (Yu *et al*., 2008). Protocatechuic acid is a polyphonic compound detected in both plants (Elansary *et al*., 2020; Semaming *et al*., 2015) and microbial extracts (Eze *et al*., 2019). Also, I*n vitro* studies have shown PCA to have broad-spectrum antimicrobial activities and also exert synergistic interaction when combined with certain antibiotics against resistant pathogens (Semaming *et al.,* 2015; Liu *et al*., 2008). Following the analyses of the crude extract of endophytic fungus isolated from *Psidium Guajava*, Enyi *et al*., (2019), reported the marked antimicrobial activity of the crude extract.

Similarly, Yu *et al*., (2008) reported the detection of scopularides A and B, in the extract of *Scopulariopsis brevicaulis*, a fungus isolated from *Tethya aurantium* a marine sponge. However, a weak activity was observed against Gram-positive bacteria.

The results of this study showed that the use of glucose as a source of carbon in optimizing the culture medium triggered the activation of the gene responsible for the biosynthesis of protocatechuic acid, citreoisocoumarinol, and scopularide(Table 1; Figure 1). Similarly, Reen *et al*., (2015), highlighted the enhancement of secondary metabolites production by microorganisms cultured on glucose optimized media.

Our results therefore revalidate the previous reports on the production of different compounds by a single strain of microorganism (OSMAC-approach) subjected to different modulation conditions and support early culture improvement/manipulation approach, when a fungus is actively growing avoiding possible alterations that may occur in the metabolic profile when re-cultured.

1. **CONCLUSION**

In this study, we observed that the incorporation of glucose into the rice medium mediated the induction of specific genes that led to the biosynthesis of different molecules by *L. theobromae* detected by the high-performance liquid chromatography system. The difference in the compounds produced by *L. theobromae* pre- and post-induction validates possibilities of inducing gene-specific chemical entity hither not expressed when the same fungus is cultured under normal (standard) conditions.

Also, it provides insight into the availability of a vast yet unexplored arsenal of bioactive secondary metabolites linked to the biosynthetic pathways of microorganisms attributed to the absence of corresponding biosynthetic enzymes and further validates *L. theobromae* as an understudied species for modulation of novel diverse bioactive compounds.

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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