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適用鰻苗循環水養殖系統之高成長膏狀飼料開發

Development of palatable glass eel paste feed with high growth rate in the recirculating aquaculture system

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本論文係林昱廷 (姓名) D09B45005 （學號）在國立臺灣大學漁業科學 (系/所/學位學程)完成之博士學位論文，於民國113年5月22日承下列考試委員審查通過及口試及格，特此證明。

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中文摘要

目前臺灣之鰻魚養殖業者多以赤蟲及絲蚯蚓作為鰻苗初期之開口餌料，取其對鰻苗具有強誘引性及良好適口性。但二種餌料皆生活於富含有機質之水域，容易夾帶病原或寄生蟲進養殖環境中，特別對於封閉集約式的循環水養殖系統有嚴重威脅。所以日本及韓國目前皆以人工膏狀飼料作為鰻苗之開口與初期餌料，其誘引性及適口性與赤蟲／絲蚯蚓接近，然其缺點為進口售價過於高昂，並不符合養殖經濟效益。而當鰻苗成長進入幼鰻階段後，目前主流使用鰻粉或浮料進行餵飼。其中鰻粉的散失性極強，容易對養殖池之水質造成嚴重影響，且易造成物理過濾器過載；浮料則由於加工過程需經過高溫膨發，無法在製程中有效添加對鰻魚成長、抗病有益之添加物如益生菌等，且初期鰻苗無法攝食。而膏狀飼料具有不易散失及低溫製作之特性，非常容易於製程中添加各種有利於鰻魚之添加物。若能研發出價格合理之鰻苗膏狀飼料，具有良好適口性及誘引性，散失性低，再加以功能性添加劑，極適合使用在室內集約循環水養殖系統，將具有龐大的商業潛力。本研究目前已研發出一種以魷魚漿搭配魚卵所製成之膏狀飼料，且發現對各類鰻苗之適口性、誘引性良好且成長表現不遜於赤蟲，更檢測不出對鰻魚有害之病原菌，極為適合搭配室內循環水系統使用。若搭配良好之機能性添加劑（碳化多銨 (CQDSpds)、納豆枯草桿菌 NTU-18)等可以進一步提升鰻苗之成長表現、抗病能力及腸道菌相，且不會影響鰻苗之腸道型態。

關鍵字: 鰻魚養殖、室內循環水系統、鰻苗飼料、膏狀飼料、飼料添加劑

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Abstract

Currently, in Taiwan, eel aquaculture farmers mostly use bloodworms as initial feed, taking advantage of their strong attractiveness and palatability to glass eel. However, both types of feed live in waters rich in organic matter, making them prone to carry pathogens or parasites into the aquaculture systems, especially to the intensive eel recirculating aquaculture systems (RAS). Therefore, Japan currently using artificial paste feed as the initial feed for glass eel, which has similar attractiveness and palatability to bloodworms. However, its excessively high price, which is not cost efficiency. As glass eel grow into the elver stage, the main using feed used is eel powder or floating feed. Eel powder is highly dispersible, easily causing serious water quality impacts in aquaculture systems, and can overload RAS physical filters. Floating feed, due to the high-temperature required during processing, cannot effectively add beneficial additives such as probiotics for eel growth and disease resistance, and a glass eel cannot consume. Paste feed, on the other hand, has the characteristics of good stability and processed under low temperatures, making it very easy to add various additives beneficial to eel growth during processing. If a paste feed with good palatability, attractiveness, low dispersibility, reasonably priced, and additives can be easily added was developed, it will be extremely suitable for use in indoor intensive RAS. This study has currently developed a paste feed made from squid paste combined with fish roes, which has been found to have good palatability and attractiveness to various species of glass eels and performance comparable growth performance with bloodworms. Furthermore, harmful pathogens for eels were not detected in the glass eel intestine, making it highly suitable for use in indoor intensive RAS. If combined with good functional additives (CQDSpds, *Bacillus subtilis natto* NTU-18), it can further enhance the growth performance, disease resistance, immune gene response and intestinal microbiota of glass eel without affecting their intestinal morphology.

Keyword: Eel aquaculture, RAS, glass eel feed, paste feed, feed additive

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1. 前言

1.1 鰻魚養殖近況

鰻魚屬於鰻鱺科(Anguillidae)，鰻鱺屬(*Anguilla*)，全世界至今共有 19 種鰻魚(Watanabe et al., 2009)，全部皆為典型之降海洄游性魚類。在東亞，目前大多以日本鰻 (*Anguilla japonica*) 為主要的養殖鰻種，取其具高營養價值 (Ahn et al., 2015) 及文化因素 (日本「土用丑日」)，使日本鰻在東亞地區具有極高之商業價值及需求 (Shahkar et al., 2015)。臺灣目前雖發現有五種鰻鱺屬物種存在 (Tzeng, 1982; Han et al., 2016)，不過至今仍以日本鰻為主要的養殖鰻種，據農委會統計資料，台灣養鰻產業在 1992 年到達高峰，靠內銷及出口日本創造出餘新台幣二百億元之產值 (農委會漁業統計資料)。唯自 1970 年代起，日本鰻苗之自然資源量逐年下滑，至 2014 時日本鰻苗之天然資源量，僅為 1970 年代時期的 5 % (Chen et al., 2014; Dekker, 2004)，並且已於 2014 年被 IUCN 列為瀕危物種 (Jacoby and Gollock, 2014)。

為了填補日本鰻苗數量銳減的缺口，近年來各國興起了異種鰻的養殖風潮。其中以歐洲鰻 (*A. anguilla*) 及美洲鰻 (*A. rostrata*) 為大宗，但歐洲鰻自 2007 年被列入 CITES 附錄二，2010 年起禁止國際貿易，養殖量迅速萎縮；自歐洲鰻禁運之後美洲鰻的苗價近年亦隨之水漲船高，且亦被列入 IUCN 紅皮書討論中，隨時皆面臨禁止國際貿易之風險 (Jacoby et al., 2014)。故近年來東亞各國皆嘗試發展

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目前 IUCN 評估無危之鱸鰻 (*A. marmorata*，俗稱花鰻) 以及太平洋雙色鰻 (*A. bicolor pacifica*，俗稱黑鰻) 的飼養 (Luo et al., 2013; Marini et al., 2021)。

1.2 熱帶性異種鰻養殖

本研究鰻種之一為鱸鰻，以其體型大及地理分布廣泛著稱 (Ege, 1939; Leander et al., 2014)，且台灣東部及宜蘭地區具有非常豐沛之鱸鰻苗資源量 (Wu, 2012; Leander et al., 2013)，低廉的苗價為其最大優勢。不過鱸鰻雖有苗價及數量之絕對優勢，但其養殖門檻相對於日本鰻困難 (Luchiari et al., 2008)。一般養殖因其體型參差大，且易罹患疾病造成大量斃死導致平均育成率不到 2 成，與日本鰻動輒平均 8 成以上的育成率相比，明顯偏低 (Thuc and Van, 2021)。且鱸鰻由鰻苗成長至上市體型 (1.2-1.5 公斤) 約需時二年半至三年以上，生長表現與其他養殖鰻種相比久上許多 (Kumai et al., 2020; Cadiz and Traifalgar, 2020)。其中特別是在由鰻苗至幼鰻養殖階段，須 6~10 個月，為日本鰻需時的兩倍以上 (林, 2019)。另一目標鰻種則為黑鰻，其肉質和日本鰻較為相近且東南亞地區有豐富鰻苗資源成為其發展優勢，不過在育成率及養殖時間上仍遜於日本鰻 (林和楊, 2016)。而目前台灣、中國及東南亞，已有不少企業與機構進行鱸鰻及黑鰻的養殖與相關研究 (林, 2016; Muthmainnah et al., 2016; Cuvin-Aralar et al., 2019)，期盼若能克服異種鰻初期養殖之瓶頸，提升成長率與育成率，臺灣異種鰻養殖將極具發展潛力 (Han, 2010; 林, 2019)。

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1.3 循環水養殖系統 (RAS)

臺灣位處於熱帶與亞熱帶的地理位置，先天環境適合水產養殖發展，但因為地狹人稠、水資源競爭、從業人口的老化、環境變化加劇及食安的注重，使得水產養殖整體發展緩慢，逐漸落後亞洲其他國家。因傳統養殖戶多採用戶外土池飼養，需抽取地下水來維持水質，進而導致地層下陷等問題，且隨著極端氣候加劇及疾病增生，不受控的室外養殖環境，常遭遇養殖生物大量暴斃造成鉅額損失。因此近年來積極推廣發展室內循環水系統養殖 (Islam and Yasmin, 2017)。室內循環水養殖系統與傳統土池相比，可提高養殖作業和生產穩定性，並不受天氣之限制 (Qiu et. al, 2016)。前人研究亦指出，使用循環水養殖系統可大幅提高養殖密度及節省用水，並進而降低引進病原之風險，因此可以減少用藥 (Deviller et al., 2005; Martins et al., 2011)。以超集約養鰻系統為例，其養殖密度可達 90 公斤/每噸水，是傳統土池的 50 倍，而用水量則僅僅是土池的 1/30，活存率更高達 95% (徐, 1997)。同時異種鰻使用循環水養殖系統也可大幅提升其成長率及育成率 (黃, 2007)。近年來因應水資源缺乏、水源污染及 永續政策的推行，許多養殖業者開始有意願投入室內循環水養殖系統的投資，不過循環水系統為一封閉式之集約系統，一旦有病原進入容易造成整個系統的崩潰 (Zhao et al., 2023)。

1.4 鰻苗飼料現況

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傳統鰻魚養殖業者多以赤蟲 (搖蚊幼蟲) 或絲蚯蚓做為鰻苗開口與初期餌料，經過數週馴養再逐漸改成鰻粉或顆粒浮性飼料 (林, 2019)。由於絲蚯蚓生活於富含有機質的水域，常受到污染而帶有各種病原，極易感染整個養殖系統。若先以抗生素等化學藥劑消毒絲蚯蚓後，再將之投餵鰻苗，則可能導致鰻魚體內藥物殘留與養殖系統內細菌抗藥性等問題 (楊和劉, 2011)。且藥物殘留及累積問題於室內循環水系統也有被放大之風險 (Martins et al., 2011; Li, 2018)。日本在 1984 年開始嘗試研發膏狀飼料以取代赤蟲當成鰻苗的開口及初期餌料，至今已完全取代絲蚯蚓。膏狀飼料為將魚肉、蝦肉、魷魚、豬肝等主材料打成漿狀黏稠物，加入各種添加劑與黏著劑成型，其嗜口性佳、易消化吸收並且方便長期冷凍保存 (林等, 2017)，並能輕易與馴餌後期的鰻粉料或是浮性顆粒料銜接 (林等, 2018)。臺灣數年前亦有業者自日本引進鰻苗膏狀飼料 (日清丸紅，日本)，但售價過高 (2000 元/公斤)，並不符合養殖成本。因此，開發本土的鰻苗膏狀飼料有其必要性。臺灣水產試驗所亦曾開發鰻魚及鰻苗之人工膏狀飼料，其成本僅為日製飼料的 1/3 到 1/4 (林等, 2017; 黃等, 2018)。而鰻苗經過此飼料餵食後，魚體腸道所含總生菌數明顯低於餵絲蚯蚓及赤蟲者，尤其是檢測不出愛德華氏菌，反觀以傳統餌料餵食組所含愛德華氏菌則高達 3.9×10^5 CFU/g 且會使鰻魚腹部明顯充血，顯示以膏狀飼料馴餌更為安全衛生，又可提高各類鰻苗活存率與育成率 (楊等, 2010; 楊等, 2011; Lin et al., 2017)。

目前赤蟲／絲蚯蚓皆以人工野外捕撈為供貨渠道，時常缺貨更常有斷貨之情況，

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2023年時絲蚯蚓售價甚至從過往的每公斤 70 元，飆漲至每公斤超過 250 元，嚴重困擾養鰻業者。不過鰻苗若以鰻粉進行餵食，又因其高散失性，對室內循環水系統之物理過濾器造成過度負擔進而導致水質快速惡化，再者鰻粉對於異種鰻苗之開口效率很差，因此並不適合用於室內循環水系統中 (Aya and Garcia, 2022)。至於浮性顆粒飼料雖不易散失又耐儲藏，但因其製程需經過高溫膨發，在此製程中，飼料中添加的益生菌、維他命、酵素等有利鰻魚生長之添加物 (Lee, 2017; Liono et al., 2019)，會被高溫製程所破壞，且其粒徑更不適於鰻苗開口，只適合幼鰻 (200尾／公斤) 以後階段使用 (林, 2019)。反觀膏狀飼料全程皆在低溫下製作，其營養價值高、生菌數低，可同時兼具鰻粉的易添加性與浮料的低散失性，極適合做為鰻魚室內循環水養殖系統之開口與初期餌料，並可望提高鱸鰻幼魚的生長率及育成率 (林, 2019)。因此，要發展室內循環水養鰻系統，必須先開發合適之適口鰻苗人工膏狀飼料取代絲蚯蚓，是此養殖過程中重要的起始關鍵 (表一)。經本實驗預實驗實際測試日清丸紅與台製三種人工膏狀飼料，實際嗜口性、誘引性以及水中安定性皆不如預期，成長率無法與赤蟲／絲蚯蚓相比，顯示此膏狀飼料仍待進一步改良 (表二)。

1.5 疾病防治

集約式循環水養殖系統中如何控制、預防由細菌、真菌、病毒及寄生蟲所引發的感染性疾病，是目前養殖業要進一步發展的一大挑戰 (Aich et al., 2020)。其中

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愛德華氏菌 (*Edwardsiella tarda*) 已被視為集約化魚類養殖中的主要病原之一 (Katya et al., 2016)。其對鰻魚所引發的鰻魚愛德華氏病之典型病徵為肝臟和腎臟的腐爛性病變，於某些嚴重案例中甚至會造成鰻魚皮膚腐敗穿孔 (Kou, 1974)，並且對鰻魚具有高度的致死率、傳染性及發病率 (Kou, 1979)。愛德華氏菌最早於 1975 年夏天在台灣有爆發紀錄，也一直是造成世界養鰻業者損失的主要原因之一 (Michael and Abbott, 1993)。其喜好存在高溫或富含有機質的水域 (張, 2001)，且根據水產動物疾病診療系統 (TDS)顯示，此病在台灣全年皆有發生紀錄 (李, 2009)。而愛德華氏菌入侵養殖環境的最可能方式，為鰻苗馴餌時所普遍使用的赤蟲／絲蚯蚓帶入養殖系統潛伏於水體、魚體中增殖，待日後爆發 (Balcazar et al., 2006; 謝, 2014)。目前對鰻魚愛德華氏病尚未具有有效之預防及清除手段，僅能通過降低飼養密度及投藥等方式抑制疾病不爆發 (Roberts, 2012; 黃等, 2015)，而雖早已有愛德華氏病之疫苗問世，但因其售價高昂又成效不佳，故台灣亦尚未引進 (Mohanty et al., 2007)。近年來，雖然陸續還是有相關抗病疫苗之研究，不過因成本考量及技術限制，大多尚且僅使用於實驗室規模 (Su et al., 2023)。

傳統養殖業者通常使用抗生素去抑制病原菌，但由前人研究指出，因為過量用藥已致使許多病菌突變出具抗病性的菌株，導致抗生素的殺菌效率逐年降低 (Pelgrift et al., 2013)。而根據前人之研究亦發現從養殖池之吳郭魚分離出之細菌對許多抗生素如：四環素，紅黴素和鏈黴素皆顯示出抗藥性 (Tuševljak et al., 2013)。且藥物殘留議題也因近年食安問題越來越受重視，所以若是能捨棄傳統

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抗生素使用新式機能性添加劑提升養殖物種之抗病力及免疫力，則一方面可以提升生長表現，另一方面更可以提供消費者安心且對環境無害的水產養殖品。本研究經過多次預實驗後挑選出兩種具發展潛力之機能性添加劑，分別為草本碳化多銨以及納豆枯草桿菌 NTU-18。

1.6 機能性添加劑

碳量子點 (CQDS) 是一種新型的含碳奈米材料，其具有優異的抗菌功效、微小的尺寸、易於合成和低細胞毒性 (Alavi et al., 2021; Zhu et al., 2012)。CQDS 因其的抗菌活性特別在治療細菌感染方面有發展潛力 (Chou et al., 2021; Miller, 2017)。亞精胺 (spd) 是一種存在於核醣體和組織中的多胺。也被發現可以有效抑制許多細菌 (Kwon & Lu, 2007)。亞精胺碳量子點 (CQDSpds) 是 Li 等人使用亞精胺合成的碳量子點 (Li et al., 2016)。其抗菌機制是 CQDSpds 對細菌膜的固體崩解作用 (Meziani et al., 2016; Jian et al., 2020)。此外，透過毒性評估，CQDSpd 對斑馬魚有高度生物相容性 (Chung et al., 2021)。其中草本炭方 (Herbmedotcin™) 為國立臺灣海洋大學林翰佳教授以及黃志清教授所領導的研發團隊合成改良之一種 CQDSpds，目前已有當成禽畜及白蝦之飼料添加劑 (炬銨生技, 臺灣)。而在水產養殖應用中，感染 WSSV (白點症病毒)的白蝦經過該 CQDSpds 處理後，可以顯著降低死亡率並增強免疫反應 (Huang et al., 2020)。不過碳化多銨對鰻魚養殖之成效如何尚未有過任何研究。

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納豆枯草芽孢桿菌從近 120 年前從發酵大豆食品中成功分離出 (Hosoi and Kiuchi, 2003)。納豆枯草桿菌已被證實無抗病性和細胞毒性 (Xu et al., 2012)。其中特別值得注意的是，納豆枯草桿菌可以承受水產飼料加工製程和生物腸道中可能遭遇之惡劣環境 (Barbosa et al., 2005; Guo et al., 2006; Shivaramaiah et al., 2011)。納豆枯草桿菌不僅透過競爭性排斥促進腸道健康，而且還產生對細菌病原體具有細胞毒性的抗菌肽 (Knap, et al., 2011；Sumi et al., 2015)。此外，納豆枯草桿菌也表現出抗發炎能力，並已被證實可作為人類、草魚和小鼠的藥物替代品和生長促進劑 (Hosoi et al., 1999; Hitosugi et al., 2015; Li et al., 2017)。此外，在先前的研究中，枯草桿菌納豆 NTU-18 表現出良好的 β-葡萄糖苷酶活性和異黃酮去糖基化效率(Kuo et al., 2012)。不過儘管納豆枯草桿菌和 CQDSpds 在水產養殖業中已被廣泛使用，但在循環水鰻魚養殖系統中評估這些機能性添加劑對生長性能、免疫反應和腸胃道影響的潛在影響仍待研究。

1.7 本研究目標

綜合以上所述，若能研發出對鰻苗具有高度適口性、高成長率且可以穩定低價大量生產之膏狀飼料，將有機會幫助重振臺灣之鰻魚養殖產業。更進一步也可以在高密度飼養之超集約循環水系統中，保障飼養鰻魚的安全以降低養殖風險。故本論文之重點在於開發高抗病 (*E. tarda*)、高成長之高品質鰻魚膏狀飼料，以及

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嘗試不同機能性添加劑，期待找出最適當之膏狀飼料配方，以提高各類鰻魚養殖初期成長率與育成率，並建立商業化標準製程，以供產業推廣之用。

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2. 材料與方法

2.1 膏狀飼料配方設計

2.1.1 膏狀飼料原料及製程

鰻苗膏狀飼料之配方，首先參考水試所配方 (林, 2019) 及實際鏡檢分析日本膏狀飼料（日清丸紅，日本）後，先後選擇以各式新鮮魚漿 (旗魚、鯊魚、金線魚、鬼頭刀等)、蝦漿 (赤尾青、糠蝦、南極蝦等) 及頭足類漿（美洲大赤魷、南魷等）作為基礎原料。製作前，所有原料皆先萃取 DNA 進行鰻魚病原菌檢測，確認皆無檢出後方能採用 (表三)。原料經過清洗後，進行打漿及去筋，增加水中穩定性及適口性。為提升膏狀飼料對鰻苗之適口性，漿料去筋後會送入擂潰機中進行擂潰攪拌，並在此步驟中添加各式必須添加劑 (關華豆膠及乳化劑等添加劑來提昇其誘引性與水中安定性)，全部製程皆維持在 4℃下。飼料成品隨後分裝並且真空冷凍於-20℃冷凍櫃保存。此成品經以下流程圖測試，其中因為每年鱸鰻苗有春夏兩波來游族群，苗量大且價格便宜容易取得，故每種不同配方之膏狀飼料預實驗皆先用鱸鰻苗來進行測試 (圖一)。本研究中不同之試作品配方提供於文末補充資料中。本研究研發之膏狀飼料目前已能完美搭配室內循環水養殖之所需。本研究中鰻苗飼養主要共可分成三個實驗，分別為: (1) 膏狀飼料與絲蚯蚓及鰻粉飼料之評比；(2) 碳化多銨機能性添加劑試驗；以及 (3) 納豆枯草桿菌 NTU-18 添加劑試驗。

2.1.2 飼料水中穩定性試驗

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為了確保膏狀飼料成品不會對室內循環水養殖系統造成污染，每種配方之膏狀飼料試作品皆會進行水中穩定性測試來評估其是否可能對物理過濾器造成負擔。水中穩定性表示方式為測試各飼料之散失率，散失率越高者代表其水中穩定性越差。試驗方法參考 Marchese 等人於 2019 對錦繡龍蝦 (*Panulirus ornatus*)膏狀飼料之水中穩定性實驗，將 50g 膏狀飼料成品置於 1000ml 裝滿養殖水並全程打氣之燒杯中，6 小時後對其進行秤重分析，並以下列公式計算得出散失率。

$$\text{散失率}(\%) = \frac{\text{6 小時後剩餘重量}(g)}{\text{膏狀飼料初始重量}(50g)} \times 100$$

2.2 實際鰻苗養殖測試

2.2.1 膏狀飼料比較分析

本實驗使用之鰻苗種類為黑鰻 (*Anguilla bicolor pacifica*)。黑鰻苗購入自花蓮漁民，捕撈地則為秀姑巒溪出海口 (23.461489°N, 121.500142°E)。鰻苗在捕撈後馬上灌氧包裝並保持18℃低溫送至國立臺灣大學漁業科學研究所育苗室。鰻苗抵達後，馬上以 2.5ppm 之過錳酸鉀 ($KMnO_4$) 溶液浸泡消毒 15 分鐘且挑出混雜其中的鱸鰻苗，並隨後移入 FRP 桶中進行為期一週之淡化。淡化過程中不進行餵食，以每天鹽度降 5‰的幅度淡化，並於淡化至鹽度 0 後進行初始體長體重量測並分缸開始實驗。實驗用之循環水系統為 40L 之魚缸共 10 缸，一缸放養 25 隻鰻魚，並且以循環水系統飼養 (週換水率 <5%)，維持水溫在 26-28℃，pH 於 6.5-7.0，溶氧 4.5-6.0 ppm，氨氮維持 0-0.1 ppm。每天餵食飼料 (赤蟲、膏狀飼料及

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鰻粉)兩次 (早晚各一次，中間間隔 8 小時)，其中鰻粉會依廠商 (福壽牌，臺灣) 建議添加 50%的水以 30RPM 速率攪拌 5 分鐘呈現煉餌狀投餵，進行三重複實驗。每週餵食 6 天，實驗為期 8 週，每次餵食量為缸內魚體總濕重的 5%。光週期為自然光週期。每兩週會定期進行全部鰻魚體長體重之量測，除分析成長表現外，亦能用來調整餵食份量。

2.2.2 碳化多銨 (CQDSpds) 添加劑

本實驗使用臺灣產量最豐富之日本鰻苗及鱸鰻苗來測試膏狀飼料及 CQDSpds 養殖成效。實驗日本鰻苗從宜蘭烏石港 (24.8691°N 121.8406°E) 向漁民直接收購，鱸鰻苗購入自花蓮漁民，捕撈地則為秀姑巒溪出海口 (23.461489 °N, 121.500142 °E)。鰻苗在捕撈後馬上灌氧包裝並保持 18℃ 低溫送至國立臺灣大學漁業科學研究所育苗室。鰻苗抵達後，馬上以 2.5ppm 之過錳酸鉀 ($KMnO_4$) 溶液浸泡消毒 15 分鐘，並隨後移入 FRP 桶中進行為期一週之淡化。淡化過程中不進行餵食，以每天鹽度降 5 ‰的幅度淡化，並於淡化至鹽度 0 後進行初始體長體重量測並分缸開始實驗。實驗用之循環水系統為 40L 之魚缸共 10 缸，一缸放養 25 隻鰻苗，並且以室內循環水系統飼養 (週換水率 <5%)，維持水溫在 26-28℃，pH 於 6.5-7.0，溶氧 4.5-6.0 ppm，氨氮維持 0-0.1 ppm。每天餵食膏狀飼料兩次 (早晚各一次，中間間隔 8 小時)，進行三重複實驗。每週餵食 6 天，日本鰻飼養實驗為期 8 週，鱸鰻則因其生長速度較緩慢故延長至 12 週。每次餵食量為缸內魚體總

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濕重的 5%。光週期為自然光週期。每兩週會定期進行全部鰻魚體長體重之量測，除分析成長表現外，亦能用來調整餵食份量。

2.2.3 納豆枯草桿菌 NTU-18 添加劑實驗

本實驗使用日本鰻苗來測試膏狀飼料及納豆枯草桿菌 NTU-18 對日本鰻之養殖成效及腸道菌相影響。實驗使用之日本鰻苗從宜蘭烏石港 (24.8691°N 121.8406°E) 向漁民直接收購。鰻苗在捕撈後馬上灌氧包裝並送至國立臺灣大學漁業科學研究所育苗室。鰻苗抵達後，馬上以 3ppm 之過錳酸鉀 ($KMnO_4$) 溶液浸泡消毒 5 分鐘，並隨後移入 FRP 桶中進行為期一週之淡化。淡化過程中不進行餵食，以每天鹽度降 5‰的幅度淡化，並於淡化至鹽度 0 後進行初始體長體重量測並分缸開始實驗。實驗用之循環水系統為 40L 之魚缸共 10 缸，一缸放養 20 隻鰻苗，並且以室內循環水系統飼養 (週換水率<5%)，維持水溫在 26-28℃，pH 於 6.5-7.0，溶氧 4.5-6.0 ppm，氨氮維持 0-0.1 ppm。每天餵食膏狀飼料兩次 (早晚各一次，中間間隔 8 小時)，進行四重複實驗。每週餵食 6 天，為期 10 週，每次餵食量為缸內魚體總濕重的 5%。光週期為自然光週期。每兩週會定期進行全部鰻魚體長體重之量測，除分析成長表現外，亦能用來調整餵食份量。納豆枯草芽孢桿菌 NTU-18（BCRC 80390）是從發酵納豆中分離出來的，並使用 LB 液態培養基 (Neogen, USA)，並按照標準方法進行培養（Kuo et al., 2012），最終添加菌液的濃度統一為 1×10^7 CFU/g。

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2.3 採樣與計算

於飼養週期結束後，先將所有鰻苗禁食 24 小時，隨後以精秤及電子游標卡尺測量計算每缸鰻苗數量及體長體重。並計算體重增加率、飼料換肉率、飼料效率、每日成長率、存活率及肥滿度 (K)。並從每缸中取出三尾魚使用乙二醇丙醚麻醉後斷頭犧牲解剖，並取其腸胃道進行組織切片；再從每缸剩餘之魚中取三尾麻醉犧牲，解剖取其頭腎及肝臟進行非專一性免疫實驗。最後再從每缸中取出三尾鰻苗，麻醉解剖後取新鮮之腸道內容物進行腸道菌相分析。

$$\text{Percentage weight gain (PWG, \%)} = \frac{\text{Final body weight (g)} - \text{Initial body weight (g)}}{\text{Initial body weight}} \times 100$$

$$\text{Feed conversion rate (FCR)} = \frac{\text{Total feed intake (g)}}{\text{Final body weight (g)} - \text{Initial body weight (g)}}$$

$$\text{Feeding efficiency ratio (FE, \%)} = \frac{\text{Final body weight (g)} - \text{Initial body weight (g)}}{\text{Total feed intake (g)}} \times 100$$

$$\text{Specific growth rate (SGR, \%/ day)} = \frac{\ln(\text{Final body weight}) - \ln(\text{Initial body weight})}{\text{rearing day}} \times 100$$

$$\text{Survival rate (SR, \%)} = \frac{\text{Final n}}{\text{Initial n}} \times 100$$

$$\text{Condition factor (K)} = \frac{\text{Body weight (g)}}{\text{Body length}^3\ \text{(cm)}} \times 1000$$

2.4 組織切片

每組隨機取樣三尾鰻苗麻醉後犧牲解剖取其腸胃道組織，隨即用 10%中性福馬林溶液浸泡固定至少 72 小時後修剪成適當大小。接下來經由酒精序列脫水後以石蠟包埋進行封片，隨後以 5μm 之厚度進行切片，染色方法使用用蘇木精及伊紅染色 (Hematoxylin-Eosin method) 並以封片膠封片後保存。腸胃道組織切片完成後經由光學顯微鏡（X200）觀察腸道型態是否完整，以及有無發炎潰爛、空

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洞化等狀態。最後透過 SPOT 5.0 (Netherlands) 軟體計算絨毛周長比 (villus circumference ratio, 公式如下)、腸道絨毛高度 (從腸壁突出的小指狀突起) 及腸胃道肌肉層厚度。

$$\text{villus circumference ratio (\%)} = \frac{\text{The highest villi length (μm)}}{\text{The circumference of villi (μm)}} \times 100$$

2.5 非專一性免疫實驗 (qPCR)

每缸取三隻鰻苗麻醉後取其頭腎 (全部鰻種) 及肝臟 (日本鰻) 進行免疫基因表現量測定。非專一性免疫基因表現量使用即時聚合酶連鎖反應 (qPCR) 進行分析，每組三至四重複。

首先 RNA 提取及 cDNA 合成之具體步驟為下：

(1) 將頭腎或肝臟組織樣本加入 800μl 的 TRIzol 試劑，用研磨棒將頭腎研磨均質化。均質化後置於室溫 (27℃)下作用 5 分鐘。

(2) 加入 Chloroform 160μL ，在 4℃下以 13,000 rpm 離心 15 分鐘。

(3) 離心後吸取最上層之液體到 1.5 mL 微量離心管中，加入 400 μL isopropyl alcohol 混合。在 4℃下以 12,000 rpm 離心 10 分鐘。

(4) 倒掉微量離心管內的液體留下底部固形物。加入 1ml 75% 酒精混合。在 4℃下以 13000 rpm 離心 5 分鐘。

(5) 倒除液體後，風乾 pellet 至半透明狀，加入 DEPC water，利用 pipette 將 pellet 混合保存。

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(6) 通過 Nano-300 分光光度計定量檢測提取的總 RNA 之 OD260nm/ OD280nm 品質和數量，根據數量進行權重配液，使各管間的 RNA 含量大致相同。

(7) 採用 PrimeScript RT 試劑盒 (Perfect Real Time, Japan) 將提取到的總 RNA 進行反轉錄合成 cDNA。

qPCR 部分，選擇 SOD、POD、LZM、IL-6 作為 qPCR 的目標基因，以 ARP 為參考基因，qPCR 所選基因和引物序列如下表所示 (表五)。在 Bio-Rad MyIQ real-time PCR 系統 (Bio-Rad, USA) 上進行 qPCR 以確定基因表達水準。各管 qPCR 反應混合物為 25μl ［12.5μl 為 2x SYBR green 混合物 (Bionova, USA)、1.0μl 正向引子、1.0 μl 反向引子、1μl 10 倍稀釋之 cDNA 樣本、9.5μl DEPC 水］。qPCR 反應條件為下：

(1) 預培養： 95°C (30 s)

(2) 擴增 40 個循環：95°C (5 s)→ 58°C (30 s)→ 72°C (30 s)

(3) 每組樣本進行 3 重複

(4) 對所有 qPCR 數據進行方差均勻性檢驗

(5) 檢驗後先將目標基因的 CT 值與 ARP 的 CT 相減，其數據為 ΔCT 值。

(6) 再將各個組別 ΔCT 值與控制組之 ΔCT 值相減，得到 ΔΔCT 值。各個基因相對於 ARP 之表現量用 $2^{-\Delta\Delta CT}$ 法求得 (Livak, 2001)

2.6 腸道菌相分析

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每缸中各取三隻鰻苗麻醉後，取其新鮮之腸道內容物進行腸道菌項分析，用以評估膏狀飼料及機能性添加劑對各類鰻苗腸道菌相的影響。使用 QIAamp DNA Micro 試劑盒 (Qiagen, Germany) 萃取胃腸道細菌 16S ribosomal DNA 的 V3-V4 區域，並使用 KAPA HiFi HotStart PCR 試劑盒 (Roche, Sweden) 以 PCR 擴增。為確保定序結果品質，最終 PCR 產物皆會使用 MultiQC 系統進行品質檢測（Ewels et al., 2016）。 MultiQC 之後，PCR 產物在 Illumina MiSeq 平台（Illumina, USA）上進行雙端定序（2 × 250 bp）。隨後，對序列資料進行多路分解並進行品質過濾。最後，透過 EasyMAP 進行 α 和 β 多樣性分析，包括主座標分析（PCoA）和線性判別分析效應大小（LEfSe）分析和所有樣本的觀測特徵、Chao1、Shannon 和 Simpson 多樣性指數，然後進行 alpha 和 beta 多樣性分析 (Hung et al., 2021; Kers and Saccenti, 2022)。

2.7 攻毒實驗

在所有樣本 (腸胃道、頭腎、肝臟及腸道菌相) 取樣都結束後，每缸剩下的鰻苗中各挑選出 10 隻平均大小 (體長體重皆無顯著差異) 之鰻苗浸泡於 500 ml 的愛德華氏菌 (*E. tarda*) 液 (濃度: 2×10^8CFU) 共 2 小時，且浸泡全程維持強打氣 (Keiichi et al., 1984)。本實驗使用之愛德華氏菌株來自臺灣大學獸醫學系陳嫩玫老師實驗室，本株菌種是由嘉義縣養殖池中受感染之日本鰻分離純化而來，並且在實驗室內使用表六配方之 Tryptic Soy Broth (TSB) (Neogen, USA))液態培養基進行擴培 (Ishihara et al., 1981)。培養條件為定溫 30℃，使用 250 RPM 轉速搖

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晃 48 小時，培養完成後進行序列稀釋並使用羊血培養基塗盤計算菌液濃度。鰻苗浸泡攻毒完成後，移回原循環水缸中並維持相同養殖條件 (水溫、溶氧等)，不過在攻毒實驗期間完全停止餵食。實驗為期 15-20 天，期間每天早晚紀錄各缸鰻苗之死亡隻數。每隻死亡的鰻苗皆會取樣其肝及鰓組織來萃取 DNA 並以 PCR 方法確定死亡鰻苗是否有感染愛德華氏菌，並於實驗結束後計算各缸存活率。

2.8 統計分析

使用 SPSS 20 for Mac (SPSS Inc., USA) 進行統計分析。所有數據結果均表示為平均值±標準差 (SD)。用 one-way ANOVA 進行單因數變方分析，若有顯著差異 ($p < 0.05$)，則使用 Tukey-HSD 多範圍檢測進行組間檢定，檢驗組間平均值是否具有顯著差異 ($p < 0.05$)。在愛德華氏菌攻毒實驗中，則使用 Mantel- Cox 檢驗比較各組的累積存活率，並採用 $p < 0.05$ 的顯著水準。

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3. 結果

3.1 膏狀飼料配方設計

3.1.1 膏狀飼料配方及製程

本實驗先後嘗試過多種不同配方，經過實際測試發現由美洲大赤魷 (*Dosidicus gigas*) 為主材料搭配魚卵當成次要原料之配方無論色澤、質地、構造、誘食性及適口性等皆最好 (表七)。其中不同配方之嘗試結果均列在補充資料中。其中在飼料形態上，本實驗研發之膏狀飼料具備和日本製膏狀飼料一樣的層疊狀結構，更加利於口器小的鰻苗進食，更進一步提升整體鰻苗養殖表現。而其餘膏狀飼料配方冷凍切割後皆為塊狀結塊，較不易使鰻苗進食。

製程方面，首先先將美洲大赤魷清肉切成 3*3*3 cm^3 之小塊，並在此過程中挑出未清除乾淨之外套膜；魚卵部分則是使用湯匙刮除卵膜，以避免筋膜卡住擂潰機軸心造成馬達過熱導致飼料在擂潰過程中變質。將上述原料分別使用 1 馬力之工業用果汁機打成漿狀，再經過筋濾機進行去筋保證飼料成品質地細緻度，避免餵食後造成循環水物理過濾器之堵塞以及影響鰻苗適口性。去筋後之漿料隨即移入擂潰機中以 50 RPM 轉速進行擂潰，並於此時加入原料總重量 7.5%之食鹽水溶液 (5%食鹽水)，一邊擂潰過程中，緩慢均勻加入關華豆膠、礦物質、維他命及機能性添加劑等添加物。擂潰過程全程以水冷法保持飼料在 4℃左右以確保飼料不會變質，並於擂潰 1 小時後將飼料成品分裝後真空冷凍保存，待日後餵食試驗時使用。製程如圖二，其餘嘗試過之配方則列於附錄補充資料中。

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3.1.2 水中穩定性

經由水中穩定性實驗，可以比較本研究研發之膏狀飼料和市售鰻粉 (福壽牌, 臺灣)、日本膏狀飼料 (日清丸紅, 日本) 及水試所膏狀飼料於水中之穩定性。從水中散失率結果中可以發現，本研究自製之膏狀飼料和水試所及日本膏狀飼料散失率無顯著差異 ($p > 0.05$) 且所有膏狀飼料皆顯著低於鰻粉組 ($p < 0.05$) (表八)。由本實驗結果中可以發現膏狀飼料之水中穩定性較傳統鰻粉好，此特性在實際養殖中亦發現和室內循環水系統搭配性良好，不易造成堵塞及水質污染，並且可以簡單觀察鰻魚進食情況以及撈除殘餌。其餘嘗試過之不同膏狀飼料配方之散失率亦列於補充資料中。

3.2 鰻苗生長表現

3.2.1 膏狀飼料比較分析

本實驗之膏狀飼料配方同表七，冷凍赤蟲為購入自屏東鹽埔鄉之採集者，鰻粉則為福壽牌之幼鰻粉。在實驗開始之前，所有組別之黑鰻苗的 IBW 並無顯著差異 ($p > 0.05$)。經過 8 週飼養實驗之成長表現呈現於表九。與鰻粉組相比，膏狀飼料及赤蟲組之黑鰻苗表現出較佳的 FBW、PWG、SGR、FE 和 FCR ($p < 0.05$)。存活率方面以膏狀飼料顯著高於其餘兩組，而赤蟲組之存活率則顯著低於膏狀飼料及鰻粉組 ($p > 0.05$)。在 K 值方面，膏狀飼料及赤蟲之肥滿度皆顯著高於鰻粉組 ($p > 0.05$) (表九)。

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3.2.2 碳化多銨 (CQDSpds)添加實驗

表十為碳化多銨 (CQDSpds) 實驗中膏狀飼料的配方比例。表十一為鱸鰻苗之生長表現，本組鱸鰻苗之初始體重並無顯著差異 ($p > 0.05$)，不過在 12 週之養殖實驗結束後 0.5 ppm 組別之鱸鰻在最終體重 (FBW)、體重增加率 (PWG)、每日生長率 (SGR)、飼料效率 (FE) 及飼料換肉率 (FCR) 上皆有顯著較高之表現 ($p < 0.05$)。此外，各組鱸鰻苗的肥滿度 (K) 和存活率並無顯著差異 ($p > 0.05$)。

表十二則為日本鰻苗之生長表現，一樣初始體重並無顯著差異 ($p > 0.05$)。在經過 8 週的養殖實驗結束後，1 ppm 組的 FBW 顯著高於控制組、0.25 ppm 和 2 ppm 組 ($p < 0.05$)，但與 0.5 ppm 組沒有顯著差異 ($p > 0.05$)。0.5 ppm 和 1 ppm 組的 PWG、SGR 和 FCR 在控制組、0.25 ppm 和 2 ppm 組中表現出顯著差異 ($p < 0.05$)，但與 0.5 ppm 組沒有顯著差異 ($p > 0.05$)。此外，各組日本鰻苗的 K 值和存活率並沒有顯著差異 ($p > 0.05$)。

3.2.3 納豆枯草桿菌 NTU-18 添加劑

表十三為納豆枯草桿菌 NTU-18 實驗中膏狀飼料的配方比例。在實驗開始時，所有組別的日本鰻苗 IBW 和初始體長 (TL) 並沒有顯著差異 ($p > 0.05$) (表十四)。經過 10 週的餵食試驗後，與其他組別相比，0.5%和 1%組之日本鰻苗表現出顯著最高的 FBW、PWG、SGR、FE 和 FCR ($p < 0.05$)。此外，所有枯草桿菌納豆 NTU-18 添加組的生長表現均顯著高於控制組 ($p < 0.05$)。而各組日本鰻苗間的 K

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值和存活率均無顯著差異 ($p > 0.05$) (表十四)。

3.3 組織切片

3.3.1 膏狀飼料比較分析

餵食不同飼料的黑鰻苗經過 10 週試驗後，腸道形態出現顯著差異，其中餵食膏狀飼料之腸道柱狀體完整、並且絨毛無空泡化或發炎現象，腸壁肌肉亦無增厚。餵食赤蟲之黑鰻苗腸壁可以明顯看出柱狀體發炎、破損及潰爛，腸壁肌肉層亦有增厚現象；餵食鰻粉之黑鰻苗腸道柱狀體雖完整，不過有空泡化和萎縮現象，腸壁肌肉層亦顯著厚於膏狀飼料組 (圖三)。其中餵食膏狀飼料之黑鰻苗絨毛長度顯著高於餵食赤蟲及鰻粉組別 ($p < 0.05$)，且膏狀飼料組別中的腸壁肌肉層厚度亦顯著低於赤蟲及鰻粉組 ($p < 0.05$) (表十五)。

3.3.2 碳化多銨 (CQDSpds)添加實驗

在碳化多銨之實驗中，鱸鰻苗和日本鰻苗之腸胃道在不同濃度之碳化多銨處理下皆無觀察到受損情形 (圖四、圖五)，並且測量腸絨毛高度及絨毛周長比亦無顯著差異 ($p > 0.05$) (表十六、表十七)。添加 CQDSpds 之所有組別中的所有腸道樣本均未觀察到絨毛狀況異常 (褶皺斷裂、擴張、絨毛矮化) 等情形。

3.3.3 納豆枯草桿菌 NTU-18 添加實驗

以不同濃度的納豆枯草桿菌 NTU-18 添加之膏狀飼料餵食日本鰻苗 10 週後，

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腸道形態也沒有顯著差異 (圖六)，且各組間絨毛平均長度和肌肉層厚度無顯著差異 (p> 0.05) (表十八)。

3.4 免疫分析

3.4.1 膏狀飼料比較分析

圖七顯示了黑鰻苗肝臟中免疫相關基因的表現量。結果顯示，膏狀飼料組之 SOD 和 POD 表現量顯著高於其他兩組（p< 0.05），而赤蟲組和鰻粉組之間差異無顯著差異（p> 0.05）。至於 HSP70，在赤蟲組中的表現量顯著高於膏狀飼料組和鰻粉組 (p< 0.05)，但膏狀飼料組和鰻粉組之間並無差異 (p> 0.05)。然而，在 IL-6 結果中，膏狀飼料組的表達量在各組中顯著最低 (p< 0.05)，而赤蟲組和鰻粉組間則沒有顯著差異 (p> 0.05)。餵食不同飼料組別之間的 LZM 表現量則都無顯著差異（p> 0.05）（圖七）。

3.4.2 碳化多銨 (CQDSpds) 添加實驗

在碳化多銨 (CQDSpds) 之實驗中，鱸鰻苗於 12 週之實驗結束後，四種免疫表現相關基因 (SOD、POD、LZM、IL-6) 之 qPCR 結果如圖八。在 SOD 表現量中，0.5 ppm 之組別顯著低於 2 ppm 之組別 (p< 0.05)；POD 之表現量中 1 ppm 及 2 ppm 組別顯著低於控制組及 0.5 ppm (p< 0.05)；LZM 之表現量 0.5 ppm 及 1 ppm 組別顯著高於控制組 (p< 0.05)，不過跟 0.25 ppm 和 2 ppm 無顯著差異 (p> 0.05)；IL-6 之表現量則呈現控制組及 0.25 ppm 顯著高於 0.5 ppm、1 ppm 及 2 ppm 組 (p< 0.05) (圖八)。

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而日本鰻苗再經過 8 週之飼養實驗後，其免疫相關基因之 qPCR 結果如圖九。在 SOD 表現量中，1 ppm 之組別顯著低於控制組 ($p< 0.05$)；POD 之表現量中 0.5 ppm 及 1 ppm 組別顯著低於控制組 ($p< 0.05$)；LZM 之表現量 1 ppm 組別顯著高於控制組及 2 ppm ($p< 0.05$)；IL-6 之表現量則呈現控制組及 0.25 ppm 顯著高於 0.5 ppm 及 2 ppm 組 ($p< 0.05$) (圖九)。

3.4.3 納豆枯草桿菌 NTU-18 添加實驗

以不同濃度的納豆枯草桿菌 NTU-18 添加之膏狀飼料餵食日本鰻苗 10 週後，肝臟中免疫相關基因的表達如圖十所示、頭腎中之免疫相關基因的表達如圖十一所示。其中不管在肝臟或頭腎，所有高於 0.25%添加組均表現出比控制組顯著更高的 IgM 表現量 ($p< 0.05$)。然而在肝臟中，各實驗組間的 SOD、CAT、POD 和 HSP90 表現量並沒有顯著差異 ($p> 0.05$) (圖十)。另外，當膏狀飼料中枯草桿菌納豆 NTU-18 濃度高於 0.5%時，肝臟及頭腎中 HSP70 的表現量也顯著高於控制組 ($p< 0.05$)，且頭腎中 CAT、POD、HSP90 各組間無顯著差異 ($p< 0.05$)。不過僅頭腎中之 SOD 表現量在 0.5%組中和其他組間有顯著差異 ($p< 0.05$) (圖十一)。

3.5 攻毒實驗

3.5.1 膏狀飼料比較分析

餵食不同飼料之黑鰻苗在 15 天攻毒實驗結束後的存活率如圖十二。其中餵食膏狀飼料之黑鰻苗存活率 (SR: 60.58%)顯著高於餵食赤蟲 (13.28%) 及鰻粉組

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(SR: 35.44%) ($p < 0.05$)；又餵食鰻粉之黑鰻苗存活率亦顯著高於赤蟲組 ($p < 0.05$) (圖十二)。

3.5.2 碳化多銨 (CQDSpds) 添加實驗

對食不同添加量碳化多銨 (CQDSpds) 之鱸鰻苗經過 20 天愛德華氏菌攻毒實驗之存活率結果顯示於圖十三。攻毒結果顯示 0.5 ppm 及 1.0 ppm 組別之累積存活率顯著高於控制組及 0.25 ppm ($p < 0.05$) (圖十三)。餵食不同添加量 CQDSpds 之日本鰻苗愛德華氏菌攻毒實驗之生存率結果則顯示於圖十四。所有餵食添加 CQDSpds 膏狀飼料之組別存活率皆顯著高於控制組 ($p < 0.05$)；且添加組中又以 1.0 ppm 組別中之存活率顯著高於 0.25 ppm、0.5 ppm 及 2.0 ppm ($p < 0.05$) (圖十四)。

3.5.3 納豆枯草桿菌 NTU-18 添加實驗

餵食納豆枯草桿菌 NTU-18 之日本鰻苗在 20 天攻毒實驗結束後的存活率如圖十五。其中餵食含有 0.5% (SR : 59.5%) 和 1% (SR : 52.3%) 納豆芽孢桿菌 NTU-18 添加之膏狀飼料鰻苗存活率顯著高於控制組 (SR: 19.7 %) 和 2 % (SR: 23.4 %) 組 ($p < 0.05$) (圖十五)。

3.6 腸道菌相

3.6.1 膏狀飼料比較分析

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α 多樣性的結果如圖十六所示。餵食赤蟲及鰻粉組別之黑鰻苗腸道菌相的 Simpson、Shannon 及 Chao1 指數顯著高於餵食膏狀飼料者 ($p< 0.05$) (圖十六)。圖十七表示了黑鰻苗腸道菌在屬層級上的相對豐度，其中膏狀飼料及鰻粉組別之腸道菌相組成較為相近，而赤蟲組之黑鰻腸道內發現相當高比例之鰻魚病原菌產氣單胞菌屬 (*Aeromonas*) 菌種 (圖十七)。

β 多樣性分析中，主座標分析 (PCoA) 結果表明，赤蟲組與其他組別 (膏狀飼料、鰻粉組) 間存在顯著差異，且自成一群 (圖十八)；且加權 UniFrac Heat map 亦顯示了赤蟲組與其他組別 (膏狀飼料、鰻粉組) 間存在顯著差異 (圖十九)。

3.6.2 納豆枯草桿菌 NTU-18 添加實驗

α 多樣性的結果如圖二十所示。控制組、0.25%、0.5%和 1%添加組日本鰻苗的腸道菌相 Simpson 和 Shannon 指數顯著高於 2%組 ($p< 0.05$) ；而 Chao1 和 Observed features 則是以 0.5%組最高 (圖二十)。圖二十一表示了日本鰻苗腸道菌在屬層級上的相對豐度。除 2%組外，其他組別 (控制組，0.25%、0.5%和 1%) 的腸道菌相組成比例相近；而在 2%添加組中則發現鯨桿菌屬 (*Cetobacterium*) 的比例要高得多 (圖二十一)。

β 多樣性分析中，主座標分析 (PCoA) 結果表明，2%組與其他組別 (控制組、0.25%、0.5%和 1%) 之間存在顯著差異 (圖二十二)；且不管加權或未加權 UniFrac Heat map 亦顯示了 2% 組與其他組別 (控制組、0.25%、0.5% 和 1%) 間存在顯著差異 (圖二十三、圖二十四)。

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4. 討論

4.1 膏狀飼料比較分析

4.1.1 成長表現

本研究為首次針對不同開口飼料對黑鰻苗的影響評估。在投餵三種不同開口飼料的黑鰻苗組別中，膏狀飼料組和赤蟲組的生長表現並沒有顯著差異，且均顯著優於鰻粉組（表九）。這項結果與先前的研究一致，顯示鰻苗的口器仍未發育完全，難以攝取粉末飼料，從而影響生長性能（Degani, 1986; Heinsbroek, 1991; McKinnon et al., 2002）。本研究發現，在三種開口飼料中，鰻粉的誘食性最差，這一結果亦和有關人工飼料和天然飼料對歐洲鰻鰻苗（*A. anguilla*）誘食性的研究結果一致，研究發現赤蟲和魚卵等成分對玻璃鰻的誘食性和適口性最高（Kamstra and Heinsbroek, 1991; Knights, 1996）。前人研究表明，具有高誘食性和適口性的開口飼料可以提高鰻苗的生長性能和新陳代謝表現（Heinsbroek and Kreuger, 1992）。相反的，不被鰻苗所嗜食的開口飼料則會增加鰻苗對疾病的易感性，導致死亡率升高（Ingram et al., 2001）。在三種飼料餵食的黑鰻苗中，膏狀飼料和赤蟲組之成長表現並無顯著差異，且皆顯著較鰻粉組來得高 (表九)。這和前人研究中鰻粉製成之煉餌型態，因鰻苗之口器發育尚未發達，較難以開口及攝食 (林, 2019)。

黑鰻苗對鰻粉之誘食性相較膏狀飼料及赤蟲明顯低落許多，可能原因為鰻粉原料為魚粉及澱粉等，相比使用新鮮原料的膏狀飼料而言對鰻苗之吸引性低上許多

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(林, 2019)。反觀膏狀飼料的誘食性和鰻苗成長表現與赤蟲相當，這可能是由於添加了魚卵和南極蝦（De Silva et al., 2001; Rodríguez et al., 2005）。而赤蟲之成長表現雖和膏狀飼料組無顯著差異，不過赤蟲組之存活率顯著低於膏狀飼料及鰻粉組，這可能是因為赤蟲本身採集自富含有機質之水域，極為容易攜帶病原菌致使鰻苗感染 (Xu and Zhang, 2014)。且前人對不同種類鰻苗的研究也表明，與投餵人工飼料的玻璃鰻相比，投餵赤蟲的鰻苗更容易爆發疾病（Dainys et al., 2017; Hsu et al., 2018; Kusumawatyet al., 2023）。此外，適口性不佳的飼料殘餌亦會迅速增加 RAS 中的氨氮濃度，並可能導致鰻苗生長遲緩甚至死亡（Sadler, 1981; Tseng and Wu, 2004）。

黑鰻苗的腸道組織和形態在膏狀飼料組和赤蟲組及鰻粉組之間存在顯著差異（表十五; 圖三）。在膏狀飼料組中，絨毛和肌肉層均處於健康狀態，這與先前對印尼雙色鰻苗消化道形態之研究結果一致（Nasruddin et al., 2014）。然而，在赤蟲組中，絨毛表現出明顯的斷裂皺褶和發炎、潰爛表現，這很可能是由胃腸道中的產氣單胞菌等病原菌所引起的（圖三；圖十七）。前人研究中，感染了產氣單胞菌的歐洲鰻和草魚腸道中也有觀察到類似症狀（Chen et al., 2020; Xiong et al., 2020）。在鰻粉組，雖然腸道型態相對完整，但絨毛有萎縮和空洞化的現象（圖三）。這可能是由於鰻粉對黑鰻苗之適口性不佳，導致鰻苗營養不良所致（McKinnon et al., 2002 年）。此外，赤蟲組和鰻粉組的腸道肌肉層厚度顯著厚於膏狀飼料組，這可能表明莖氣單胞菌引起了發炎或飼料不合適導致了鰻苗營養不

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良所致（Song et al., 2014; Božić et al., 2021）。相較之下，本研究中新開發之膏狀飼料對太平洋雙色鰻苗顯示出絕佳的適口性，且在腸道中未發現有引入致病性疾病（圖十七），顯示其作為鰻苗開口飼料的巨大潛力。且根據成本效益分析，目前赤蟲之售價 1 公斤約 250-280 元，本膏狀飼料每公斤成本約僅 150 元，顯見本膏狀飼料之發展優勢。

4.1.2 鰻苗免疫表現

熱休克蛋白（HSPs）通常被用作養殖魚類的 stress 指標（Cara et al., 2005; Swirplies et al., 2019）。前人研究表明，HSP70 對鰻鱺屬物種的壓力反應更為準確敏感（Lee et al., 2013; Lin et al., 2024）。與膏狀飼料組和鰻粉組相比，赤蟲組的 HSP70 表現量顯著較高（$p < 0.05$）（圖七）。這結果可能是由於腸道菌相中存在大量的產氣單胞菌 (*Aeromopnas*)（圖十七）。此結果也和先前的日本鰻之研究結果一致，即感染產氣單胞菌後，鰻魚肝臟中的 HSP70 表現量會顯著提升（Liang et al., 2016）。

溶菌酶（LZM）是魚類先天免疫的重要指標，廣泛存在於生物體內（Saurabh and Sahoo, 2008）。 LZM 也可作為養殖魚類的 stress 指標（Demers and Bayne，1997 年）。然而，在本研究中，經過八週的餵食試驗後，餵食不同飼料的黑鰻苗之 LZM 表現量並沒有顯著差異，這表明開口飼料對黑鰻苗之 LZM 表現量可能較無影響。白細胞介素-6（IL-6）是一種多功能細胞因子，在介導適應性免疫反應中發揮關鍵作用，並具有與免疫系統相關的廣泛生物活性（Hirano, 1998）。 IL-

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6 常被用作水產養殖魚類的發炎指標（Chen et al., 2012; Lin et al., 2023）。在近本研究中，與赤蟲組和鰻粉組相比，膏狀飼料組之 IL-6 表現量顯著較低（$p< 0.05$）（圖七）。赤蟲組和鰻粉組中 IL-6 表現量較高與這些組之腸道組織學中觀察到的發炎相關，而膏狀飼料組中則沒有發現這種發炎（圖三）。前人對江鯉（*Cyprinus carpio* var. Jian）的研究也發現了類似的結果，顯示腸道發炎反應導致 IL-6 表達增加（Jiang et al., 2015）。

SOD 和 POD 等抗氧化酶在保護魚類免受氧毒性和缺氧壓力方面發揮至關重要的作用（Wang et al., 2017；Li et al., 2022）。與赤蟲組和鰻粉組相比，膏狀飼料組的 SOD 和 POD 表現量顯著提升（$p< 0.05$）（圖七）。先前的研究表明，感染病原體或營養不良的魚類由於代謝失衡導致抗氧化酶活性降低，SOD 和 POD 表達水平明顯降低（Olsvik et al., 2005）。在赤蟲組和鰻粉組中觀察到的 SOD 和 POD 表現量較低。本研究在赤蟲和鰻粉組中觀察到的顯著較低 SOD 和 POD 表現量與先前對受到產氣單胞菌挑戰的大西洋鮭魚之結果一致，這也與腸道菌相組成有關（圖十七）（Du et al., 2015）。此外，膏狀飼料組中 SOD 和 POD 表現量的圖生表明對鰻苗的免疫表現亦有正向影響，這可以在愛德華氏菌攻毒實驗中觀察到明顯較高的存活率獲得支持（圖十二），且這也和先前對不同鰻苗的研究結果類似（Lin et al., 2023; Lin et al., 2024）。

4.1.3 腸道菌相及型態分析

餵食赤蟲和鰻粉黑鰻苗的腸道菌相 α 多樣性顯著高於餵食膏狀飼料者（圖十

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七)。這可能是因為赤蟲本身攜帶較多病原菌使黑鰻苗攝食後於腸道中繁殖(Dainys et al., 2017)；而鰻粉組的多樣性增高可能是因為溢散於水中之鰻粉造成飼養水體中的細菌具有豐富營養源大量在缸壁孳生菌膜等黏著物（特別鰻粉中之澱粉含量高，具充沛碳源）而導致（林, 2019)。其中值得注意的是赤蟲組之黑鰻苗的腸道菌相組成中產氣單胞菌屬（*Aeromonas*）佔據不少比例（圖十七)，而前人研究已指出由產氣單胞菌屬菌種所導致的鰻魚赤鰭病為目前鰻魚養殖中嚴重疾病之一（Guan et al., 2011; Qin et al., 2014)。而此屬菌之存在也是導致赤蟲組之存活率為顯著最低者。前人研究中在鰻苗階段餵食赤蟲和鰻粉的日本鰻苗中也觀察到類似的結果，其中赤蟲組的腸道微生物多樣性也較高（Iida et al., 1984）。α多樣性較高的原因可能是血蟲的採集地點主要在富含有機物的水域，將各種細菌引入魚體內（Simangunsong et al., 2023）。此外，糊狀飼料組的α多樣性較低，這與餵食經檢疫之人工開口餌料的美洲鰻鰻苗（*A. rostrata*）的研究結果一致（Liang et al., 2023）。膏狀飼料組中腸道菌相組成主要是 *Cetobacterium* 和 *Plesiomonas*（圖十七），該兩屬菌種在先前研究中顯示出和歐洲鰻之成長表現存在正相關（Shi et al., 2020; Yajima et al., 2023）。雖然鰻粉組的腸道菌相組成與膏狀飼料組相似，但 *Clostridium* 的豐度較高（圖十七）。該屬菌種的存在將導致鰻苗的生長表現降低以及和胃腸道病變，這與生長表現 (表九)和腸道形態（圖三）的結果一致（Hsieh et al., 2018; Shi et al., 2020）。

而雖然 α 多樣性鰻粉跟赤蟲皆高於膏狀飼料組，在 β 多樣性方面則呈現赤蟲

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自己獨立一組而鰻粉和膏狀飼料較為相近之趨勢（圖十九、圖二十)。這可以解釋人工飼料（膏狀飼料、鰻粉）等相較天然生物餌料（赤蟲、絲蚯蚓等）對鰻苗腸道菌相的影響，故呈現出人工飼料一群、赤蟲一群之結果。在β多樣性分析中，赤蟲組的 LDA 差異最大，而膏狀飼料組和鰻粉組中具有顯著 LDA 差異的物種相對更接近，這與 PCoA 結果一致（圖十八）。兩個β多樣性結果都表明，投餵人工開口飼料（膏狀飼料和鰻粉）和投餵天然初始飼料（赤蟲）的鰻苗腸道菌相群之間存在顯著差異，這與先前對不同水產養殖魚類物種的研究結果一致（Iida et al., 1984; Dhanasiri et al., 2011; Mai et al., 2023）。

其中餵食赤蟲之黑鰻苗腸道切片出現明顯的破損及發炎情形，這可能和其腸道內含有如產氣單胞菌等魚類病原菌所造成（圖三)。前人於感染產氣單胞菌之歐洲鰻和草魚之腸道中亦有觀察到類似病徵 (Chen et al., 2020; Xiong et al., 2020)。而在鰻粉組中，雖然腸道型態完整，不過有出現萎縮及空洞化等狀態（圖三)，這可能是因為鰻粉之型態對黑鰻苗尚不好進食利用，導致營養不良而產生（林, 2019)。

4.2 碳化多銨 (CQDSpds) 添加實驗

4.2.1 成長表現

在鱸鰻苗成長表現方面，發現添加 0.5 ppm 之組別具有顯著最佳之成長表現；而在日本鰻苗中則顯示了 0.5 ppm 至 1 ppm 的添加劑量都可以顯著提升日本鰻苗之成長表現。而且從腸道切片圖皆未觀察到進食 CQDSpds 對兩種鰻苗之腸胃道

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造成破壞。可見適量添加之 CQDSpds 等機能性添加劑可提升對鰻苗之成長表現及育成率，並且不會影響腸胃道型態。本研究中對兩種鰻魚生長促進作用可能是由於膏狀飼料中的 CQDSpds 增強了兩種鰻魚的飼料攝取、食物吸收、新陳代謝和免疫相關基因表現 (Bae et al., 2012)。飼養結果還顯示出，兩種鰻苗皆會受到過量添加的 CQDSpds (2 ppm) 的影響，前人關於美洲鰻苗飼料添加劑的研究也發現了類似的現象 (Zhang et al., 2022)，顯示機能性添加物超過最適濃度可能會對鰻苗的生長性能產生負面影響。

4.2.2 鰻苗免疫表現

前人研究指出 SOD 及 POD 可以當作魚類面對壓力之反應物 (Abele et al., 2004; Oliva et al., 2012)，在魚類面對壓力及器官受損之情況下表現量會上升 (Liu et al., 2015)。餵食添加 0.5 ppm 添加量之 CQDSpds 之膏狀飼料的鱸鰻苗在此二種免疫基因之表現量皆顯著低於控制組 (圖八)；而日本鰻苗在餵食 0.5 ppm 及 1 ppm 組中之 POD 和 1 ppm 之 SOD 表現量顯著最低 (圖九)。故可以推論食用 0.5 ppm 組之鱸鰻苗及 0.5-1 ppm 組之日本鰻苗應可以有效降低環境壓力對其的影響或是提升了其免疫反應減少活性氧之攻擊，進而呈現出更佳之成長表現。此實驗結果也和前人在虹鱒及斑馬魚之研究結果相符 (Zhou et al., 2018; Vazirzadeh et al., 2020)。而過度的碳化多銨添加則會造成 SOD 及 POD 之表現量上升，此結果可能如同前人於草魚之實驗中導致魚類器官之損壞甚至死亡 (Liu et al., 2013)。

而在 LZM 的表現量中，可以發現兩種鰻苗在 0.5 ppm 及 1 ppm 之組別中表現

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量顯著高於控制組 ($p < 0.05$)。而 LZM 在魚類維持自身免疫防禦機制中有重要作用，更參與魚類許多下游之免疫反應 (Saurabh et al., 2008)。在本實驗中不論日本鰻苗或鱸鰻苗在 0.5 ppm 及 1 ppm 之組別呈現出了較高之 LZM 表現量，而兩種鰻苗也分別在該添加量組別中呈現了最佳之生長表現，該結果和前人於日本鰻及虹鱒研究中一致 (Shahkar et al., 2018; Vazirzadeh et al., 2020)。而更高的 LZM 表現量亦可以提升鰻苗之免疫表現，此結果亦可解釋攻毒實驗 0.5 ppm 及 1 ppm 之累積存活率顯著高於其他組 (圖十三、圖十四)。先前的研究亦表明，在補充維生素 E 後，日本鰻的 LZM 活性明顯提高（Shahkar et al., 2018）；而在虹鱒飼料中添加海藻也會增加 LZM 的表達（Vazirzadeh et al., 2020）。且在餵食了添加中草藥萃取物之飼料，一具有抗菌效果之飼料添加劑，的鱸鰻中也顯行出了最佳的 LZM 活性和生長表現（解, 2022）。本實驗結果與上述結果一致，雖然 CQDSpds 具有抑菌劑之功能不過在適量添加之劑量下，仍可以提升鰻苗之 LZM 表現量。

IL-6 是經由免疫系統在生物體受到感染時所分泌之物質，其可以幫忙促進免疫細胞之啟動，不過 IL-6 之濃度增加則可能引起細胞風暴，且表示生物體正處於發炎狀態中 (Tanaka et al., 2014; Stefan et al., 2017)。故 IL-6 常被用為受感染與否的篩查標示。從本實驗結果來看，所有餵食 CQDSpds 之鰻苗皆呈現了較低之 IL-6 表現量，可能代表膏狀飼料中添加 CQDSpds 可以有效降低養殖系統中鰻苗被感染之可能性，進而造就更佳之成長、免疫表現 (Copaescu et al., 2020)。

先前的研究指出，可以在飼料中添加有效的機能性添加劑來防止愛德華氏菌造

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成鰻魚養殖業者的損失 (Mohanty and Sahoo, 2007; Lee et al. 2018)。結果顯示，所有餵食含有 CQDSpds 飼料的鰻魚也比對照組表現出更好的愛德華氏菌抗病能力(圖十四、圖十五)。然而，當 CQDSpds 的濃度達到 2 ppm 時，存活率低於 1 ppm，雖然存活率仍高於對照組，但超劑量的抑菌劑如：CQDSpds 及抗生素等可能會削弱鰻苗的腸道和免疫系統，導致生長表現下降 (Bae et al., 2012)。根據結果，CQDSpds 的添加展現出可以提升鰻苗對愛德華氏菌的抗病潛力。

4.3 納豆枯草桿菌 NTU-18 添加實驗

4.3.1 成長表現

本實驗是首次對鰻苗（5000 P/kg）階段進行飼料益生菌添加的成效評估，先前大部分的研究是在幼魚階段 (50-100 P/kg) 進行 (Zheng et al., 2019；Park et al., 2020)，又或者在柳葉鰻階段 (Jang et al., 2023)。統整前人研究和本實驗結果，鰻魚飼料中添加不同益生菌均顯示出可以顯著增強生長表現以及各種免疫相關反應 (Zheng et al., 2019；Park et al., 2020；Jang et al., 2023)。本研究中，在膏狀飼料中添加納豆枯草桿菌 NTU-18 顯著改善了日本鰻苗的生長表現，顯示其作為優良鰻魚飼料添加劑的潛力。同時在飼餵添加枯草芽孢桿菌 WB60 的飲食的日本鰻魚中也發現了類似的生長促進作用 (Lee et al., 2018)。而在添加 0.5% 和 1% 納豆枯草桿菌 NTU-18 的組別中觀察到最佳的生長表現 (表十三)，這與先前的研究結果一致，皆顯示鰻魚飼料中枯草芽孢桿菌類的最佳添加劑量為 1×10^{7}~1×10^{8} CFU/g kg^{-1} (Lee et al., 2017; Park et al., 2020)。生長表現的增加可能

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因為納豆枯草芽孢桿菌 NTU-18 對日本鰻苗消化過程的影響，此有助於提升鰻苗之生長和營養吸收 (Nayak, 2021)。而在腸道形態方面，本研究中並未觀察到添加劑對日本鰻苗腸道形態造成影響，顯示添加枯草芽孢桿菌納豆 NTU-18 有利於日本鰻苗的生長表現，且不會引起腸道形態的變化。

4.3.2 免疫表現

在本研究中，所有餵食添加納豆枯草桿菌 NTU-18 膏狀飼料之日本鰻苗的肝臟和頭腎中 IgM 的表達均顯著高於對照組。先前對金頭鯛 (*Sparus aurata*) 餵食添加益生菌的實驗也觀察到類似的結果 (Cuesta et al., 2004)，顯示枯草芽孢桿菌納豆 NTU-18 可以作為免疫調節劑來增加 IgM 表現。此外，肝臟和頭腎中 IgM 表現的增加也可以增強日本鰻抵抗外來病原體和疾病的能力 (Sahoo et al., 2021)。

熱休克蛋白 (HSP) 可以根據其分子量進行分類，包括 HSP110、HSP90、HSP70、HSP60、HSP40、HSP10 等 (Feder and Hofmann, 1999)。其中，HSP70 和 HSP90 可是經由 stress 誘發，故常被用作水產養殖生物是否感受 stress 之指標 (Cara et al., 2005; Cerezuela et al., 2012)。與控制組相比，在添加量 0.5%枯草芽孢桿菌納豆 NTU-18 以上組別的日本鰻苗肝臟和頭腎中 HSP70 的表現量增加；然而各組間 HSP90 的表現量並無顯著差異 (圖十、圖十一)。此結果和前人研究一致，顯示在日本鰻飼料中添加枯草芽孢桿菌可以增加 HSP70 的表達，但對 HSP90 沒有顯著差異，這表明 HSP70 對於日本鰻可能是更好的 stress 指標 (Lee et al., 2013; Lee et al., 2018)。此外，添加枯草芽孢桿菌 0.5%和 1%組在愛德華氏菌攻毒試驗

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後表現出最好的存活率 (圖十四)，這說明納豆枯草桿菌 NTU-18 可能透過增強非特異性免疫來提昇日本鰻苗對愛德華氏菌的抗性 (Guzik et al., 1999; Lee et al., 2013)。

抗氧化酶，如過氧化物酶 (POD)、超氧化物歧化酶 (SOD) 和過氧化氫酶 (CAT) 在防止細胞內氧化壓力的有害影響方面，發揮重要的作用 (Fattman et al., 2003)。SOD-CAT 系統透過抑制活性氧自由基的形成來對抗氧毒性壓力。它經常被用作評估硬骨魚類氧化壓力的標記 (Liu, 2011)。在本實驗中，只有 0.5%組的肝臟中 SOD 表現量顯著增加 (圖十)，而肝臟或頭腎中其他抗氧化相關酶 (CAT 和 POD) 的表達沒有顯著差異組間 (圖十、圖十一)。類似結果也顯示在餵食枯草芽孢桿菌的日本鰻觀察到，顯示枯草芽孢桿菌類群可能無法有效刺激日本鰻的 SOD 表達 (Ai et al., 2011; Lee et al., 2018)。其中僅在 0.5%添加組的肝臟中發現 SOD 表達的顯著提升，這可能表示 0.5%是改善肝臟免疫反應的最適添加劑量，且這也和 0.5%組之愛德華氏菌攻毒實驗之存活率顯著最高結果相符 (圖十五)。

在愛德華氏菌攻毒實驗中，0.5%和 1%組中觀察到顯著較高的存活率 (圖十五)。根據免疫相關基因的結果，納豆枯草桿菌 NTU-18 提高了日本鰻苗 HSP70、SOD 和 IgM 的表達，這些酵素有助於更有效地抵抗愛德華氏菌 (Lee et al., 2013)。這顯示納豆枯草桿菌 NTU-18 可以增強日本鰻苗之免疫反應，並可減少 RAS 中細菌性疾病感染的損失。

4.3.3 腸道菌相分析

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在枯草芽孢桿菌 2%添加組的腸道菌相 α 和 β 多樣性顯著降低 (圖二十～圖二十四)，這可能是由於 2%處理組的鰻魚腸道內過量的納豆枯草桿菌 NTU-18 造成其抑制其他腸道細菌。而雖然沒有顯著差異，但 2%組日本鰻苗的生長表現相對其他添加組較低，這項結果可能是因腸道微生物群多樣性的減少，其中主要以梭桿菌屬 (*Fusobacteriia*) 和梭菌屬 (*Clostridia*) 為主，這兩屬菌種的增多已在前人研究中表示和腸胃道病變有關 (Hsieh et al., 2018)。然而，2%組仍表現出明顯高於對照組的成長率。這種差異可能是由於鯨桿菌 (*Cetobacterium*) 的在腸道菌相中的變化，在先前的研究中，其的存在與鰻魚的生長表現呈正相關 (Yajima，2023)。鯨桿菌被指出可生產高濃度的維生素 B12 以促進鰻魚的代謝速度，並降低飼料轉換率(Qi et al., 2023)。本研究結果顯示了透過餵食含納豆枯草桿菌 NTU-18 的飼料來增強鰻苗的腸道酵素活性是有潛力的。而 2%之添加量可能過量，導致對日本鰻苗的生長表現、免疫相關基因和抗病性沒有進一步改善。

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5. 結論

臺灣之鰻苗養殖時至今日仍以赤蟲、絲蚯蚓及鰻粉等傳統餌料為主。故因為水質惡化或生物餌料造成養殖鰻魚大量生病斃死之情事仍常有通報。若能研發新型鰻苗初期餌料，如膏狀飼料等，將可以對鰻魚養殖產業有極大的貢獻及提升。

綜合本研究所有實驗之結果，所研發之新型膏狀飼料對鰻苗之誘食性及成長表現表現，並不遜於傳統使用之赤蟲及絲蚯蚓之成效。而根據成本分析，本飼料之預售價格遠低於日本製膏狀飼料，亦低於赤蟲價格，並且不會造成鰻苗腸道受損，顯見其具市場發展潛力。並且根據鰻苗腸道型態及菌相分析，其不會帶入病原至鰻苗體內或循環水養殖系統中。

對鰻苗而言，膏狀飼料之成長表現顯著高於鰻粉。且本膏狀飼料之水中穩定性極高，比起傳統使用之鰻粉更加適合搭配室內循環水系統使用。其中膏狀飼料之新型層疊狀組織比起鰻粉之煉餌型態對鰻苗具有更好的適口性，亦可以大幅減少殘餌污染水質及滋生菌膜之事情。對比現行常用之傳統鰻苗餌料，本膏狀飼料之高適口、誘食性且低散失率、低成本，將有望成為循環水養鰻系統推廣關鍵之一。

在機能性添加劑方面，適量添加之碳化多銨 (CQDSpds) 及納豆枯草桿菌 NTU-18 皆表現出可以提升各式鰻苗生長表現、免疫基因及抗病能力之能力。特別是兩種機能性添加劑皆展現出對愛德華氏菌具有良好的抵抗能力，可以有效控制循環水養殖系統中疾病爆發之損失。

本研究之膏狀飼料希望可以搭配適合之循環水養鰻系統，降低水質污染以及提

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升鰻苗成長表現。有潛力成為推廣超集約化循環水養鰻系統之重要助力，為臺灣永續養殖盡一份心力。

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1. **Lin, Y. T.**, & Han, Y. S.* (2024). Species diversity of freshwater glass eel (Anguilliformes, Anguillidae) of Yilan, Taiwan, with remark on two new records. *Zookeys*, *In press*. (第一作者, Zoology 69/180, 39.1%)

2. **Lin, Y. T.**, Hung, Y. C., Chen, L. H., Lee, K. T., & Han, Y. S.* (2024). Effects of adding *Bacillus subtilis natto* NTU-18 in paste feed on growth, intestinal morphology, gastrointestinal microbiota diversity, immunity, and disease resistance of *Anguilla japonica* glass eels. *Fish & Shellfish Immunology*, 109556.

(第一作者, VETERINARY SCIENCES 5/144, 3%; FISHERIES 5/56, 9%)

3. **Lin, Y. T.**, Pan, Y. F., & Han, Y. S.* (2023). Effects of adding spermidine carbon quantum dots in feed on growth, intestinal morphology, immunity and disease resistance of *Anguilla japonica* and *Anguilla marmorata*. *Aquaculture Reports*, 33, 101847. (第一作者, FISHERIES 8/59, 13%)

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(B) 研討會論文

1. Hung, W. C., Yang, J. C., Hsu, H. Y., Huang, Y. C., **Lin, Y. T.**, & Han, Y. S.* (2020). Growth potential of *Anguilla marmorata* glass eels in different seasons in eastern Taiwan. 臺灣水產學會刊, 47(3), 163-168. (共同作者)

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(C) 技術報告及其他

1. 專利：具浮性之水產養殖用的飼料結構。 發明人：**林晏廷**、韓玉山, 新型第 M641214 號

(D) Under review

1. **Lin Y. T.***, Hsu H. Y., & Han, Y. S. (2024). Evaluation of different initial feed on growth performance, intestinal morphology and microbiota diversity, immunity response, and disease resistance of pacific short-finned glass eel, *Anguilla bicolor pacifica*. (第一作者)

2. **Lin Y. T.***, Lin, C. H., & Han, Y. S. (2024). A southernmost record of the Pacific black scabbardfish *Aphanopus arigato* (Scombriformes, Trichiuridae) from South China Sea. (第一作者)

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Table

表一、各式鰻苗飼料之優缺點比較

飼料種類	絲蚯蚓	赤蟲	鰻粉	浮料	膏狀飼料
適口性	佳	佳	佳	不佳	佳
散失性	低	低	極強	低	低
添加物	無法添加(活體)	不易添加	易添加	不易添加(裹粉)	易添加
易帶病原	是	是	否	否	不易帶原
營養比重	低	低	高	高	高
RAS 適性	不佳(易帶病原)	不佳(易帶病原)	不佳(易污染)	佳	佳

表二、鱸鰻苗餵食赤蟲、日製膏狀飼料與三種台製膏狀飼料之成長與存活比較

餵食 20 日後	赤蟲	日本	台製	台製	台製
平均體長	51.4±2.4[a]	49.3±3.1[a]	47.9±4.1[b]	48.1±4.6[b]	48.9±3.9[b]
平均體重 (g)	0.18±0.07[a]	0.13±0.05[ab]	0.11±0.04[b]	0.09±0.03[b]	0.10±0.02[b]
存活率 (%)	82±5.5[b]	92±3.6[a]	85±4.1[ab]	80±5.3[b]	87±2.7[a]

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表三、膏狀飼料原料病原菌檢驗

病原菌	Flexibacter columnaris	Vibrio anguillarum	Aeromonas hydrophila	E. tarda
病名	爛鰓病	鰻弧菌	赤鰭病	愛德華氏病
絲蚯蚓	V	V	V	V
赤蟲	V		V	V
南極蝦				
糠蝦				
赤尾青				
魚漿				
魷魚				
魚卵				

表四、各式膏狀飼料型態和對鱸鰻苗誘食性、適口性分析

膏狀飼料	魷魚(自製)	日本製	水試所	魚漿(自製)	蝦漿(自製)
色澤	粉橘紅	粉橘紅	褐色	灰褐色	灰褐色
冷凍質地	軟	軟	硬 (冰晶多)	硬 (冰晶多)	軟
斷面構造	層疊狀	層疊狀	塊狀	塊狀	塊狀
誘食性	佳	佳	適中	不佳	佳
適口性	佳	佳	不佳	不佳 (筋膜多)	不佳 (細殼多)

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表五、用於 qPCR 之鰻苗非專一性免疫基因之引子

Genes	Primer	Sequences
ARP (reference gene)	Forward	5′-GTGCAGCTCATTAAGACCGG-3′
	Reverse	5′-GGCGATATTCCTCACACCCT-3′
SOD	Forward	5′-TAACGTACGACTATGGGGCC-3′
	Reverse	5′-GCCGCCACCATTAAACTTCA-3′
POD	Forward	5′-GACATCACCCGTTTCTGCAA-3′
	Reverse	5′-GTGGATGAAGGAGGGGAACA-3′
LZM	Forward	5′-TGCTGGAATGGATGGATACC-3′
	Reverse	5′-GTAATCGCAGTGCTGATGTC-3′
IL-6	Forward	5′-CCAGATGTCGCTTCACTTCG-3′
	Reverse	5′-ACTTGGATGTCGTCACCCAT-3′
HSP-70	Forward	5′-CCATCCTGACCATCGAAGAC-3′
	Reverse	5′-GTTCTCTTGGCCCTCTCACA-3′
HSP-90	Forward	5′-GTGGTGGACTCTGAGGAT-3′
	Reverse	5′-CGAGACACTTCTTGACGATA-3′
IgM	Forward	5′-CGGTTCTTCTGACAATCG-3′
	Reverse	5′-TCGGGCACAGTAATACAC-3′
CAT	Forward	5′-ATGGTGTGGGACTTCTGGAG-3′
	Reverse	5′-AGTGGAACTTGCAGTAGAAACG-3′

表六、愛德華氏菌 TSB 培養基配方

Tryptic Soy Broth (TSB)	Total: 1000ml
Tryptone	17g
NaCl	5.0g
Soytone	3.0g
K_2HPO_4	2.5g
Dextrose	2.5g
ddH_2O	970ml

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表七、本實驗設計之膏狀飼料配方

原料	比例	備註
魷魚漿 (%)	75	去筋膜
魚卵 (%)	15	去卵膜
鹽水 (%)	7.5	5%食鹽水溶液
Guar gum (%)	2.3	
Vitamins premix (%)	0.1	
Mineral premix (%)	0.1	

表八、本實驗膏狀飼料和日本、水試所研發之膏狀飼料散失率對比

飼料	膏狀飼料 (自製)	膏狀飼料 (日清丸紅)	膏狀飼料 (水試所配方)	鰻粉 (福壽牌)
散失率 (%)	10±2.3 [a]	10±1.1 [a]	11±3.7 [a]	30±5.2 [b]

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表九、餵食不同飼料黑鰻苗之成長表現

	Blood worm	Paste feed	Powder
Initial TL (mm)	53.0±1.10 [a]	52.9±0.97 [a]	52.9±1.03 [a]
Final TL (mm)	78.5±7.21 [a]	75.4±9.17 [a]	67.6±5.80 [b]
IBW (g)	0.153±0.01 [a]	0.152±0.01 [a]	0.152±0.01 [a]
FBW (g)	0.87±0.27 [a]	0.80±0.32 [a]	0.49±0.16 [b]
SGR (%)	3.10±0.03 [a]	2.97±0.04 [a]	2.09±0.11 [b]
PWG (%)	468.1±8.31 [a]	426.3±10.9 [a]	223.53±19.7 [b]
FE (%)	5.84±0.53 [a]	7.09±0.28 [a]	4.39±0.18 [b]
FCR	17.2±0.71 [a]	14.1±0.12 [a]	22.8±0.99 [b]
SR (%)	68.3±0.03 [c]	95.5±0.01 [a]	86.67±0.03 [b]
K	1.80±0.08 [a]	1.86±0.09 [a]	1.57±0.05 [b]

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表十、碳化多銨 (CQDSpds)實驗中各組膏狀飼料配方

原料	Control	0.25ppm	0.5ppm	1ppm	2ppm
魷魚漿 (%)	75	75	75	75	75
魚卵 (%)	15	15	15	15	15
鹽水 (%)	7.5	7.5	7.5	7.5	7.5
Guar Gum (%)	2.3	2.3	2.3	2.3	2.3
Vitamins premix (%)	0.1	0.1	0.1	0.1	0.1
Mineral premix (%)	0.1	0.1	0.1	0.1	0.1
CQDSpds (ppm)	0	0.25	0.5	1	2

表十一、碳化多銨 (CQDSpds)實驗中各組鱸鰻苗之成長表現 (Lin et al., 2023)

CQDSpds	Control	0.25ppm	0.5ppm	1ppm	2ppm
Initial BW (g)	0.092±0.001^{a}	0.091±0.003^{a}	0.092±0.001^{a}	0.092±0.002^{a}	0.091±0.002^{a}
Final BW (g)	0.222±0.048^{b}	0.231±0.017^{ab}	0.266±0.082^{a}	0.245±0.051^{ab}	0.232±0.011^{ab}
PWG (%)	144.68±13.95^{b}	153.51±13.17^{ab}	183.86±24.16^{a}	167.79±27.12^{ab}	153.86±6.49^{b}
SGR (%/day)	1.05±0.08^{b}	1.11±0.05^{ab}	1.26±0.12^{a}	1.17±0.08^{ab}	1.12±0.08^{b}
FE (%)	22.74±1.53^{b}	24.35±3.23^{b}	30.18±1.82^{a}	26.46±6.73^{a}	24.37±2.67^{b}
FCR	4.39±0.23^{a}	4.11±0.46^{a}	3.31±0.33^{b}	3.78±0.57^{a}	4.10±0.46^{a}
K	1.06±0.07^{a}	1.11±0.06^{a}	1.24±0.10^{a}	1.16±0.12^{a}	1.11±0.03^{a}
SR (%)	61.67±7.07^{a}	60.00±6.01^{a}	68.33±7.07^{a}	70.00±9.42^{a}	65.00±11.78^{a}

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表十二、碳化多銨 (CQDSpds)實驗中各組日本鰻苗之成長表現

(Lin et al., 2023)

CQDSpds	Control	0.25ppm	0.5ppm	1ppm	2ppm
IBW (g)	0.396±0.01[a]	0.400±0.02[a]	0.394±0.02[a]	0.390±0.03[a]	0.399±0.01[a]
FBW (g)	0.918±0.12[a]	0.928±0.15[a]	1.056±0.26[b]	1.109±0.37[b]	0.976±0.23[a]
PWG (%)	131.82±6.21[a]	131.77±8.45[a]	169.37±9.58[b]	185.56±2.47[b]	144.65±6.96[a]
SGR (%/day)	1.00±0.03[a]	1.00±0.04[a]	1.17±0.06[b]	1.24±0.31[b]	1.06±0.03[a]
FE (%)	46.61±9.75[a]	47.10±7.35[a]	59.42±7.34[b]	62.24±5.21[b]	51.52±1.43[ab]
FCR	2.14±0.21[a]	2.12±0.17[a]	1.68±0.16[b]	1.61±0.11[b]	1.94±0.07[ab]
K	0.95±0.05[a]	0.93±0.02[a]	0.93±0.01[a]	0.94±0.03[a]	0.94±0.02[a]
SR (%)	62.00±2.83[a]	64.00±5.66[a]	64.00±5.66[a]	70.00±2.83[a]	66.00±2.83[a]

表十三、納豆枯草桿菌 NTU-18 實驗中實驗中各組膏狀飼料配方

Ingredients	Control	0.25ppm	0.5ppm	1ppm	2ppm
Squid mince (%)	70	70	70	70	70
Fish mince (%)	20	20	20	20	20
Krill (%)	5	5	5	5	5
Guar gum (%)	4.8	4.55	4.3	3.8	2.8
Vitamins premix (%)	0.1	0.1	0.1	0.1	0.1
Mineral premix (%)	0.1	0.1	0.1	0.1	0.1
Bacillus subtilis natto NTU-18(%)	0	0.25	0.5	1	2

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表十四、納豆枯草桿菌 NTU-18 實驗中各組日本鰻苗之成長表現

(Lin et al., 2024)

	Control	0.25 %	0.5 %	1 %	2 %
Initial TL (mm)	59.89±0.81 [a]	60.00±0.45 [a]	60.12±0.83 [a]	60.06±0.78 [a]	59.89±0.46 [a]
Final TL (mm)	94.07±17.1 [a]	99.75±8.7 [b]	103.19±12.4 [b]	104.60±10.1 [b]	101.85±9.4 [b]
IBW (mg)	184.9±7.2 [a]	185.6±9.5 [a]	187.9±9.7 [a]	186.4±8.8 [a]	187.7±12.1 [a]
FBW (mg)	687.6±39.3 [a]	832.0±61.0 [b]	933.4±72.4 [c]	954.7±32.7 [c]	854.8±63.5 [b]
SGR (%)	1.87±0.43 [a]	2.14±0.25 [b]	2.29±0.31 [c]	2.33±0.37 [c]	2.16±0.31 [b]
PWG (%)	271.8±38.4 [a]	348.2±52.1 [b]	396.8±43.7 [c]	412.2±57.1 [c]	355.8±65.2 [b]
FE (%)	62.39±14.2 [a]	72.58±16.5 [b]	81.27±16.3 [c]	84.32±11.4 [c]	74.34±14.7 [b]
FCR	1.60±7.04 [a]	1.38±6.06 [b]	1.23±6.13 [c]	1.19±8.77 [c]	1.35±6.80 [b]
SR (%)	98.75±0.01 [a]	98.75±0.01 [a]	98.75±0.01 [a]	98.75±0.01 [a]	100±0.00 [a]
Final K	0.83±0.11 [a]	0.84±0.18 [a]	0.85±0.07 [a]	0.83±0.10 [a]	0.85±0.12 [a]

表十五、餵食不同飼料黑鰻苗之腸胃道參數測量

	Blood worm	Paste feed	Powder
Villi length (***μ***m)	14.7±2.05 [b]	26.8±0.59 [a]	14.2±1.82 [b]
Muscular layer length (***μ***m)	5.67±1.01 [b]	3.57±0.21 [a]	5.34±0.93 [b]

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表十六、CQDSpds 添加實驗中各組鱸鰻苗之腸胃道參數測量

(Lin et al., 2023)

CQDSpds	0ppm	0.25ppm	0.5ppm	1ppm	2ppm
Villus height (μm)	61.18±4.98[a]	61.43±6.21[a]	61.79±5.80[a]	60.98±7.37[a]	60.73±4.36[a]
Villus circumference ratio (%)	10.7±2.10[a]	10.3±1.41[a]	10.5±3.67[a]	10.8±1.92[a]	10.7±4.35[a]

表十七、CQDSpds 添加實驗中各組日本鰻苗之腸胃道參數測量 (Lin et al., 2023)

CQDSpds	0ppm	0.25ppm	0.5ppm	1ppm	2ppm
Villus height (μm)	93.78 ±5.98[a]	94.75±4.29[a]	94.33±7.14[a]	95.14±5.67[a]	95.02±3.02[a]
Villus circumference ratio (%)	13.3±1.71[a]	13.1±3.12[a]	12.9±5.26[a]	13.0±6.40[a]	13.1±1.42[a]

表十八、納豆枯草桿菌 NTU-18 添加實驗中日本鰻苗之腸胃道參數測量

(Lin et al., 2024)

B. subtilis natto NTU-18	Control	0.25%	0.5%	1%	2%
Villi length (μm)	45.1± 5.6[a]	48.5 ± 4.1[a]	44.5 ± 4.3[a]	49.2 ± 5.0[a]	45.4 ± 4.5[a]
Muscular layer thickness (μm)	6.85± 1.02[a]	7.05± 1.16[a]	7.12± 0.96[a]	7.09± 0.88[a]	6.92± 1.04[a]

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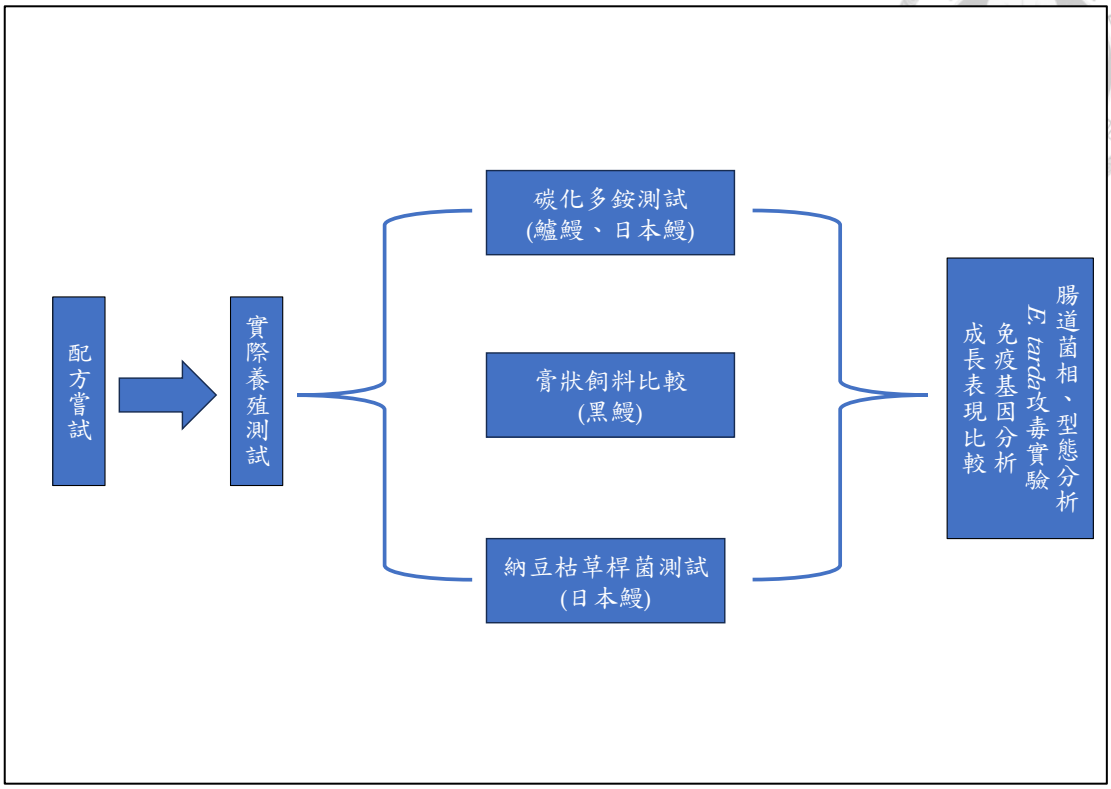
Figure



圖一、實驗流程圖

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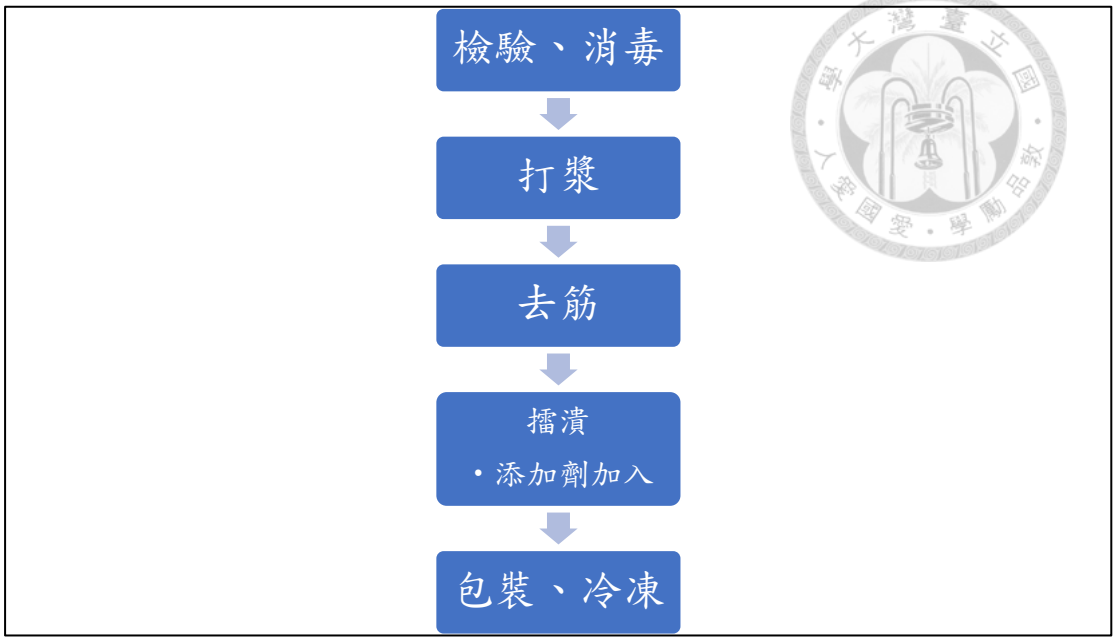
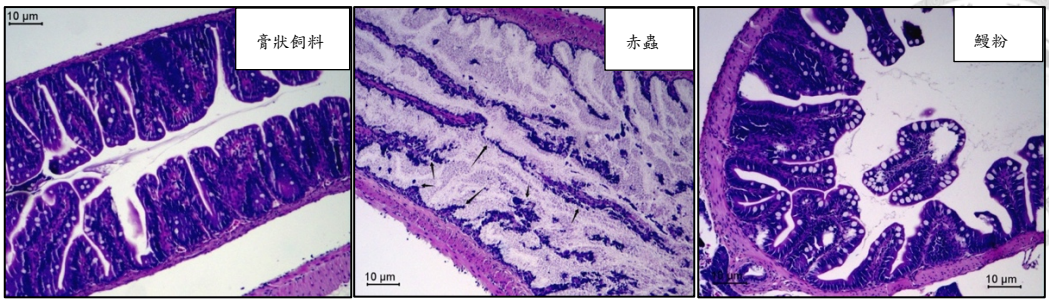


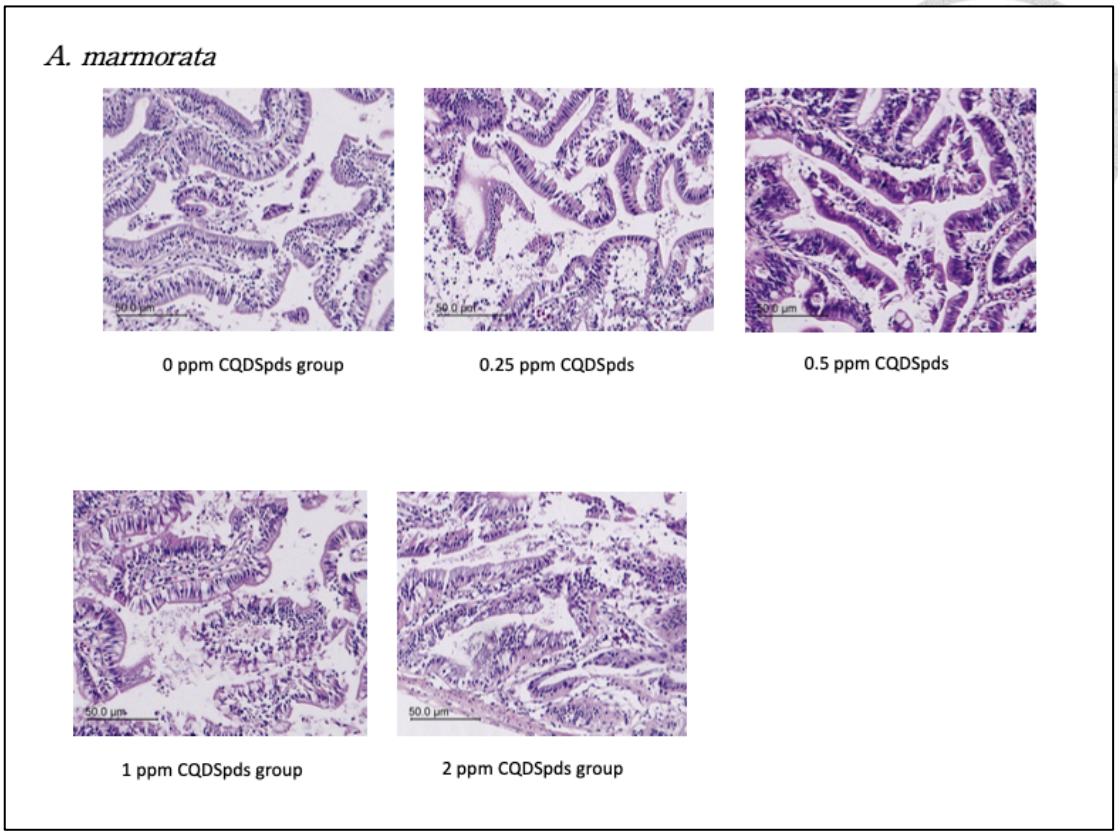
圖二、膏狀飼料製程圖

doi: 10.6342/NTU202402135



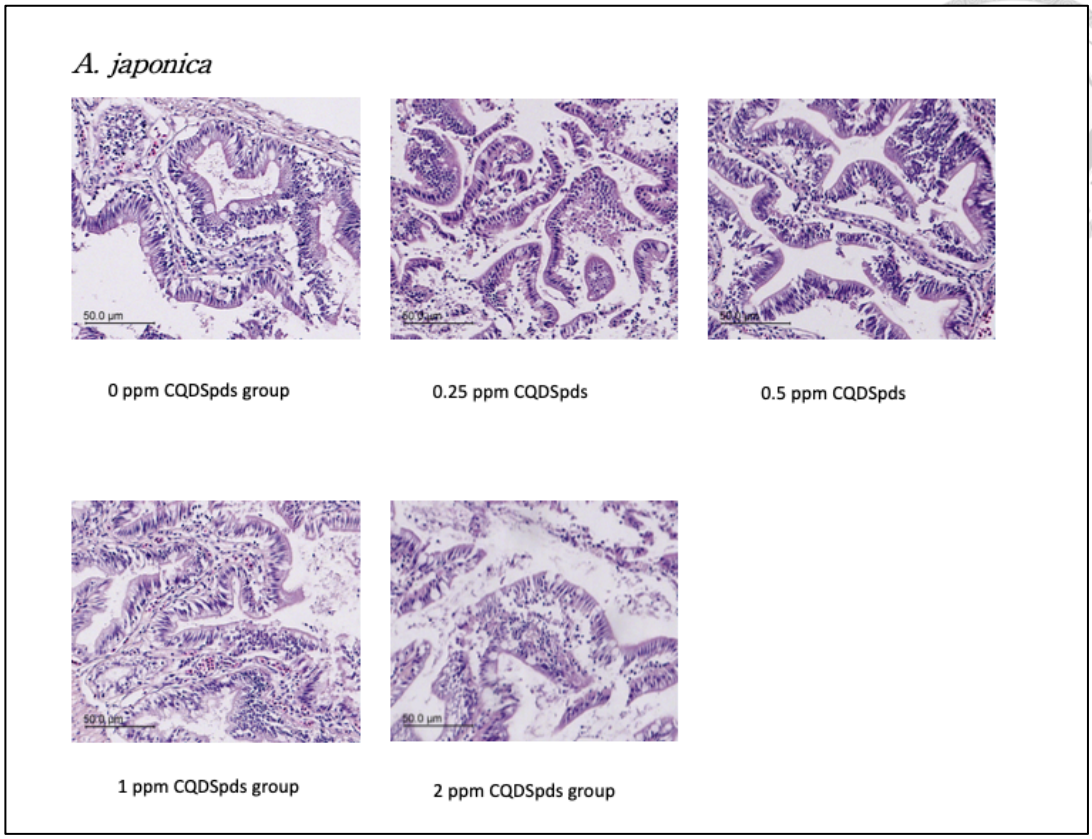
圖三、餵食不同飼料黑鰻苗之腸胃道切片圖

doi: 10.6342/NTU202402135



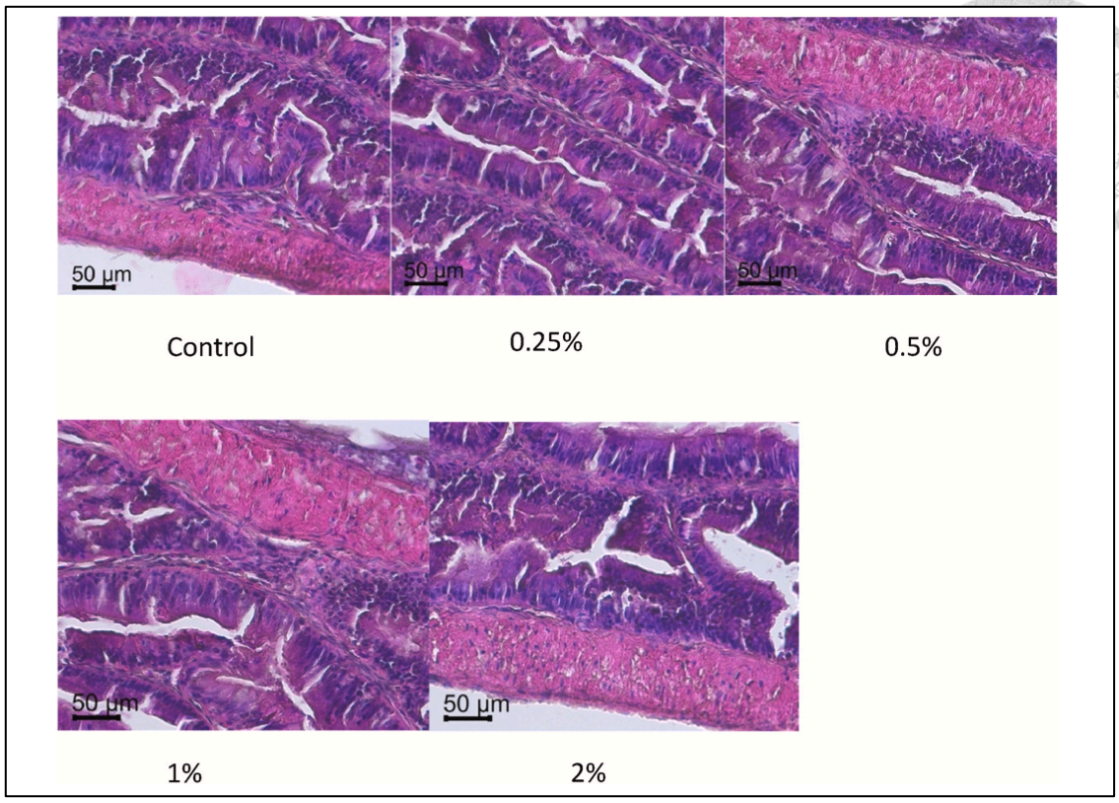
圖四、CQDSpds 添加實驗中鱸鰻苗之腸胃道切片圖 (Lin et al., 2023)

doi: 10.6342/NTU202402135



圖五、CQDSpds 添加實驗中日本鰻苗之腸胃道切片圖 (Lin et al., 2023)

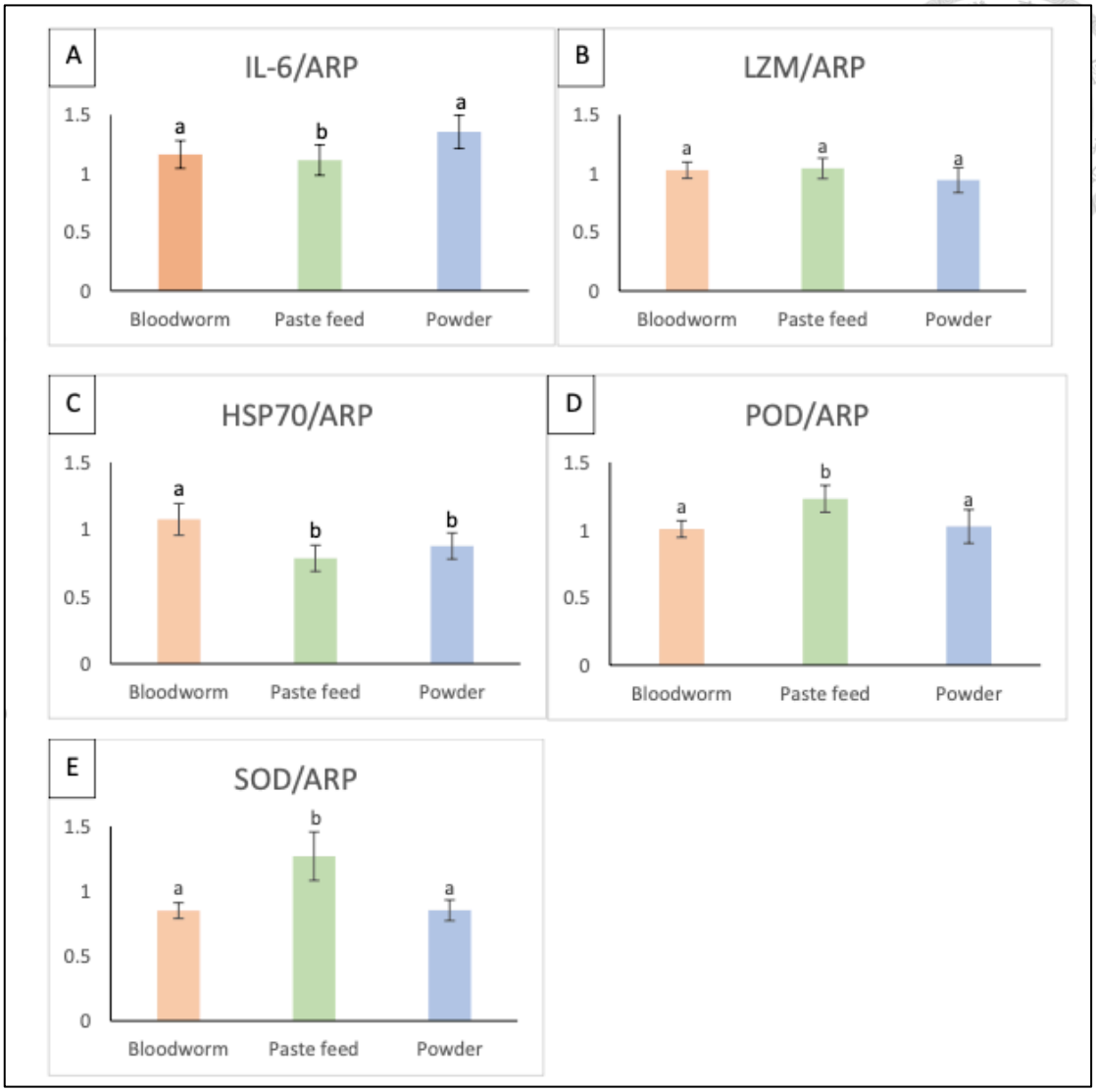
doi: 10.6342/NTU202402135



圖六、納豆枯草桿菌 NTU-18 添加實驗中日本鰻苗之腸胃道切片圖

(Lin et al., 2024)

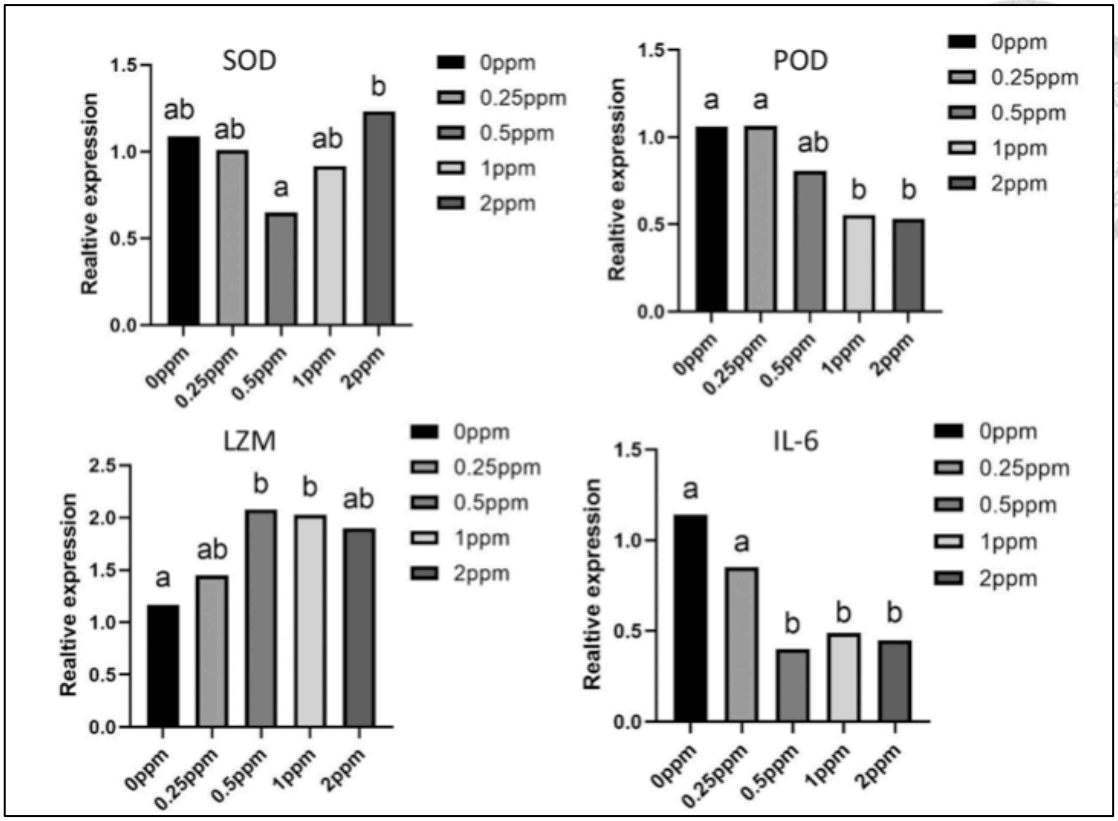
doi: 10.6342/NTU202402135



圖七、餵食不同飼料黑鰻苗之免疫基因表現量

(A:IL-6/ARP; B: LZM/ARP; C: HSP70/ARP; D: POD/ARP; E: SOD/ARP)

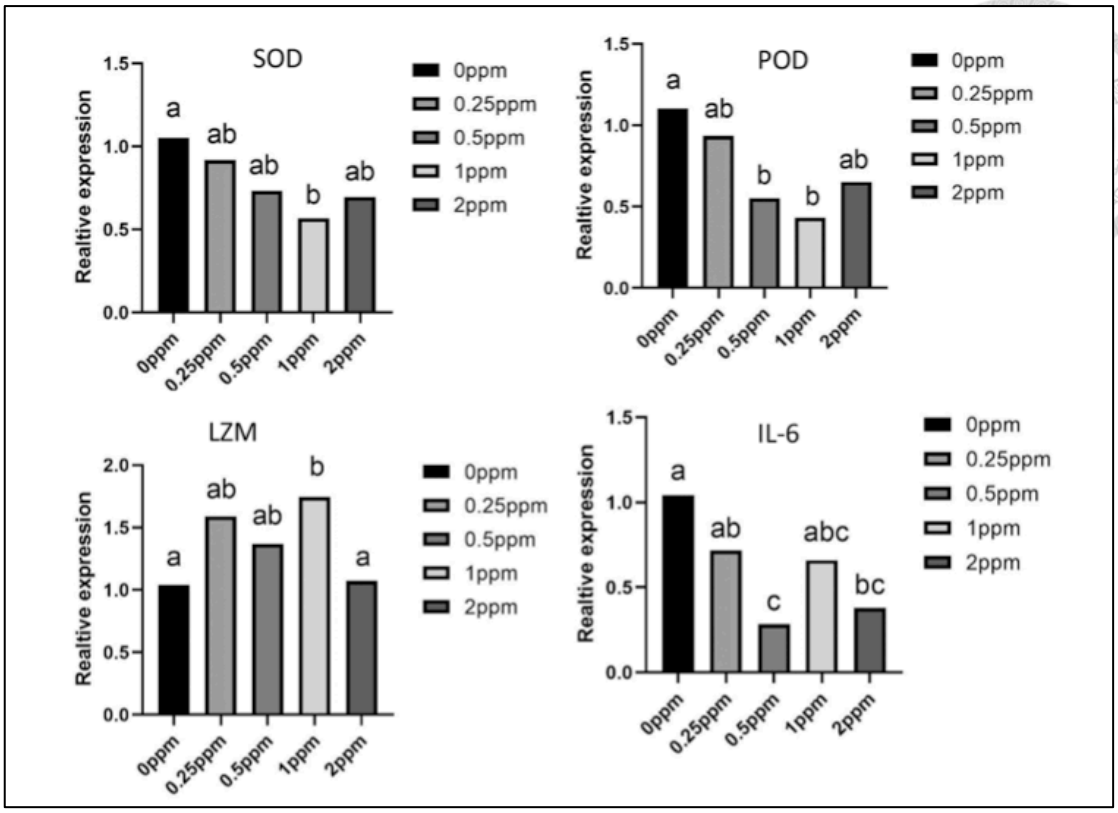
doi: 10.6342/NTU202402135



圖八、碳化多銨 (CQDSpds)實驗中各組鱸鰻苗之免疫基因表現量

(Lin et al., 2023)

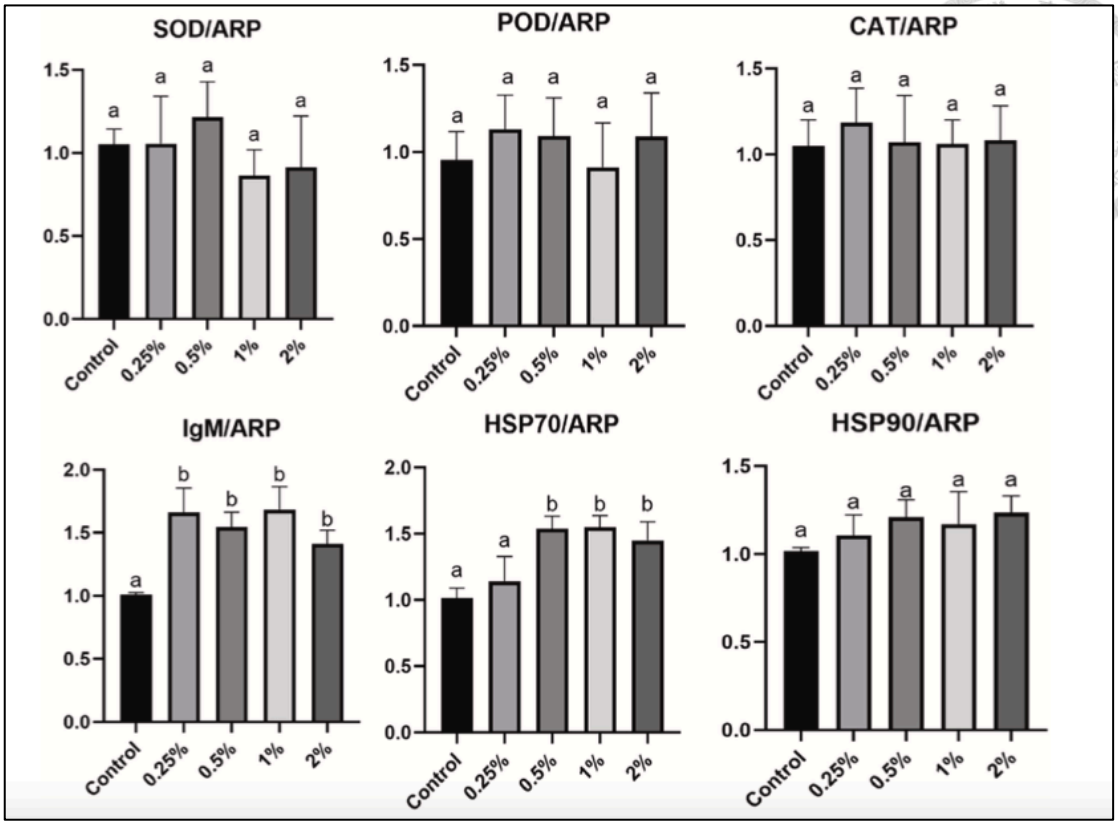
doi: 10.6342/NTU202402135



圖九、碳化多銨 (CQDSpds)實驗中各組日本鰻苗之免疫基因表現量

(Lin et al., 2023)

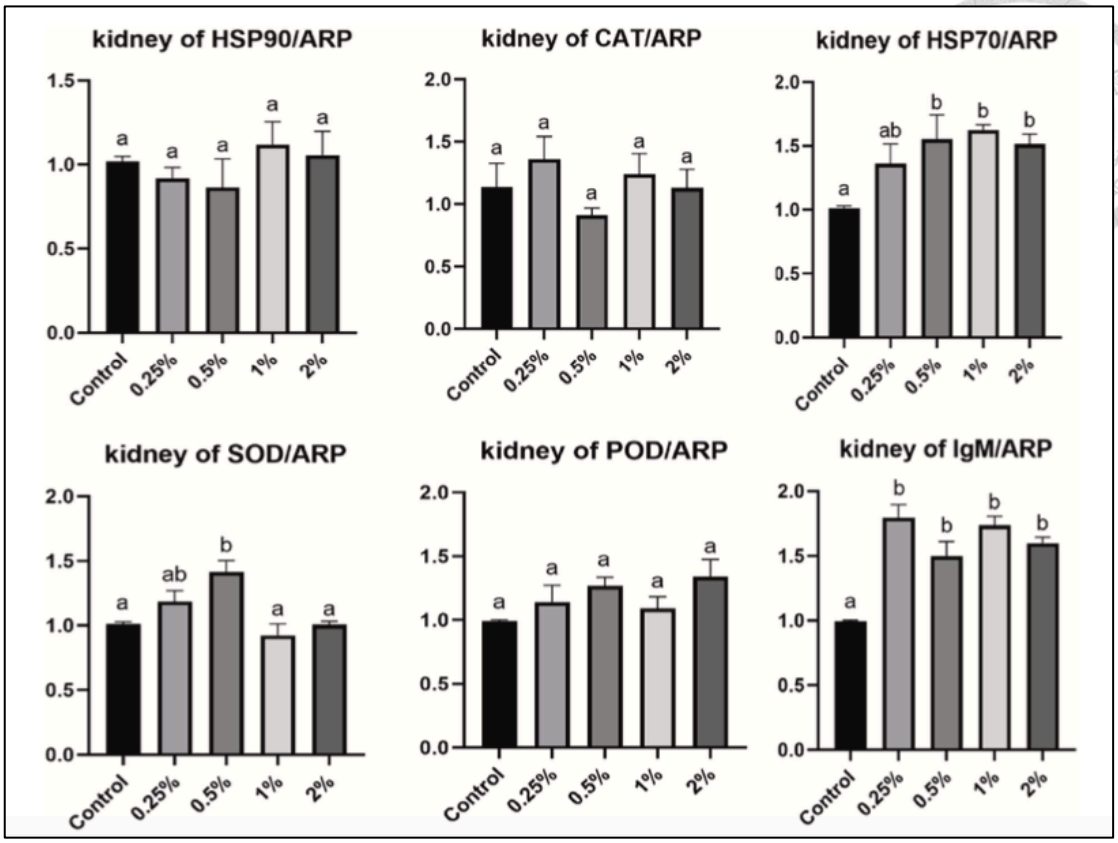
doi: 10.6342/NTU202402135



圖十、納豆枯草桿菌 NTU-18 實驗中各組日本鰻苗肝臟之免疫基因表現量

(Lin et al., 2024)

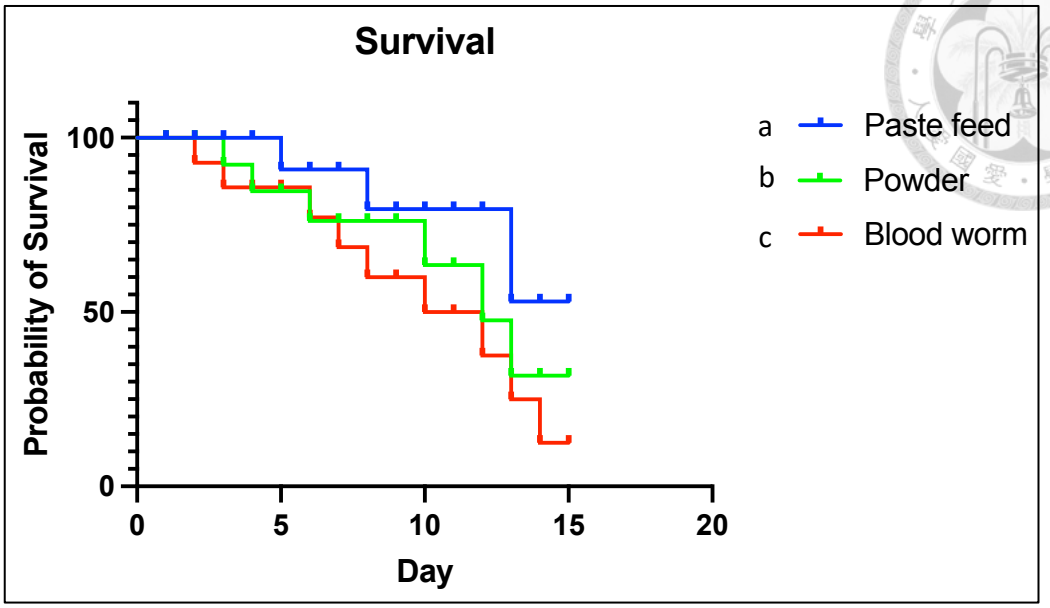
doi: 10.6342/NTU202402135



圖十一、納豆枯草桿菌 NTU-18 實驗中各組日本鰻苗頭腎之免疫基因表現量

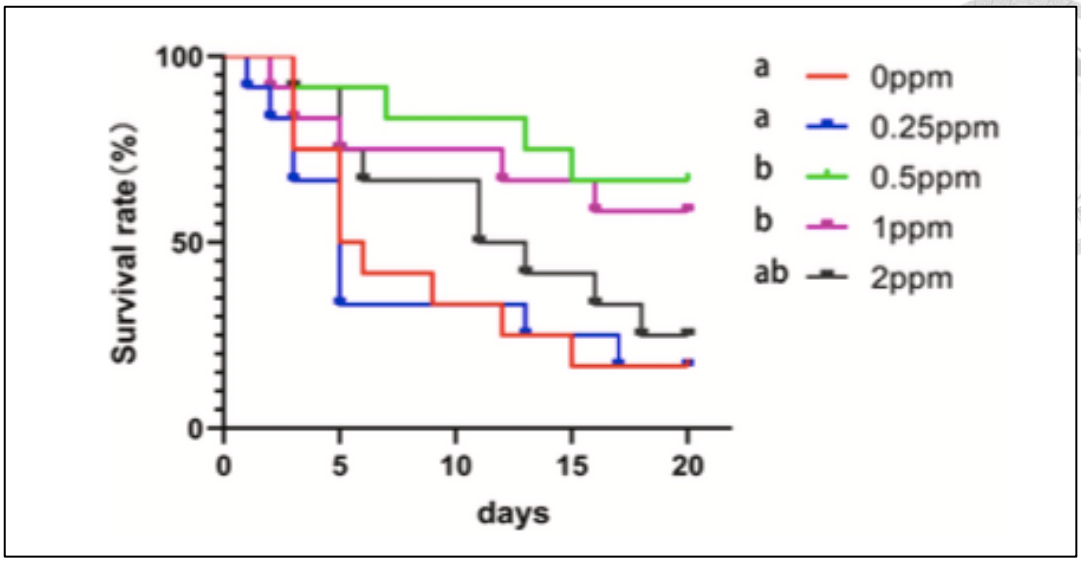
(Lin et al., 2024)

doi: 10.6342/NTU202402135



圖十二、餵食不同飼料之黑鰻苗經過 15 天攻毒實驗累計存活率

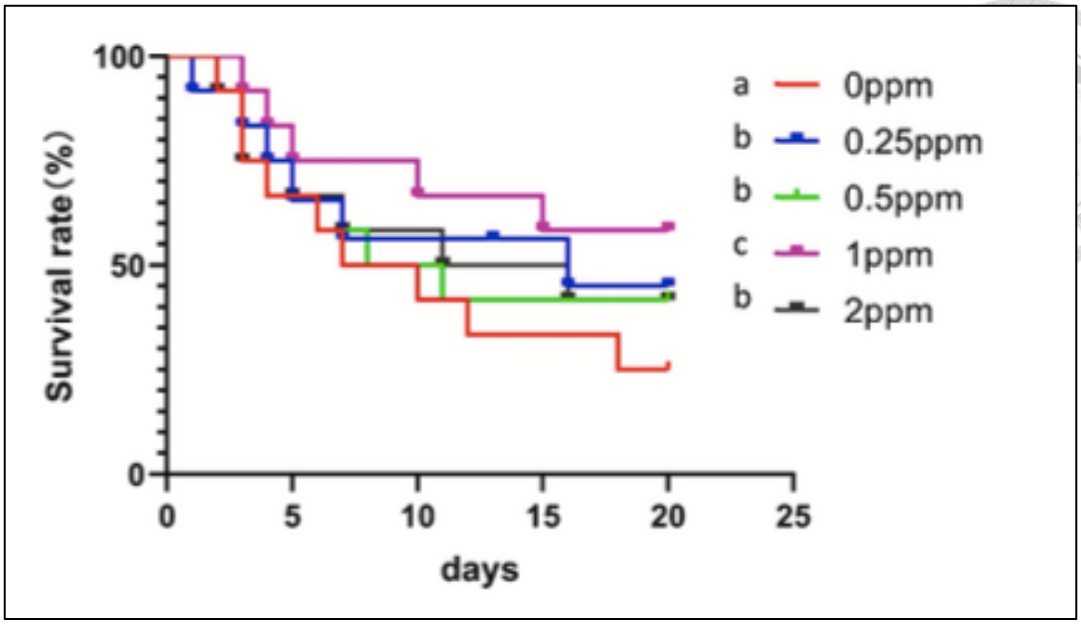
doi: 10.6342/NTU202402135



圖十三、碳化多銨 (CQDSpds)實驗中經過 20 天攻毒實驗之鱸鰻苗累計存活率

(Lin et al., 2023)

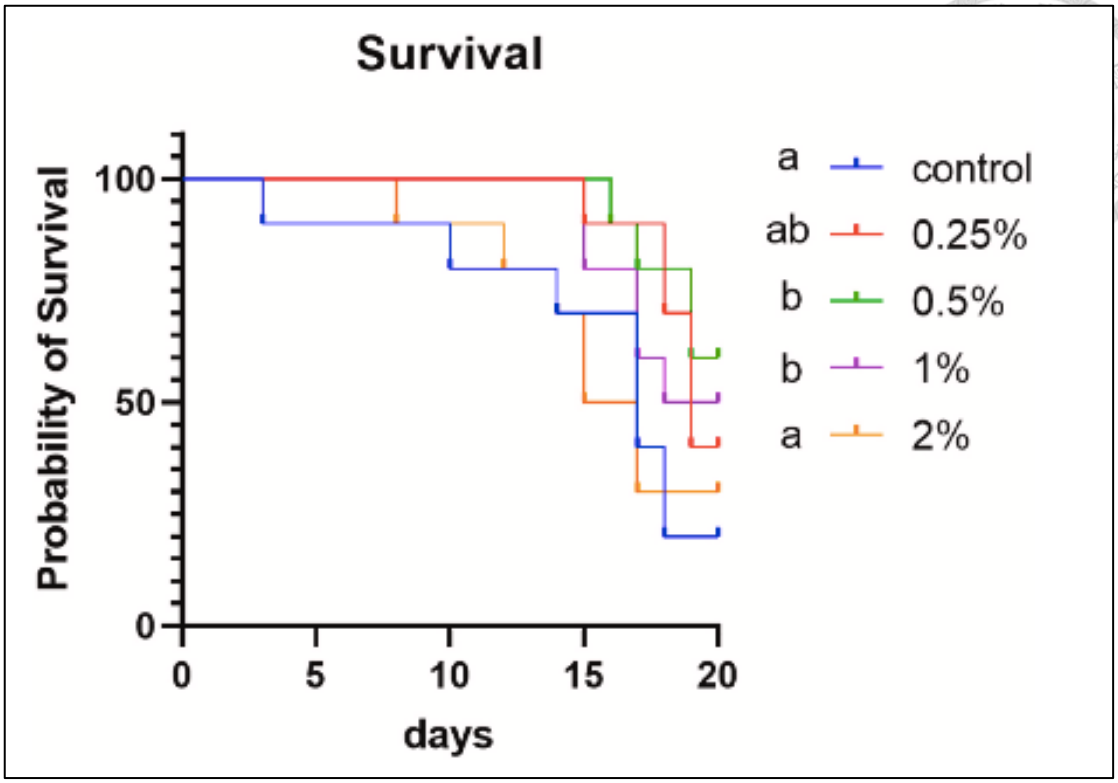
doi: 10.6342/NTU202402135



圖十四、碳化多銨 (CQDSpds)實驗中經過 20 天攻毒實驗之日本鰻苗累計存活率

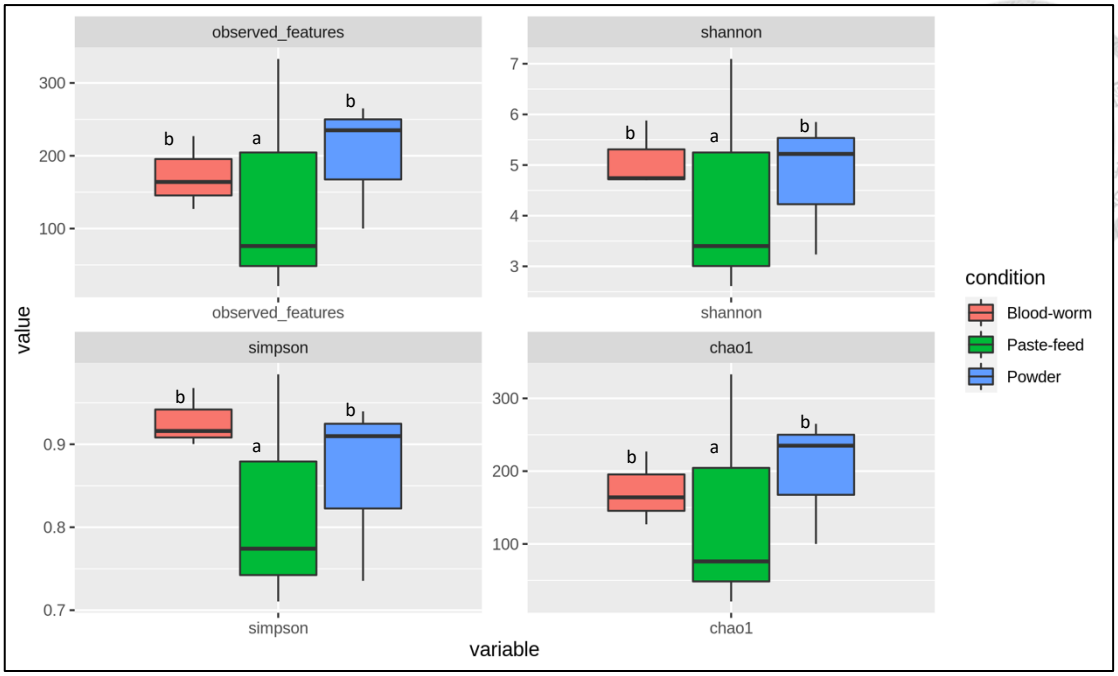
(Lin et al., 2023)

doi: 10.6342/NTU202402135



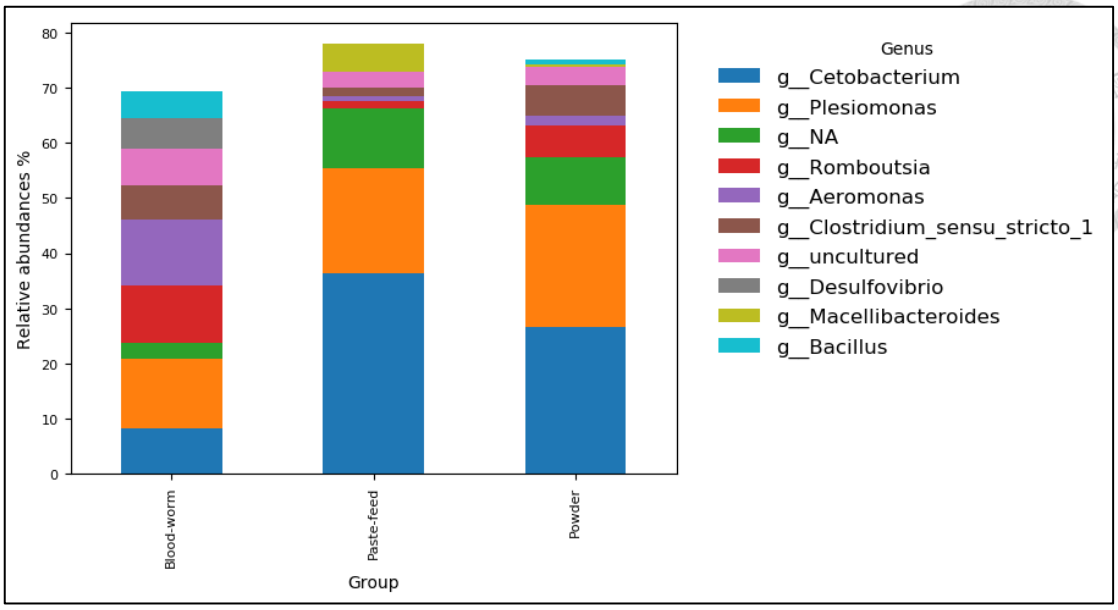
圖十五、納豆枯草桿菌 NTU-18 實驗中經過 20 天攻毒實驗之日本鰻苗累計存活率 (Lin et al., 2024)

doi: 10.6342/NTU202402135



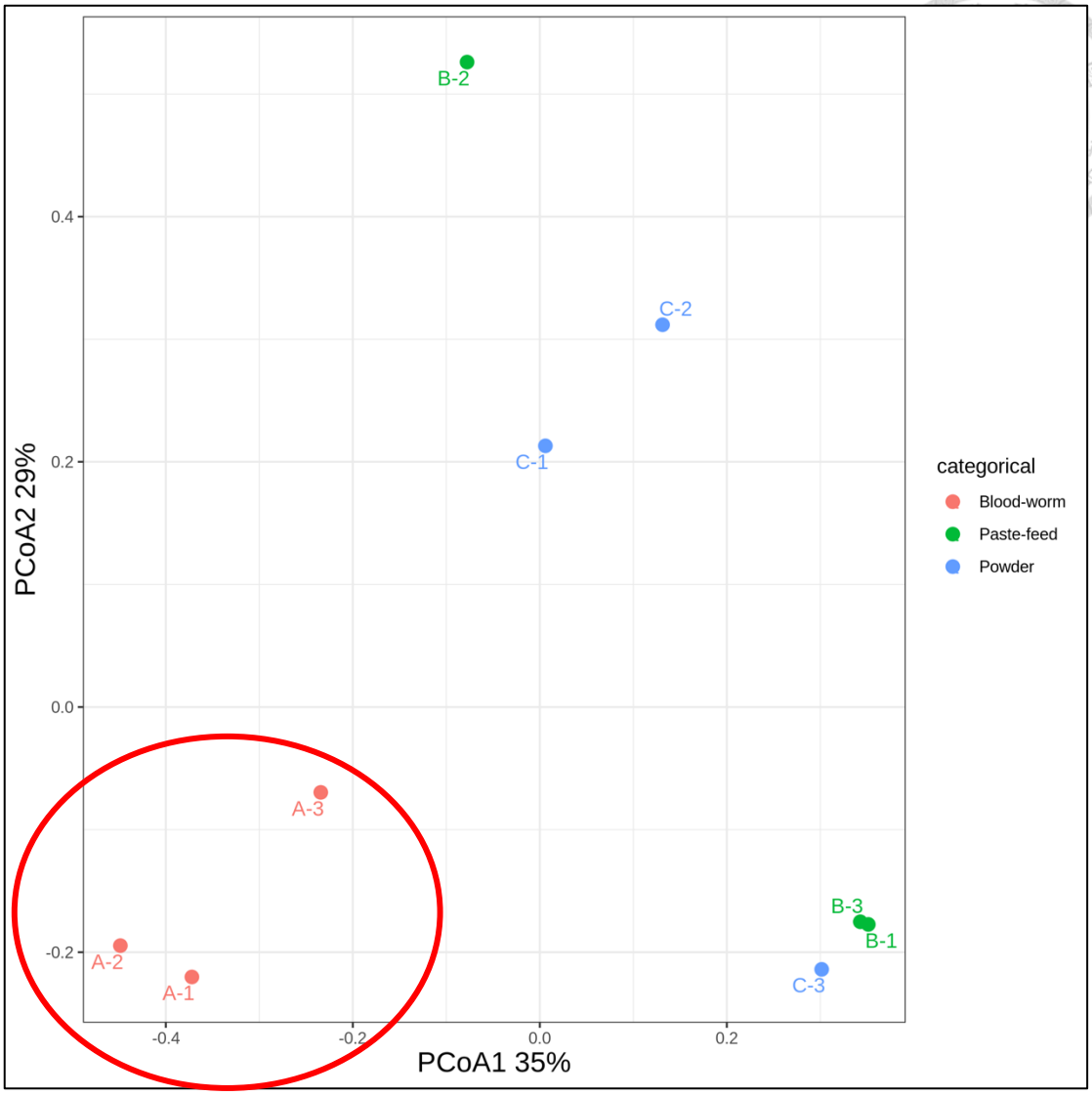
圖十六、餵食不同飼料之黑鰻苗腸道菌相 α 多樣性參數

doi: 10.6342/NTU202402135



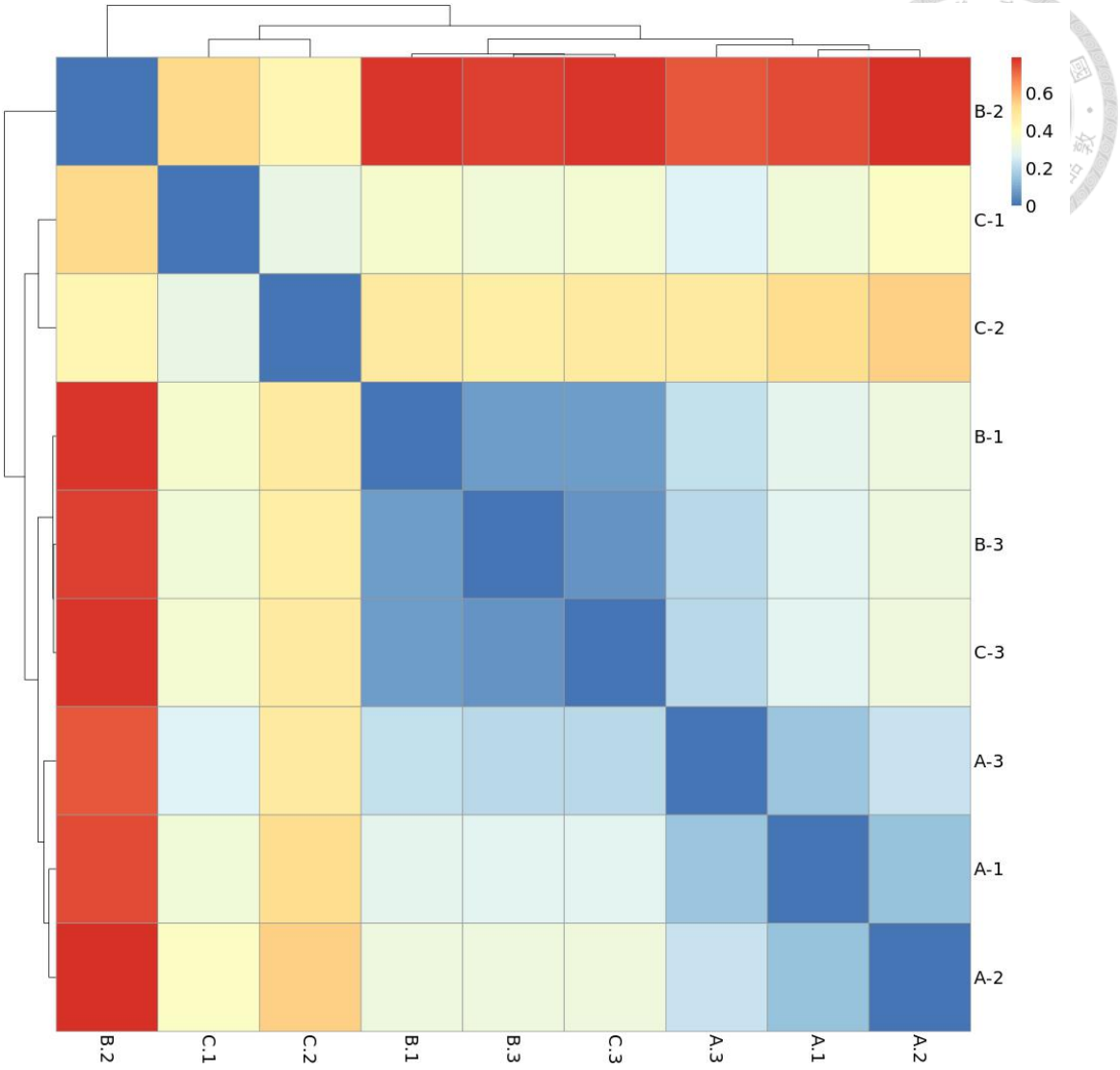
圖十七、餵食不同飼料之黑鰻苗腸道菌相組成（屬層級）

doi: 10.6342/NTU202402135



圖十八、餵食不同飼料之黑鰻苗腸道菌相主座標分析 (PCoA)

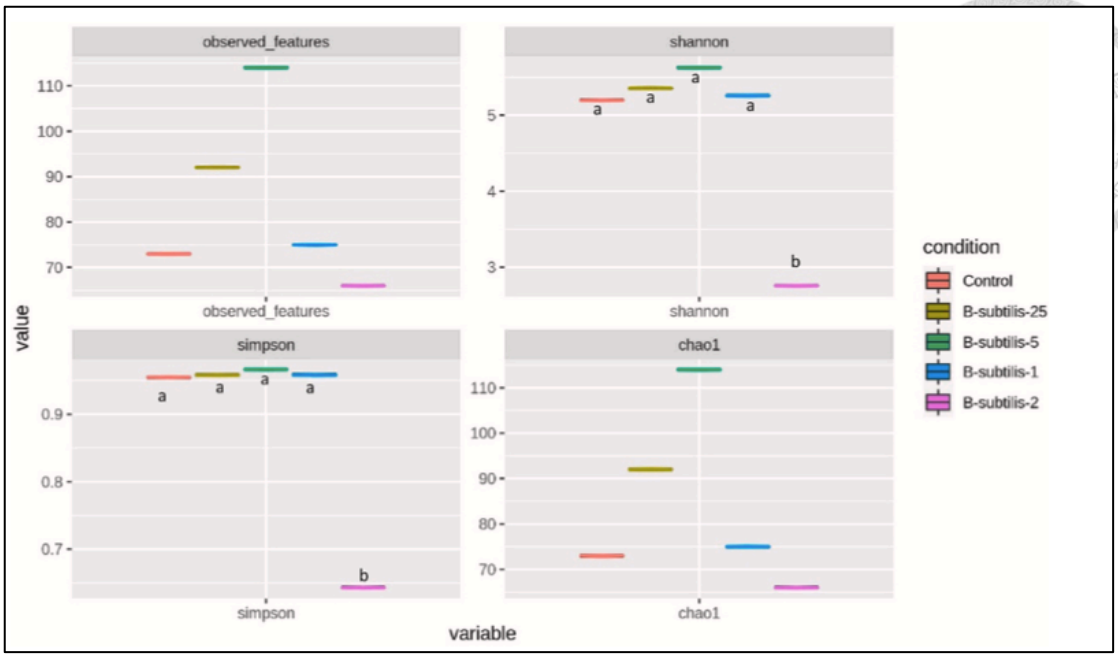
doi: 10.6342/NTU202402135



圖十九、餵食不同飼料之黑鰻苗腸道菌相加權 UniFrac heatmap

(A: 赤蟲、B: 膏狀飼料、C: 鰻粉)

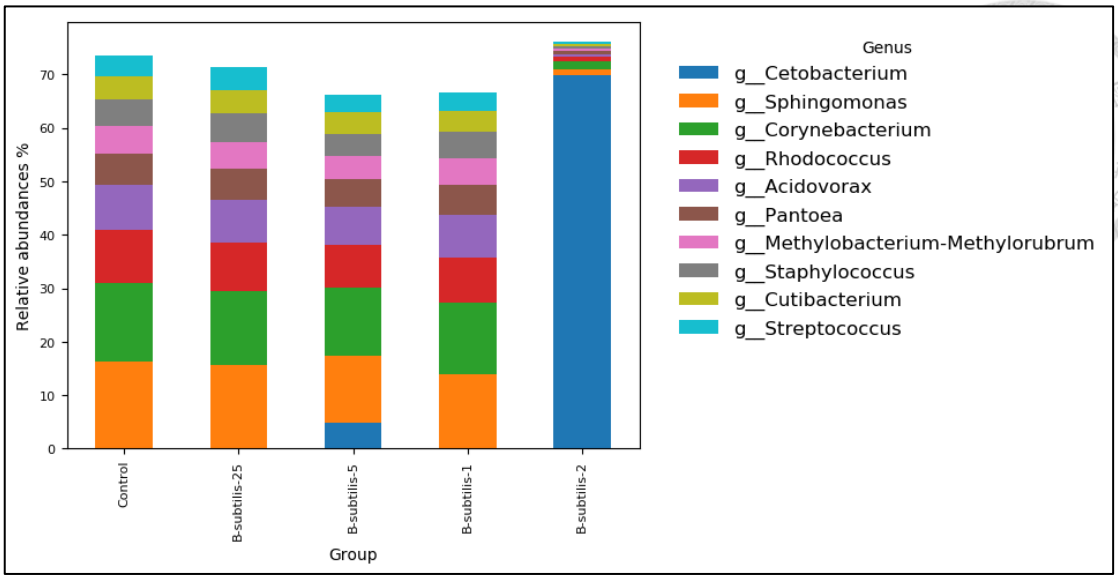
doi: 10.6342/NTU202402135



圖二十、餵食納豆枯草桿菌 NTU-18 之日本鰻苗腸道菌相 α 多樣性參數

(Lin et al., 2024)

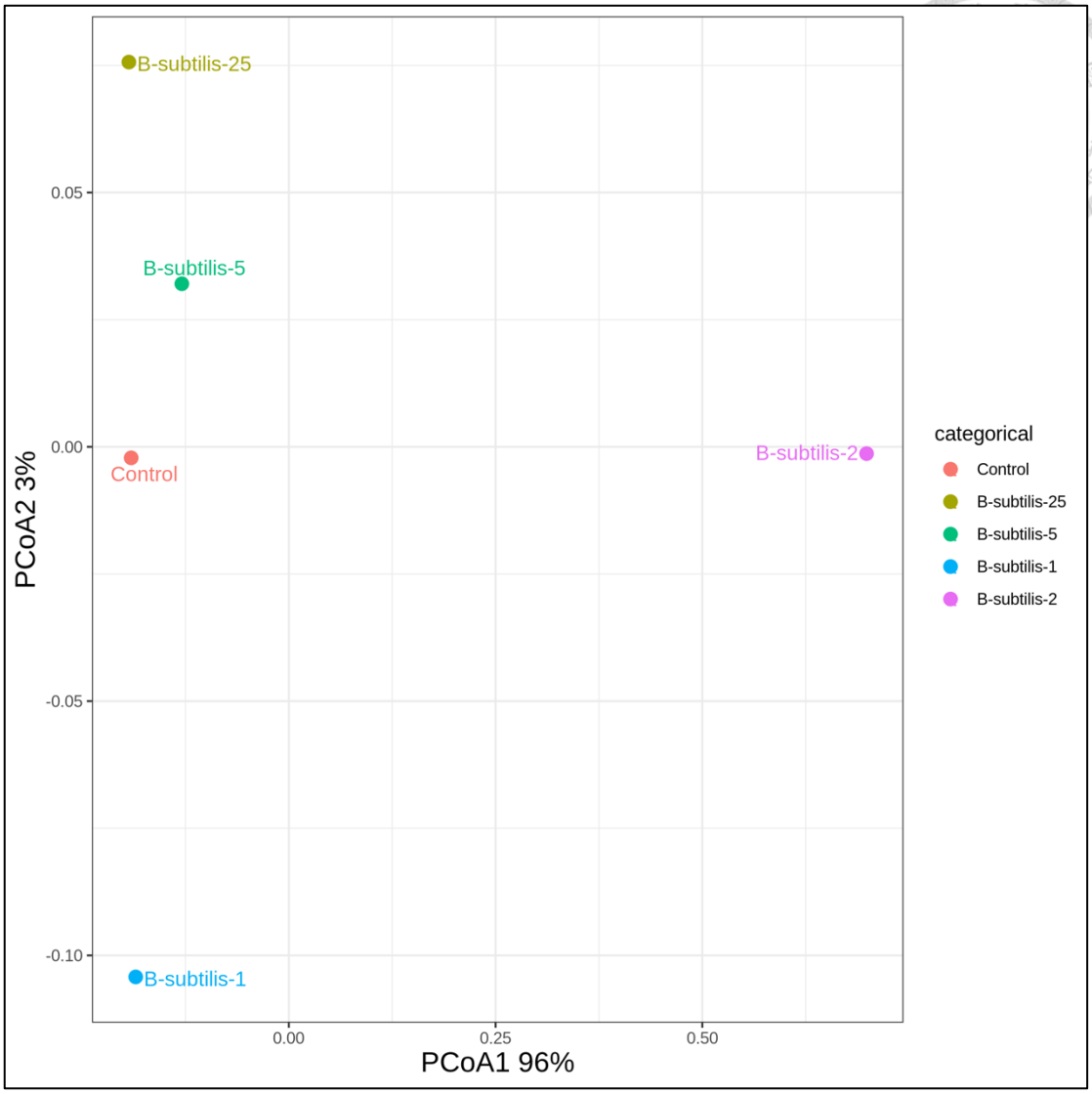
doi: 10.6342/NTU202402135



圖二十一、餵食納豆枯草桿菌 NTU-18 之日本鰻苗腸道菌相組成（屬層級）

(Lin et al., 2024)

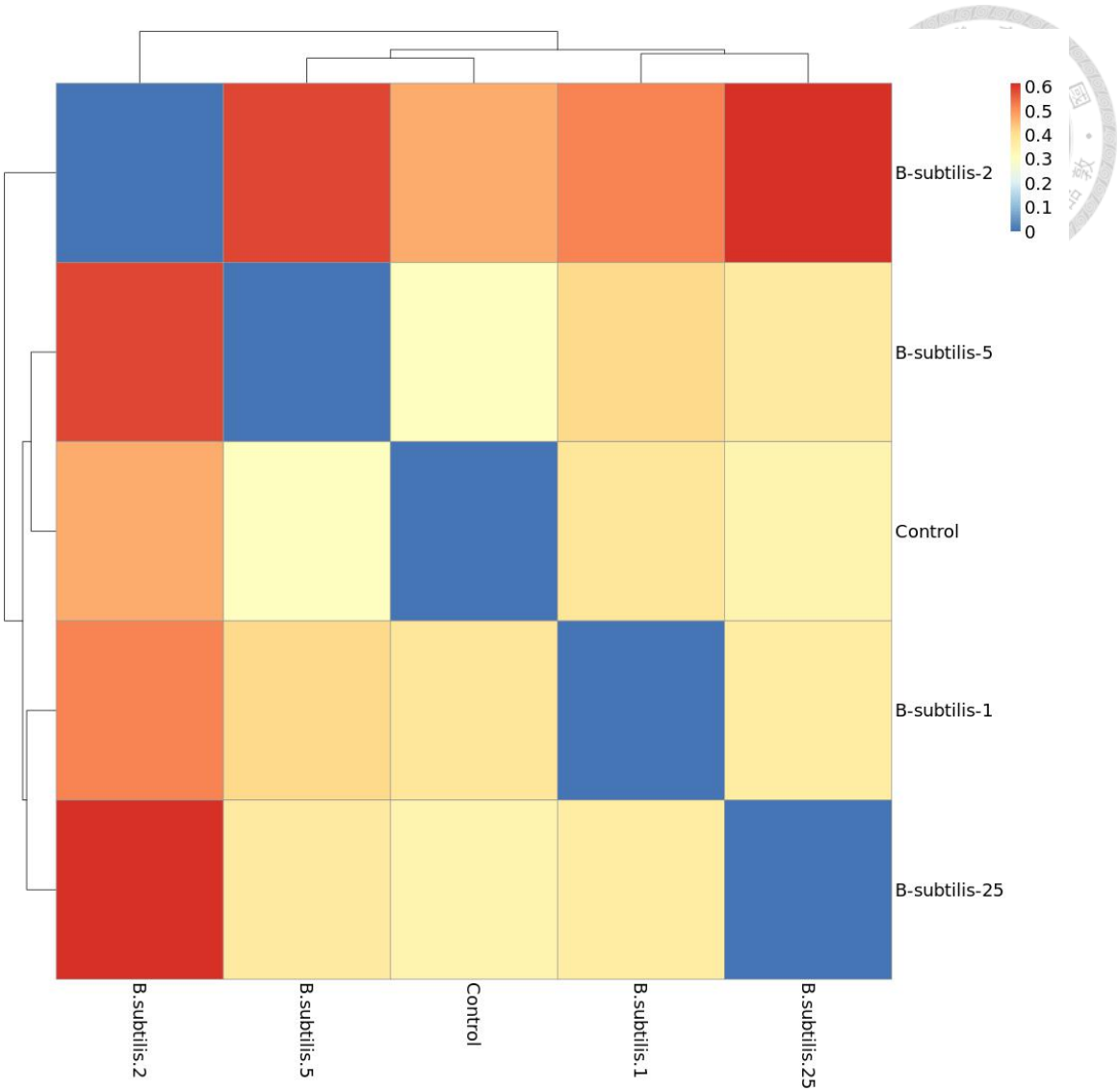
doi: 10.6342/NTU202402135



圖二十二、餵食納豆枯草桿菌 NTU-18 之日本鰻苗腸道菌相主座標分析 (PCoA)

(Lin et al., 2024)

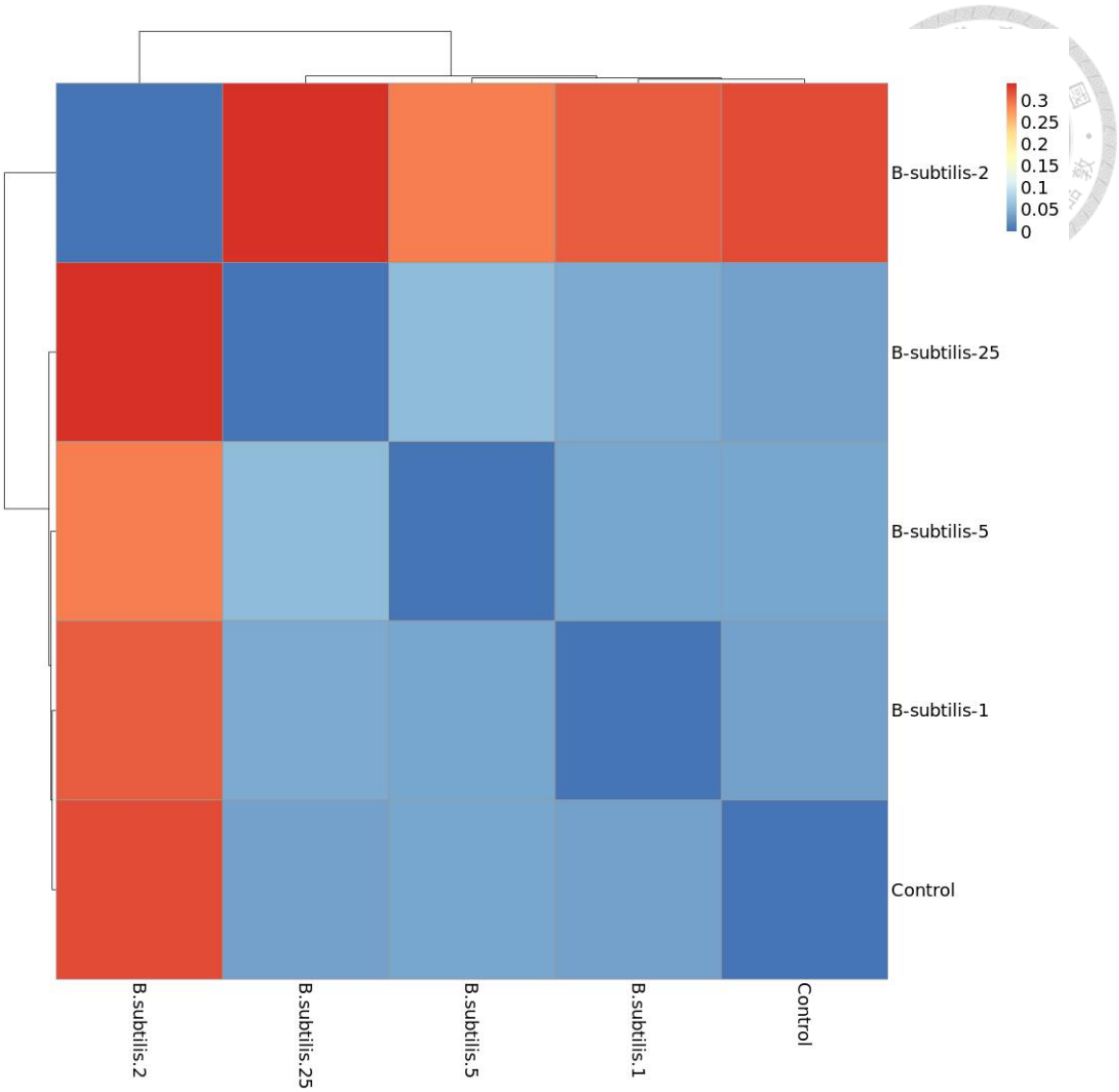
doi: 10.6342/NTU202402135



圖二十三、餵食納豆枯草桿菌 NTU-18 之日本鰻苗腸道菌相

未加權 UniFrac heatmap (Lin et al., 2024)

doi: 10.6342/NTU202402135



圖二十四、餵食納豆枯草桿菌 NTU-18 之日本鰻苗腸道菌相

加權 UniFrac heatmap (Lin et al., 2024)

doi: 10.6342/NTU202402135

補充資料：

一、原料測試：

補表一、水試所配方

原料	
下雜魚漿 (%)	80
豬肝 (%)	10
蛋黃 (%)	5
Guar gum (%)	4.8
Vitamins premix (%)	0.1
Mineral premix (%)	0.1
散失率 (%)	36
鱸鰻苗適口性	差

補表二、蝦漿配方 (南極蝦)

原料	
南極蝦漿 (%)	85
南極蝦粉 (%)	10
Guar gum (%)	4.8
Vitamins premix (%)	0.1
Mineral premix (%)	0.1
散失率 (%)	40
鱸鰻苗適口性	適中，但易污染水質

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補表三、蝦漿配方 (赤尾青)

原料	
赤尾青漿 (%)	80
白魚粉 (%)	15
Guar gum (%)	4.8
Vitamins premix (%)	0.1
Mineral premix (%)	0.1
散失率 (%)	25
鱸鰻適口性	不佳，有過多碎殼

補表四、白肉魚漿配方 (鯊魚、旗魚、鬼頭刀)

原料	
白肉魚漿 (%)	75
白魚粉 (%)	15
魚油 (%)	2.5 2.5
乳化劑 (%)	
Guar gum (%)	4.8
Vitamins premix (%)	0.1
Mineral premix (%)	0.1
散失率 (%)	16
鱸鰻苗適口性	以旗魚表現最佳， 其他魚種易有筋膜殘留

doi: 10.6342/NTU202402135

補表五、混合漿 (魚、蝦、頭足類混合)配方

原料	
白肉魚漿 (%)	50
赤尾青漿 (%)	30
魷魚漿 (%)	10
魚油 (%)	2.5
乳化劑 (%)	2.5
Guar gum (%)	4.8
Vitamins premix (%)	0.1
Mineral premix (%)	0.1
散失率 (%)	14
鱸鰻適口性	適中，但效果不及日本製

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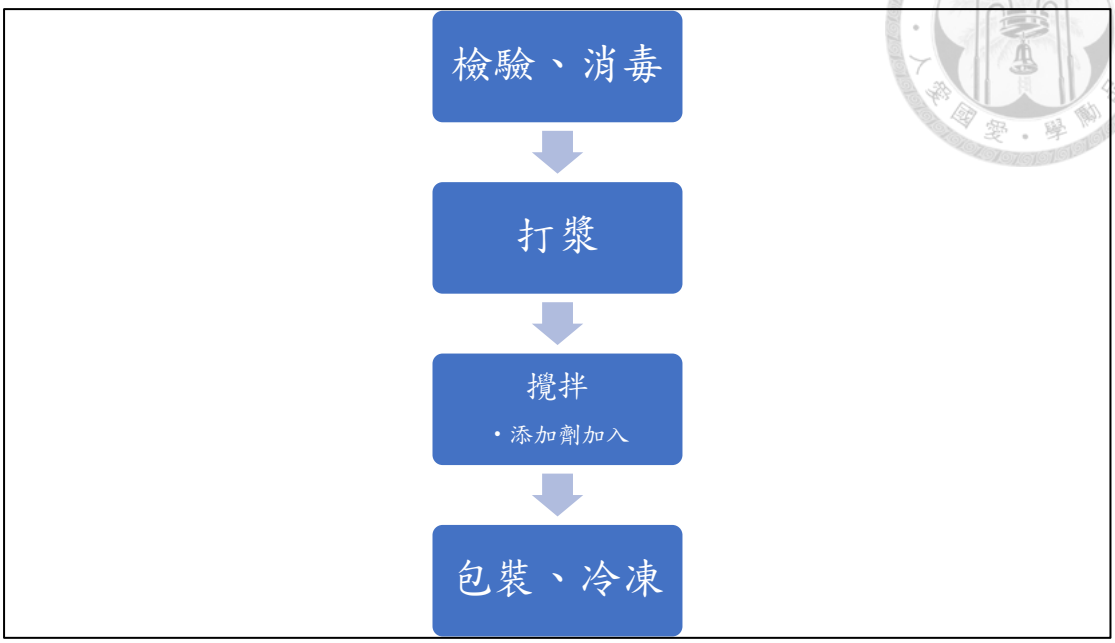
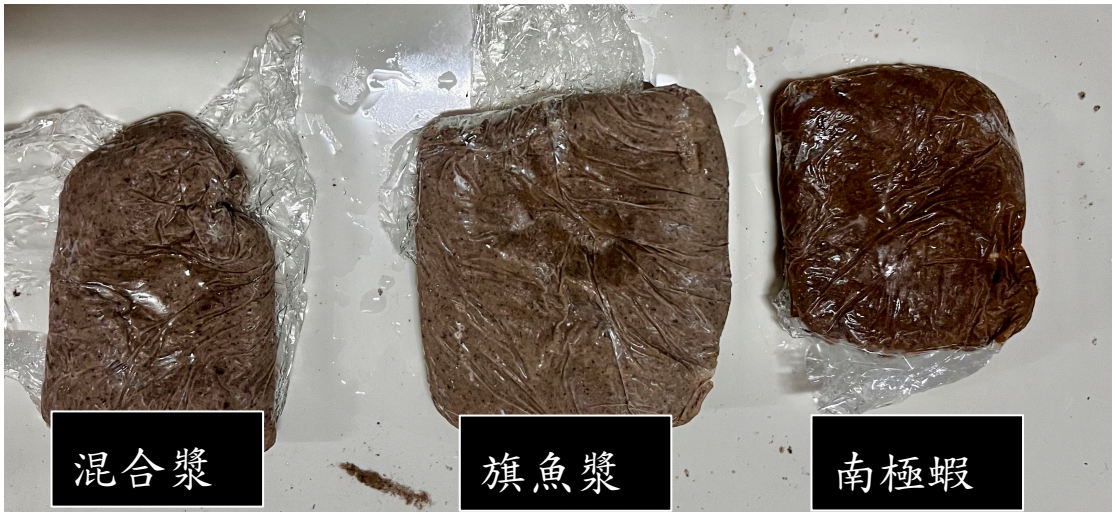
二、製程改良



補圖一、最原始製程，尚未使用擂潰及筋濾機。

成品之水中穩定性及質地一致性皆不佳，鱸鰻苗適口性及成長表現

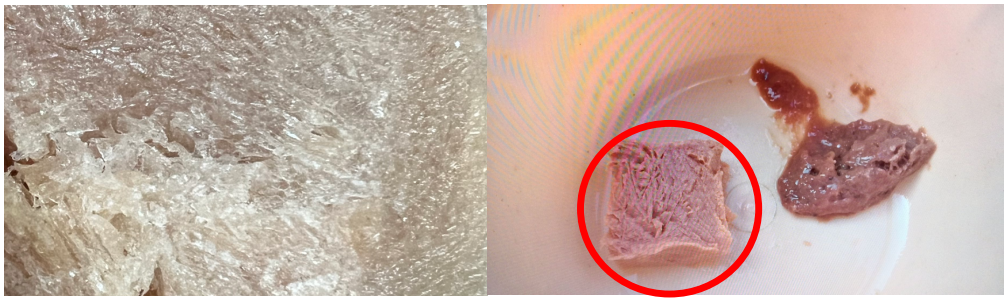
皆遠遜於日本膏狀飼料及赤蟲



補圖二、各式膏狀飼料試做原型

doi: 10.6342/NTU202402135

補圖三、大規模養殖之鰻苗搶食最終版本之膏狀飼料



補圖五、膏狀飼料之層疊狀結構呈現（中間紅圈處為日清膏狀飼料）

doi: 10.6342/NTU202402135

已發表期刊論文全文

1. **Lin, Y. T.**, & Han, Y. S.* (2024). Species diversity of freshwater glass eel (Anguilliformes, Anguillidae) of Yilan, Taiwan, with remark on two new records. *Zookeys*, *In press*. (p.100-109)

2. **Lin, Y. T.**, Hung, Y. C., Chen, L. H., Lee, K. T., & Han, Y. S.* (2024). Effects of adding *Bacillus subtilis natto* NTU-18 in paste feed on growth, intestinal morphology, gastrointestinal microbiota diversity, immunity, and disease resistance of *Anguilla japonica* glass eels. *Fish & Shellfish Immunology*, 109556. (p. 110-121))

3. **Lin, Y. T.**, Pan, Y. F., & Han, Y. S.* (2023). Effects of adding spermidine carbon quantum dots in feed on growth, intestinal morphology, immunity and disease resistance of *Anguilla japonica* and *Anguilla marmorata*. *Aquaculture Reports*, 33, 101847. (p. 122-130)

4. **Lin, Y. T.**, Hung, W. C., Yeh, Y. F., Lu, K. M., Cherng, D. H., & Han, Y. S.* (2023). Effects of different LED light spectra on growth and immunity of the Japanese eel (*Anguilla japonic*a) and giant mottled eel (*A. marmorata*). *Zoological Studies*, 62. (p. 131-142)

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ZooKeys @@@: #–## (2024)
DOI: 10.3897/zookeys.@@@.125590

Research Article

Species diversity of freshwater glass eel (Anguilliformes, Anguillidae) of Yilan, Taiwan, with remark on two new records

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Abstract

Yilan, Taiwan is the first place in East Asia where freshwater glass eels, the juvenile stage of *Anguilla* species, arrive by ocean currents. We collected glass eels by fyke net in Lanyang River estuary twice a month from July 2010 to November 2023. By morphological examination and sequencing of the mitochondrial cytochrome b gene, we identified seven species of *Anguilla*. Most of the glass eels captured in Yilan belonged to the species *A. japonica*, *A. marmorata*, and *A. bicolor pacifica*. Only a few were *A. luzonensis*, and two *A. celebesensis* were recorded. In addition, two species were recorded for the time time from Taiwan; *A. interioris* and *A. borneensis* were confirmed by cytochrome b sequencing. Thus, we increase the number of *Anguilla* species in Taiwan from five to seven.

Key words: *Anguilla borneensis*, *Anguilla interioris*, glass eel, new records

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Introduction

The freshwater eel (*Anguilla* spp.) comprises 16 species and three subspecies (Arai 2016a). All *Anguilla* species are catadromous fish, meaning they migrate to the ocean to spawn (Arai and Chino 2012). The leaf-like larvae of *Anguilla* species, known as leptocephali, are carried by ocean currents and undergo metamorphosis into eel-like juveniles, which are known as glass eels (Tsukamoto et al. 2002; Hatakeyama et al. 2022). Glass-eel fishing is crucial for the eel aquaculture industry, as there are no artificial reproduction techniques for commercial purposes (Okamura et al. 2014). In Taiwan, the dispersal of glass eels is primarily influenced by the Kuroshio Current (Hsiung et al. 2022b). Yilan, Taiwan, is renowned as the largest glass-eel fishing ground in Taiwan due to its proximity to the Kuroshio. Notably, Yilan holds the distinction of being the first location in East Asia where glass eels arrive, establishing it as a significant hub for this crucial stage in the eel life cycle (Han et al. 2016a).

To date, five *Anguilla* species have been identified and recorded in Taiwan (Leander et al. 2012; Han et al. 2016b). Among these, *A. japonica*, *A. marmorata*, and *A. bicolor pacifica* are the most prevalent species (Han 2001; Hsu et al. 2019), while *A. luzonensis* and *A. celebesensis* are notably very rare and primarily observed as glass eel in Taiwan (Teng et al. 2009; Han et al. 2016b). Previous studies suggest that *A. japonica*, *A. marmorata*, and *A. bicolor pacifica* share a

common spawning area near the southern West Mariana Ridge (Kuroki et al. 2009; Arai 2016b), whereas other tropical eel species (*A. celebesensis*, *A. borneensis*, *A. luzonensis*, and *A. interioris*) have been identified near southern Mindanao Island as their spawning grounds (Aoyama et al. 2003; Wouthuyzen et al. 2009; Arai 2014, 2016b). Due to the morphological challenges in distinguishing tropical eel glass eels (Minegishi et al. 2005), DNA barcoding techniques, as highlighted by Wibowo et al. (2021), provide a precise method for the identification of species. Previous research also indicates that mitochondrial cytochrome b gene fragments are suitable for the identification of freshwater eels (Han et al. 2008). This study aims to analyse glass-eel samples captured in Yilan from July 2010 to November 2023. Through DNA sequencing, the goal is to confirm the number of freshwater glass-eel species transported to Taiwan during this period.

Materials and methods

Sample collection

Glass eels were collected twice a month at night using a fyke net positioned in the estuary of the Yilan River (24.7162°N, 121.8352°E) from July 2010 to November 2023. Following the capture, all the samples were immersed in a 95% ethanol solution for measurement and preservation. All freshwater glass-eel specimens were deposited in the Institute of Fisheries, National Taiwan University (**NTUIFS**). Recent research adhered to ethical regulations set forth by the Institutional Animal Care and Use Committee (IACUC) under approval number NTU-110-EL-00152.

Morphological measurement

The method for morphological identification of anguillid glass eels was adapted from Han et al. (2012), and the description of the pigmentation stage followed Fukuda et al. (2013). Four morphological parameters were measured using digital callipers with an accuracy of 0.1 mm: total length (TL), head length (HL), pre-dorsal length (PDL), and pre-anal length (PAL). The fin-difference ratio was then calculated using the formula shown below. Glass eels with fin differences exceeding 13% in Yilan were consistently identified as *A. marmorata* (Han et al. 2012). Therefore, specimens displaying black pigment on the tail and fin differences <13% were chosen for mitochondrial cytochrome b gene sequencing.

$$\text{Fin Difference Ratio}\,(\%) = \frac{PAL(\text{mm}) - PDL(\text{mm})}{TL(\text{mm})} \times 100$$

Mitochondrial cytochrome b gene sequencing

Freshwater glass-eel specimens with a fin-difference ratio <13% were DNA sequenced for precise identification; these amounted to 281 samples. Genomic DNA was extracted from the dorsal-fin tissue of the glass eels using the FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen, Taiwan). Polymerase chain reaction (PCR) was carried out to amplify a segment of mitochondrial cytochrome b using forward primer: cytb-F (5'-GAT GCC CTA GTG GAT CTA CC-3') and reverse primer: cytb-R (5'-TAT GGG TGT TCT ACT GGT AT-3'), which was adapted from Han et al. (2008). The resulting PCR product (approximately

1000 bp) was sequenced using the primers cytb-F or cytb-R (by Genomic Biotech Inc., Taiwan), following protocols from Han et al. (2008). Sequencing results were submitted to the National Center for Biotechnology Information (**NCBI**) GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm species.

Results

Diversity of freshwater glass eel

A total of 29,442 freshwater glass eels were collected between July 2010 and November 2023. The composition of freshwater glass-eel species is shown in Table 1. *Anguilla japonica* and *A. marmorata* were the most prevalent species, comprising 95.4% of our captures (Table 1). Although *A. bicolor pacifica* and *A. luzonensis* were infrequently captured, they still represented 3.9% and 0.7% of all specimens, respectively, and two *A. celebesensis* were also recorded (Table 1). Additionally, two species were found in Taiwan for the first time: *A. interioris* and *A. borneensis* (Table 1).

New *Anguilla* records from Taiwan

The sequencing results of the two new records, total two specimens (NTUIFS IL13'0812-76 and NTUIFS IL21'0715-207), and the best matched BLAST results are shown in Table 2.

Table 1. Number of species (*n*) and percentage contributions of freshwater glass eels collected in Yilan.

Species	*n*	Percentage contributions
A. japonica	14217	48.3
A. marmorata	13864	47.1
A. bicolor pacifica	1152	3.9
A. luzonensis	205	0.7
A. celebesensis	2	<0.01
A. interioris	1	<0.01
A. borneensis	1	<0.01

Table 2. Sequencing BLAST results of three new records freshwater glass eel.

Specimen	Species	Percent identity (%)	NCBI accession
NTUIFS IL13'0812-76	*Anguilla interioris*	99.4	HG965574.1
NTUIFS IL21'0715-207	*Anguilla borneensis*	99.4	NC_006536.1*

*NC_006536.1 was identified as *A. malgumora*, which is a junior synonym of *A. borneensis* according to Minegishi et al. (2005).

Family Anguillidae

***Anguilla interioris* Whitley, 1938**
Figs 1, 2, Table 3

Material examined. NTUIFS IL13'0812-76, 46 mm TL, off the estuary of the Yilan River, Yilan, northeastern Taiwan (24.7162°N, 121.8352°E), 12 August 2013, fyke net, collected by Yu-San Han.

Short description. PDL 29.3% in TL; PAL 39.1% in TL; fin-difference ratio 9.78%. Body elongate, head length 13.1% TL. The specimen was in fresh condition, with black pigment distributed on the caudal fin and slightly on the caudal peduncle; pigmentation stages V_{B2} (Fig. 2).

Distribution. New Guinea (Aoyama et al. 2000); Philippines (glass eel only, Wibowo et al. 2021); Indonesia (leptocephalus only, Kuroki et al. 2006; all stages, Zan et al. 2022); Taiwan (glass eel only, present study).

Remarks. The distribution of *A. interioris* has been primarily known from only New Guinea (Aoyama et al. 2000). However, a study by Kuroki et al. (2006) documented the leptocephalus of *A. interioris* in the Indonesian Archipelago, marking the first expansion of the species beyond its then-known range. Additionally, records of *A. interioris* have been identified using DNA sequencing from Indonesia and southern Mindanao, Philippines (Wibowo et al. 2021; Zan et al. 2022). Herein, we present the first record of *A. interioris* glass eel from Taiwan.

Figure 1. *Anguilla interioris* (NTUIFS IL13'0812-76), 46 mm TL. Preserved in 95% alcohol. Scale bar: 10 mm.

Figure 2. Pigmentation on the tail tip of *Anguilla interioris* (NTUIFS IL13'0812-76).

Table 3. The morphological parameters of seven freshwater glass eel collected in Yilan.

Species	TL (mm)	PDL (mm)	PAL (mm)	Fin-difference ratio (%)
A. japonica	61.1±2.5	15.1±0.9	20.1±0.7	9.2±1.3
A. marmorata	51.4±2.7	11.8±0.8	19.4±1.1	15.5±0.8
A. bicolor pacifica	49.2±2.3	18.3±1.6	18.5±1.6	0.5±0.5
A. luzonensis	52.9±2.7	13.7±0.7	19.4±1.0	11.4±1.1
A. celebesensis	45.3	12.8	17.5	10.4
A. interioris	46.0	13.5	18.0	10.1
A. borneensis	49.5	13.0	18.0	9.8

***Anguilla borneensis* Popta, 1924**
Figs 3, 4, Table 3

Material examined. NTUIFS IL21'0715-207, 49.5 mm TL, off the estuary of the Yilan River, Yilan, northeastern Taiwan (24.7162°N, 121.8352°E), 15 July 2021, fyke net, collected by Yen-Ting Lin.

Short description. PDL 26.3% in TL; PAL 36.4% in TL; fin-difference ratio 10.1%. Body extremely elongate, head length 10.1% TL. The specimen was in fresh condition, with black pigment distributed on the caudal peduncle and caudal fin; pigmentation stages V_A (Fig. 4).

Distribution. Indonesia (Watanabe et al. 2014); Taiwan. (glass eel only, present study).

Remarks. The best-matched GenBank accession number for NTUIFS IL21'0715-207 was found to be NC_006536.1, which corresponds to *A. malgumora* submitted by Minegishi et al. (2005). However, it is noteworthy that *A. malgumora* was identified as a junior synonym of *A. borneensis* by Minegishi et al. (2005). Based on the comprehensive examination by Minegishi et al. (2005) and the detailed de-

Figure 3. *Anguilla borneensis* (NTUIFS IL21'0715-207), 49.5mm TL. Preserved in 95% alcohol. Scale bar: 10 mm.

Figure 4. Pigmentation on the tail tip of *Anguilla borneensis* (NTUIFS IL21'0715-207).

scription provided in NC_006536.1, we can confidently affirm that our specimen NTUIFS IL21'0715-207 is *A. borneensis*. Herein, we report the first record of *A. borneensis* outside of the Indonesia (Watanabe et al. 2014).

Discussion and conclusion

Leptocephali and glass eels primarily rely on ocean currents for transport (Kuroki et al. 2016). In Taiwan, the main current responsible for transporting glass eels is the North Equatorial Current (NEC), followed by the Kuroshio, which is known to carry the most abundant anguillid species (*A. japonica* and *A. marmorata*) to the region (Hsiung et al. 2022a). Additionally, other tropical eels (*A. bicolor pacifica*, *A. luzonensis*, and *A. celebesensis*) may reach Taiwan via the bifurcation region of the NEC near the Philippine coast, which could potentially transport glass eels from southern Mindanao Island to the Kuroshio (Aoyama et al. 2015; Rudnick et al. 2015). The two species identified in our study align with previous research on the diversity of tropical glass eels (*A. celebesensis*, *A. interioris*, and *A. borneensis*) in southern Mindanao (Shirotori et al. 2016).

Previous research based on differences in Sr:Ca ratios in the leptocephalus otoliths has shown the presence of two populations of *A. interioris*, with one population in the Indian Ocean and another in the Pacific Ocean (Kuroki et al. 2006). Furthermore, leptocephali of the Pacific Ocean population of *A. interioris* potentially are transported to Taiwan via the Mindanao Current which ultimately forms a connection with the Kuroshio and the Mindanao Eddy (Kuroki et al. 2006).

The distribution of leptocephali and glass eels of the Indonesian *A. borneensis*, which is considered the most basal *Anguilla* species, remains unclear (Aoyama et al. 2001). The spawning area of *A. borneensis* may overlap with other basal tropical eels (*A. celebesensis*, *A. interioris*, *A. marmorata*, and *A. bicolor bicolor*) in Indonesia in the western Pacific Ocean (Arai and Abdul Kadir 2017); this suggests the possibility that a similar pathway to Taiwan is followed, as by *A. interioris* and *A. celebesensis* (Han et al. 2016b).

Alternatively, it is possible that if *A. borneensis* and *A. interioris* establish a new population in the western Pacific Ocean, their larvae could be carried to Taiwan via the North Equatorial Current (NEC) and the Kuroshio. Additionally, some alien freshwater eel species have escaped from aquaculture ponds and have been reported to have similar migration behaviour of native eel in East Asia (Okamura et al. 2002). Examples include *A. rostrata*, which has been discovered in Taiwanese waters (Han et al. 2002), and the European eel, *A. anguilla*, which was captured in the East China Sea and Japanese waters (Aoyama 2000; Okamura et al. 2002). Therefore, the possibility of alien eel species establishing new populations in the West Pacific Ocean cannot be discounted, whether caused by human activities (*A. rostrata* and *A. anguilla*) or by natural phenomenon (*A. interioris* and *A. borneensis*) (Aoyama 2000; Han et al. 2002).

Although there are seven species of freshwater glass eel recorded in Taiwan, only elvers of *A. japonica*, *A. marmorata*, *A. luzonensis*, and *A. bicolor pacifica* had been found in streams (Tzeng and Tabeta 1983; Watanabe et al. 2013; Hsu et al. 2019). The existence of the elvers and adults of *A. celebesensis*, *A. interioris*, and *A. borneensis* still need confirmation in the field.

In conclusion, the present study increases the number of freshwater glass-eel species in Taiwan from five to seven (Leander et al. 2012; Han et al. 2016b), with the addition two new species records in this paper.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

The experiment and sample collecting were performed by Yu-San Han and Yen-Ting Lin. Yen-Ting Lin write the manuscript. Yu-San Han designed and supervised the experiments. All authors participated in manuscript writing and interpretation of results. All authors read and approved the final manuscript.

Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

NTUIFS IL13'0812-76 sequencing data

Authors: Yen-Ting Lin, Yu-San Han

Data type: txt

Explanation note: The sequencing results of NTUIFS IL13'0812-76.

Link: https://doi.org/10.3897/zookeys.@@@.125590.suppl1

Supplementary material 2

NTUIFS IL21'0715-207 sequencing data

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Full length article

Effects of adding *Bacillus subtilis natto* NTU-18 in paste feed on growth, intestinal morphology, gastrointestinal microbiota diversity, immunity, and disease resistance of *Anguilla japonica* glass eels

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ABSTRACT

Japanese eel, *Anguilla japonica*, holds significant importance in Taiwanese aquaculture. With the intensification of eel farming, the impact of *Edwardsiella tarda* has become increasingly severe. Consequently, the abusive use of antibiotics has risen. *Bacillus subtilis natto* NTU-18, a strain of *Bacillus* with a high survival rate in feed processing, plays a crucial role in promoting intestinal health through competitive rejection, enhancing immune responses against bacterial pathogens, and improving intestinal health by modulating gastrointestinal microbiota to produce beneficial metabolites of mice and grass carp, *Ctenopharyngodon idella*. This study investigated the effects of different proportions (control, 0.25 %, 0.5 %, 1 %, and 2 %) of *B. subtilis natto* NTU-18 added to paste feed on the growth performance, intestinal morphology, and microbiota, expression of immune-related genes, and resistance to *E. tarda* in Japanese glass eel. The results indicated that the growth performance of all groups with *B. subtilis natto* NTU-18 added was significantly higher than that of the control group and did not impact the villi morphology. The expression of immune-related genes in the kidney, specifically HSP70 and SOD, was significantly higher from 0.5 % and above than the control; however, no significant differences were observed in CAT, POD, and HSP90. In the liver, significant differences were found in HSP70 and IgM above 0.25 % compared to the control group, with no significant differences in SOD, CAT, POD, and HSP90 among all groups. Additionally, intestinal microbiota analysis revealed that the 2 % additional group had significantly lower diversity than other groups, with *Cetobacterium* as the dominant species. The challenge test observed that the survival rates of the 0.5 % and 1 % groups were significantly higher. This research suggests that adding 0.5 % and 1 % of *B. subtilis natto* NTU-18 to the diet is beneficial for Japanese glass eel's immunity, growth performance, and disease resistance.

1. Introduction

The Japanese eel, *Anguilla japonica*, holds a prominent position as an economically vital species in the aquaculture industry within East Asia [1]. In the 1990s, Taiwan played a significant role in supplying eels to the Japanese market; however, the eel-culture industry has faced challenges and has declined since then [2]. Traditional outdoor ponds were found to be vulnerable to environmental factors such as rainfall, typhoons, and temperature fluctuations. Responding to these challenges, indoor recirculating aquaculture systems (RAS) gained popularity for eel cultivation due to their resilience against environmental uncertainties [3,4]. Nevertheless, the closed and high-density conditions in highly intensive RAS make them prone to rapid disease transmission [5]. Prolonged exposure to elevated cortisol concentrations, resulting from disease resistance efforts and confined spaces, has been observed to have adverse effects, such as growth retardation and damage in young fish within RAS [6]. Consequently, there is a need to address these challenges and enhance the immunity and growth performance of cultured eels to bolster the competitiveness of RAS in eel aquaculture.

Eels are carnivorous fish that require up to 40–60 % protein in their feed [7]). Traditionally, in the initial stages of eel culturing, farmers often utilize *Chironomus* sp. larvae to feed glass eels [8]. However, this practice has a heightened risk of introducing pathogens into the culturing system [9]. A promising alternative is "paste feed", a novel eel

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feed primarily composed of quarantined fish, squid, and krill [10]. Its high protein content ensures safe and ample nutrition for optimal growth of glass eels [11]. Among various eel feeds, paste feed exhibits lower water pollution compared to powder feed and superior palatability compared to floating feed [12]. Notably, paste feed offers the advantage of easy and uniform incorporation of feed additives, making it highly suitable for experiments and RAS.

Edwardsiella tarda is a significant cause of losses in eel aquaculture for eel farmers [13,14]. The incidence of *E. tarda* outbreaks is prevalent throughout the year due to temperature variations, poor water quality, and a lack of preventive measures, leading to a rapid increase in infection rates [15]. Antibiotics are often used to mitigate losses to combat the vitality and frequent occurrences of *E. tarda*, resulting in the indiscriminate use of these medications [16]. Unfortunately, improper antibiotic use can foster the development of drug-resistant strains, particularly in aquatic animals [17]. The overuse of antibiotics has significant drawbacks, including the emergence of antibiotic resistance, a decline in fish immunity, and concerns related to food safety [18].

Probiotics have emerged as a promising additive to enhance disease resistance in cultured species without resorting to antibiotics [19,20]. Previous studies have highlighted the immune-boosting effects of *Bacillus subtilis* and *B. licheniformis* in *Oreochromis niloticus* [21]. Additionally, *Dicentrarchus labrax* fed with a *Vagococus fluvialis*-supplemented diet exhibited inhibition of *Vibrio anguillarum* proliferation [22]. *Bacillus* spp. has also been recognized for its ability to absorb organic matter, contributing to improved water quality in aquaculture [23], making it a popular choice in the industry. *Bacillus* spp. can survive various conditions, including the gastrointestinal tract of teleosts, since that it is a common additive in aquaculture [24]. Within the *Bacillus*, the strain *B. subtilis natto* has been selected for nearly 120 years since its successful isolation from fermented soy foods [25]. It has been demonstrated that *B. subtilis natto* exhibits no transferable antibiotics and cytotoxicity [26]. Notably, *B. subtilis natto* can withstand feed processing steps and endure the severe environment encountered in the intestine [27–29]. *B. subtilis natto* not only promotes intestinal health through competitive rejection but also produces antimicrobial peptides (AMPs) that are cytotoxic to bacterial pathogens [30–32]. Additionally, *B. subtilis natto* has exhibited anti-inflammatory effects and serves as a drug substitute and growth promoter in humans, *Ctenopharyngodon idella*, and mice [33–36]. Moreover, in previous study *B. subtilis natto* NTU-18 showed good β-glucosidase activity and isoflavone deglycosylation efficiency [37]. Although, the wide range used of *B. subtilis natto* in the aquaculture industry, still a minor concern about evaluating the potential effects of *B. subtilis natto* on growth performance, immune response, and gastrointestinal impacts in the recirculating Japanese glass eel culturing system.

This research aimed to estimate the effects of *B. subtilis natto* NTU-18 on the growth performance, gastrointestinal morphology and microbiota, immune-related genes, and resistance to *E. tarda* in Japanese glass eel. And determined whether adding *B. subtilis natto* NTU-18 to eel paste feed could serve as a potential alternative supplement of antibiotics, aiming to simultaneously enhance the growth and immune performance of Japanese glass eel.

2. Materials and methods

2.1. Experimental diet and probiotic

The experimental paste feed was formulated using a combination of squid mince, fish mince, krill, essential nutrient additives. And *Bacillus subtilis natto* NTU-18 (BCRC 80390, Bioresource Collection and Research Center, Taiwan) was isolated from fermented natto and cultivated following the method by Luo et al. (2006). The final product was uniformized at a concentration of 1×10^7 CFU/g. All fresh ingredients were carefully quarantined by PCR before processing to ensure they were pathogen-free. Following quarantine, the ingredients were ground using a grinder. Then underwent a mixing procedure by an electric mixer for 60 min. The temperature was maintained under 5 °C to keep freshness in whole process. The composition of the diet utilized in this research is detailed in Table 1. Five graded concentrations of *B. subtilis natto*, ranging from 0 % (control) to 0.25 %, 0.5 %, 1 %, and 2 %, were added to the experimental diet during the mixing procedure for Japanese eel. To maintain freshness and pathogen-free condition, all paste feed was stored at −20 °C with vacuum packaging and defrost before every feeding trail.

2.2. Experimental animals and feeding trail

400 Japanese glass eels were captured from Wushi Harbor (24.8691°N 121.8406°E), Yilan, Taiwan, between November 2021 and February 2022 by local fishermen. Upon purchase, the eels were carefully transported to the Institute of Fisheries, National Taiwan University, Taipei, Taiwan, with packaging filled with oxygen and maintained at a constant water temperature of 15 °C. To prevent the introduction of pathogens or parasites into the recirculating aquaculture system (RAS), all eels underwent disinfection by soaking in a 3 ppm potassium permanganate (KMnO4) solution for 5 min. The eels were then divided into 20 indoor RAS tanks (30 × 30 × 45 cm), with each tank accommodating 20 eels for a one-week acclimation, and paste feed with 0 % *B. subtilis natto* NTU-18 (control) was supplied during the acclimation time. Initial body weight (IBW) and total length of the Japanese glass eel were measured to ensure there were no significant differences among the groups (59.9 ± 0.7 mm, 187.7 ± 7.9 mg) (Table 2.) before the feeding trial. The eels were then divided into five groups, each fed with different concentrations of *B. subtilis natto* NTU-18: 0 % (control), 0.25 %, 0.5 %, 1 %, and 2 %. The experiment was conducted with four replicates.

The feeding trial was conducted twice daily, at 10 a.m. and 6 p.m., six days a week. The feeding amount was set at 5 % of the total eel wet weight, and any residual bait and feces were cleaned up 1 h after each feeding session. The photoperiod was maintained at 12 h of light (7:00–19:00) and 12 h of darkness (19:00–07:00). The indoor RAS was maintained in optimal conditions for Japanese eel, with dissolved oxygen reaching almost saturation through complete aeration by solid aeration. The water temperature was kept between 22 and 24 °C, pH levels were maintained at 7.0–7.2, ammonia nitrogen was kept below 0.05 ppm, and nitrite levels below 0.02 ppm. The water exchange rate was approximately 30 L/h, and the exchange water was flowing through a UV light for disinfection. Also, 5 % of filtered water was added to the RAS daily to maintain the water quality. The Japanese eels were reared for 10 weeks under the feeding trial. All experiments adhered to the procedures and guidelines by the Institutional Animal Care and Use Committee (IACUC) for the welfare of laboratory animals, as the approval number “NTU110-00152”.

2.3. Sample collection

Body length and weight were measured every 14 days, and starved for 1 day before the measurement to reduce the error. When measuring, the eels were first anesthetized in 150 ppm of 2-Phenoxyethanol solution for 15 min. Initial body weight (IBW) was measured before the

Table 1
Composition of the experimental paste feed.

Ingredients	Control	0.25 %	0.5 %	1 %	2 %
Squid mince (%)	70	70	70	70	70
Fish mince (%)	20	20	20	20	20
Krill (%)	5	5	5	5	5
Guar gum (%)	4.8	4.55	4.3	3.8	2.8
Vitamins premix (%)	0.1	0.1	0.1	0.1	0.1
Mineral premix (%)	0.1	0.1	0.1	0.1	0.1
Bacillus subtilis natto NTU-18 (%)	0	0.25	0.5	1	2

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Table 2
Effects of dietary *B. subtilis natto* NTU-18 on the growth performance of *Anguilla japonica*.

	Control	0.25 %	0.5 %	1 %	2 %
Initial TL (mm)	59.89 ± 0.81[a]	60.00 ± 0.45[a]	60.12 ± 0.83[a]	60.06 ± 0.78[a]	59.89 ± 0.46[a]
Final TL (mm)	94.07 ± 17.1[a]	99.75 ± 8.7[b]	103.19 ± 12.4[b]	104.60 ± 10.1[b]	101.85 ± 9.4[b]
IBW (mg)	184.9 ± 7.2[a]	185.6 ± 9.5[a]	187.9 ± 9.7[a]	186.4 ± 8.8[a]	187.7 ± 12.1[a]
FBW (mg)	687.6 ± 39.3[a]	832.0 ± 61.0[b]	933.4 ± 72.4[c]	954.7 ± 32.7[c]	854.8 ± 63.5[b]
SGR (%)	1.87 ± 0.43[a]	2.14 ± 0.25[b]	2.29 ± 0.31[c]	2.33 ± 0.37[c]	2.16 ± 0.31[b]
PWG (%)	271.8 ± 38.4[a]	348.2 ± 52.1[b]	396.8 ± 43.7[c]	412.2 ± 57.1[c]	355.8 ± 65.2[b]
FE (%)	62.39 ± 14.2[a]	72.58 ± 16.5[b]	81.27 ± 16.3[c]	84.32 ± 11.4[c]	74.34 ± 14.7[b]
FCR	1.60 ± 0.14[a]	1.38 ± 0.16[b]	1.23 ± 0.13[c]	1.19 ± 0.07[c]	1.35 ± 0.18[b]
SR (%)	98.75 ± 0.01[a]	98.75 ± 0.01[a]	98.75 ± 0.01[a]	98.75 ± 0.01[a]	100 ± 0.00[a]
Final K	0.83 ± 0.11[a]	0.84 ± 0.18[a]	0.85 ± 0.07[a]	0.83 ± 0.10[a]	0.85 ± 0.12[a]

TL: total length; BW: body weight; SGR: specific growth rate: PWG: percentage weight gain; FE: feeding efficiency ratio; FCR: feed conversion ratio; SR: survival rate; K: condition factor.
Different letters indicate significant differences between groups ($p < 0.05$).

experiment begins, and body length and body weight were measured every two weeks to ensure stable growth of eels. The FBL (Final body length), FBW (Final body weight), PWG (Percentage weight gain), SGR (Specific growth rate), K (Condition factor), FE (Feeding efficiency ratio), FCR (Feed conversion ratio), and SR (Survival rate) were calculated to determine the growth performance between each group. Equations as below:

$$Percentage\ weight\ gain\ (\%) = \frac{FBW\ (g) - IBW\ (g)}{IBW\ (g)} \times 100$$

$$Specific\ growth\ rate\ (\%) = \frac{\ln(FBW\ (g)) - \ln(IBW\ (g))}{70} \times 100$$

$$Condition\ factor = \frac{FBW\ (g)}{FBL^3\ (cm)} \times 1000$$

$$Feeding\ efficiency\ ratio\ (\%) = \frac{FBW\ (g) - IBW\ (g)}{Dry\ feed\ intake\ (g)} \times 100$$

$$Feed\ conversion\ ratio = \frac{Dry\ feed\ intake\ (g)}{FBW\ (g) - IBW\ (g)}$$

$$Survival\ rate\ (\%) = \frac{Final\ number\ of\ fish}{Initial\ number\ of\ fish} \times 100$$

After 10-week of feeding, four eels were selected from every group randomly and promptly immersed in a 700 ppm 2-phenoxyethanol solution before undergoing decapitation. Following sacrifice, the liver and head kidney of the four eels in the same group were put in the tissue RNA protecting reagent (Bioman Scientific, Taipei) and placed under −80 °C immediately together for subsequent real-time PCR analysis. Moreover, the intestines of the four eels were meticulously cleaned and placed in a 10 % formalin solution for histological and morphology assessment.

2.4. Intestine histology and morphology

The middle intestine of the eel was removed for observing the morphology. The method was modified from Lin et al. [38]. First, fixed the dissected intestine with 10 % formalin for 72 h. Gradually dehydrate in an aqueous alcohol solution, then embedded the tissue by paraffin. Cut with a rotary microtome to a thickness of 4 μm and spread the slices in water bath at 50 °C. After drying, underwent hematoxylin and eosin stain, at last sealing. Then observed whether there is difference in intestinal histology under light microscope and calculated the height of the villi and the thickness of the intestinal muscular layer by SPOT software (SPOT 5.0, Netherlands).

2.5. Real-time PCR (RT-PCR)

The liver and head kidney of the eel was homogenized with a grinding rod. Subsequently, 800 μl of TRIzol reagent (Invitrogen, USA) was added, and the mixture was incubated at 25 °C for 5 min. Following this, 160 μl of chloroform (Invitrogen, USA) was added, then centrifuged at 13,000 rpm for 15 min under 4 °C. The supernatant was combined with 400 μl of isopropyl alcohol and subjected to centrifugation at 12,000 rpm at 4 °C for 10 min. After careful removal of the supernatant, 1 ml of 75 % ethanol was added to wash the pellet, followed by centrifugation at 13,000 rpm for 5 min under 4 °C. Post-centrifugation, the liquid was aspirated, and the pellet was air-dried at 25 °C for 5 min. Subsequently, re-dissolution was facilitated by adding 20 μl of 60 °C RNase-free water, and the concentration was determined using a spectrophotometer (Thermo Scientific, USA). After RNA extraction, cDNA synthesis was performed using 2 μl of RNA from each group through reverse transcription with the Quant Nova Reverse Transcription Kit (Qiagen, Germany). The concentration of the synthesized cDNA was measured using a thermal cycling meter (ND-1000 spectrophotometer, Nano Drop Technologies, Inc., DE, USA).

Six immune-related genes, namely superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), heat shock protein (HSP) 70, HSP90, and immunoglobulin M (IgM), were selected as target genes for RT-PCR, with acid ribosomal protein (ARP) chosen as the reference gene. Those genes chose in RT-PCR were according to previous about Japanese eel immunology [13,38]. The RT-PCR primers are listed in Table 3. The RT-PCR reaction conditions included preincubation (95 °C, 10 min), 40 cycles of denaturation (95 °C, 15 s) and amplification (60 °C, 15 s), and a melting curve step (95 °C, 15 s). Each sample was tested in four replicates. After obtaining the CT values for each group, the ARP values were calculated using the $2^{-\Delta\Delta CT}$ analysis in the relative quantitative method to compare performance differences between the groups [39].

2.6. Antibacterial ability of B. subtilis natto

The bacteriostasis circle of *B. subtilis natto* NTU-18 on the *E. tarda*-coated agar was assessed using the inhibition zone method modified from previous study [38,40]. *B. subtilis natto* NTU-18 with gradient concentrations (0, 0.25 %, 0.5 %, 1.0 %, and 2.0 %) were applied to 8

Table 3
List of immune-related gene primers used for RT-PCR.

Genes	Primer	Sequences
ARP (reference gene)	Forward	5′-GTGCAGCTCATTAAGACCGG-3′
	Reverse	5′-GGCGATATTCCTCACACCCT-3′
SOD	Forward	5′-TAACGTACGACTATGGGGCC-3′
	Reverse	5′-GCCGCCACCATTAAACTTCA-3′
POD	Forward	5′-GACATCACCCGTTTCTGCAA-3′
	Reverse	5′-GTGGATGAAGGAGGGGAACA-3′
HSP90	Forward	5′-GTGGTGGACTCTGAGGAT-3′
	Reverse	5′-CGAGACACTTCTTGACGATA-3′
HSP70	Forward	5′-CCATCCTGACCATCGAAGAC-3′
	Reverse	5′-GTTCTCTTGGCCCTCTCACA-3′
IgM	Forward	5′-CGGTTCTTCTGACAATCG-3′
	Reverse	5′-TCGGGCACAGTAATACAC-3′
CAT	Forward	5′-ATGGTGTGGGACTTCTGGAG-3′
	Reverse	5′-AGTGGAACTTGCAGTAGAAACG-3′

ARP: acid ribosomal protein; SOD: superoxide dismutase; POD: peroxidase; HSP90: heat shock protein 70; HSP90: heat shock protein 90; IgM: immunoglobulin M; CAT: catalase.

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mm thick paper disks (50 **μl** each) and placed on Tryptic Soy Broth (TSB) agar (Neogen, USA) medium coated with *E. tarda*, then cultured for 1 day (24 h) under 30 °C in an incubator. Then determined the antibacterial ability of *B. subtilis natto* NTU-18 by the diameter of the bacteriostasis circle observed on the TSB agar.

2.7. Challenge test

To estimate the effect on disease tolerance from the *B. subtilis natto* NTU-18 supplied diet, the *E. tarda* challenge test was conducted right after the feeding trail. The *E. tarda* was obtained from the School of Veterinary Medicine, National Taiwan University, Taipei. This bacteria strain was isolated from the sicked *A. japonica* and confirm by 16S RNA sequencing. *E. tarda* was cultured in TSB (Neogen, USA) at 30 °C for 24 h before the challenge test. The growth performance of *E. tarda* was measured by plate counting by colony counter on TSB agar (Neogen, USA). The bacterial colony was undergone PCR for identification [41]. In each group, 12 eels were randomly selected and exposed to a 1×10^8 CFU ($2 \times LD_{50}$) *E. tarda* solution for 4 h with aeration [42,43]. The experiment was conducted with four replicates. Subsequently, the eels were returned to the same RAS tank for a 20-day challenge test with the same culturing condition in the feeding trail. Daily mortality was recorded, and the liver and gut samples of the dead fish were dissected for PCR analysis to confirm *E. tarda* infection as the cause of the death.

2.8. Gastrointestinal microbiota metagenomics analysis

For evaluating the effects from *B. subtilis natto* NTU-18 on Japanese glass eel intestinal microbiota, two eel per group would randomly choose after the feeding trail. Followed by one day fast, the Japanese eel midgut was dissected for the microbiota analysis. The V3–V4 regions of the gastrointestinal bacterial 16S ribosomal DNA gene were extracted using the QIAamp DNA Micro Kit (Qiagen, Germany) and amplified by PCR with the KAPA HiFi HotStart PCR Kit (Roche, Sweden). The purified amplicons were pooled equimolarly and subjected to paired-end sequencing (2×250 bp) on Illumina MiSeq platform (Illumina, USA). Raw fastq files underwent demultiplexing, quality filtering, and analysis at Genomics Inc., Taiwan. The observed features, Chao1, Shannon, and Simpson diversity parameters for all samples were calculated based on Kers and Saccenti [44], then followed with the alpha and beta diversity analysis. The index was calculated as below:

Observed features: The number of taxa, most often defined as an operational taxonomic unit (OTU) or amplicon sequence variant (ASV) observed.

Chao1: An abundance-based nonparametric estimator for taxa richness.

Shannon diversity (H): H is an estimator of taxa diversity, combining richness and evenness, which places greater emphasis on taxa richness.

Simpson's Index (D): D is an estimator of taxa diversity, combining richness and evenness, which places greater emphasis on taxa evenness. Ranging from 0 to 1, the index increases as diversity decreases.

$$Chaol = s + \frac{F_1(F_1 - 1)}{2(F_2 - 1)}$$

$$D = \frac{1}{\sum_{i=1}^{s} p_i^2}$$

$$H = -\sum_{i=1}^{s} p_i \, log(p_i)$$

s: number of OTU/ASV; F1: number of OTU/ASV with only one sequence; F2: number of OTU/ASV with two sequences; p_i: proportion of the community represented by the ith OTU/ASV.

2.9. Statistical analysis

All data are presented as mean ± standard deviation (SD). Differences in growth parameters, Shannon index, and Simpson index were analyzed using one-way ANOVA in SPSS 24.0 for Windows (IBM SPSS Statistics 24.0). If a statistically significant difference ($p < 0.05$) was observed, post-hoc analysis was performed using Tukey's Honestly Significant Difference (HSD) multiple range test to examine and compare differences between the groups. For the challenge test, the survival rates of each group were compared using the Log Rank (Mantel-Cox) test. A significance level of $p < 0.05$ was adopted for all statistical analyses.

3. Results

3.1. Growth performance

The results for FBW, PWG, SGR, K, FE, FCR, and SR are presented in Table 2. At the beginning of the experiment, all groups showed no significant differences between IBW and initial total length (TL). After 10 weeks of feeding trail, the 0.5 % and 1 % groups exhibited significantly highest FBW, PWG, SGR, FE, and FCR compared to other groups ($p < 0.05$). Additionally, all *B. subtilis natto* NTU-18 treated groups demonstrated significantly higher results than the control group ($p < 0.05$) (Table 2). There were no significant differences in K and survival rate among all groups ($p > 0.05$).

3.2. Intestinal histology and morphology

After feeding Japanese eel with different concentrations of *B. subtilis natto* NTU-18 containing paste feed for 10 weeks, there was no significant difference in the mean length of the villi and the muscular layer thickness among all groups ($p > 0.05$) (Table 4). As the results revealed in Fig. 1, there is also not significant difference in intestine morphology (Fig. 1).

3.3. Expression of immune-related genes

The expression of immune-related genes in the liver is presented in Fig. 2. It revealed a significant difference in HSP70 between the groups containing above 0.5 % *B. subtilis natto* NTU-18 and the control group ($p < 0.05$). All groups above 0.25 % showed significantly higher IgM than the control group ($p < 0.05$). However, there was no significant difference in SOD, CAT, POD, and HSP90 among each group ($p > 0.05$) (Fig. 2).

Fig. 3 shows the expression of immune-related genes in the head kidney. When the *B. subtilis natto* NTU-18 concentrations above 0.5 % in the paste feed, expression of HSP70 was significantly higher than control group in the head kidney ($p < 0.05$). All groups with *B. subtilis natto* NTU-18 above 0.25 % showed significantly higher IgM expression compared to the control group ($p < 0.05$). However, there was only a significant difference between the 0.5 % group and the other groups in SOD in head kidney ($p < 0.05$), and there was no significant difference in CAT, POD, and HSP90 between each group in the head kidney ($p > 0.05$) (Fig. 3).

Table 4
Intestinal morphology of *A. japonica* after *B. subtilis natto* NTU-18 feeding trail.

	Control	0.25 %	0.5 %	1 %	2 %
Villi length (μm)	45.1 ± 5.6[a]	48.5 ± 4.1[a]	44.5 ± 4.3[a]	49.2 ± 5.0[a]	45.4 ± 4.5[a]
Muscular layer thickness (μm)	6.85 ± 1.02[a]	7.05 ± 1.16[a]	7.12 ± 0.96[a]	7.09 ± 0.88[a]	6.92 ± 1.04[a]

Different letters indicate significant differences between groups ($p < 0.05$).

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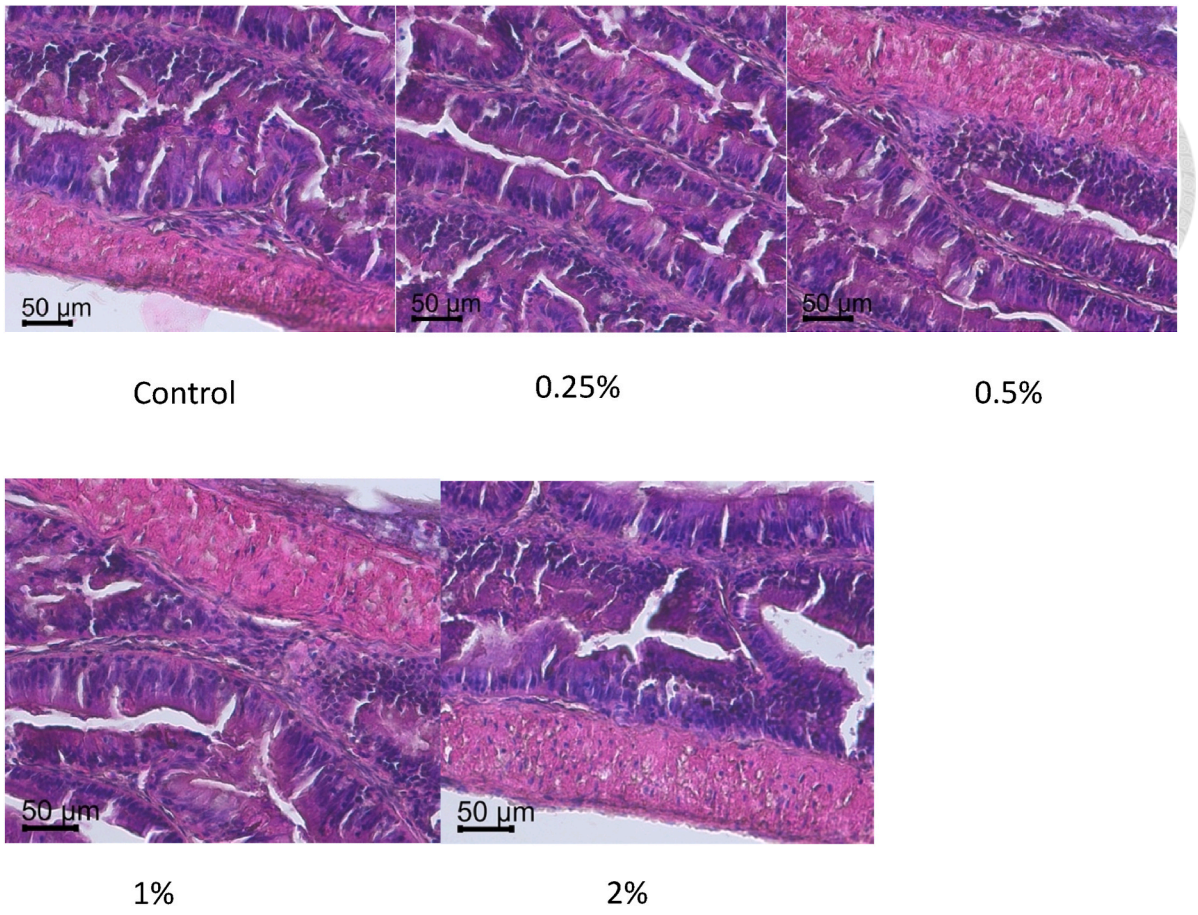

Fig. 1. Details of the intestinal histological of *A. japonica* with different concentration of *B. subtilis natto* NTU-18 after feeding trail. (Staining; scale bar = 100 μm; Original magnification ×200).

3.4. In vitro antibacterial experiment

The *E. tarda* inhibition zone of *B. subtilis natto* NTU-18 is presented in Fig. 6. Only the 2 % of *B. subtilis natto* NTU-18 had showed the antibacterial performance against to the *E. tarda* (Fig. 4).

3.5. Challenge test

Fig. 5 show the survival rate of *A. japonica* glass eels underwent challenge test by *E. tarda*. The first mortality occurred on the third day in control group of the challenge test, and DNA was extracted from the liver of the deceased eel for PCR analysis to confirm the presence of *E. tarda*. At the conclusion of the 20-day challenge test, the survival rates (SR) of fish fed with diets containing 0.5 % (SR: 59.5 %) and 1 % (SR: 52.3 %) *B. subtilis natto* NTU-18 were significantly higher than those fed with control (SR: 19.7 %) and 2 % (SR: 23.4 %) diets ($p < 0.05$) (Fig. 5).

3.6. Gastrointestinal microbiota diversity

The results of alpha diversity were showed in Fig. 6. For observed features and Chao1, the 0.5 % group exhibited the highest value among all groups. The Simpson and Shannon indices in the control, 0.25 %, 0.5 %, and 1 % groups were significantly higher than the 2 % group ($p < 0.05$) (Fig. 6).

Fig. 7 shows the relative abundance of gastrointestinal bacteria at the class level. *Acinobacteria*, *Gammaproteobacteria*, and *Alphaproteobacteria* were the main dominant bacteria in all groups except the 2 % group, which *Fusobacteriia* and *Clostridia* showed a much higher ratio (Fig. 7). The relative abundance of intestinal microbiota at the genus level is presented in Fig. 8. *Cetobacterium* was the main dominant bacteria in the 2 % group and also showed a certain percentage composition in the 0.5 % group. In the other groups (control, 0.25 %, 0.5 %, and 1 %), *Sphingomonas, Corynebacterium, Rhodococcus, Acidovorax, Pantoea, Methylobacterium, Staphylococcus, Cutibacterium*, and *Streptococcus*, in descending order of proportion, were the most dominant bacteria in the Japanese glass eel intestine (Fig. 8).

The results of beta diversity were demonstrated in Figs. 9–11. Principal coordinates analysis (PCoA) results indicated that the dissimilarity between the 2 % group and the other groups (control, 0.25 %, 0.5 %, and 1 %) (Fig. 9). Regardless the unweighted or weighted unique fraction metric (UniFrac) heatmap showed the difference of the 2 % group compare to others groups (control, 0.25 %, 0.5 %, and 1 %) (Fig. 10; Fig. 11).

4. Discussion

The recent study was the first evaluation of the probiotics supplement eel paste feed at the glass eel stage. Most of the research conducted at the elver stage (12–20 g) [45,46] and few on the leptocephalus [47]. According to above studies and our research, the different probiotics additive in the eel feed all showed a significant enhanced of the growth performance with various immune related enhancements [45–47]. In this research, adding *B. subtilis* natto NTU-18 to the paste feed significantly improved the growth performance of Japanese glass eel, indicating the potential of *B. subtilis natto* NTU-18 as an excellent eel feed additive. Similar growth-promoting effects were found in Japanese eel elvers fed with a diet supplemented with *B. subtilis* WB60 [48,49]. The most favorable growth performance was observed in the groups with

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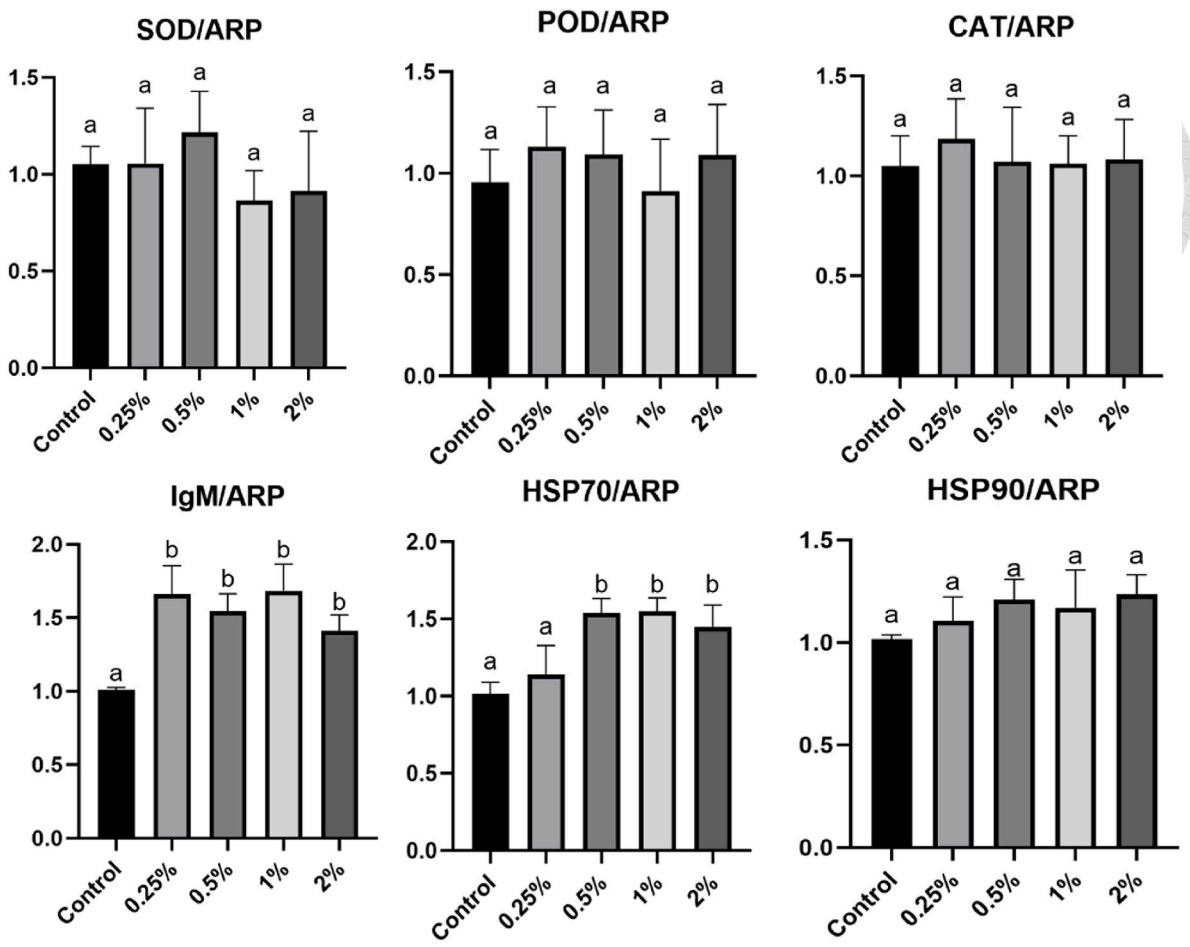

Fig. 2. SOD, POD, CAT, HSP90, HSP70, IgM expression in liver of Japanese glass eels treated with different *B. subtilis natto* NTU-18 containing diet. Different letters indicate significant differences ($p < 0.05$).
ARP: acid ribosomal protein; SOD: superoxide dismutase; POD: peroxidase; HSP90: heat shock protein 70; HSP90: heat shock protein 90; IgM: immunoglobulin M; CAT: catalase.

0.5 % and 1 % *B. subtilis natto* NTU-18 added (Table 2). This finding aligns with results from a previous study, suggesting that the optimal addition of *B. subtilis* in eel feed within the range of $1 \times 10^7 \sim 1 \times 10^8$ CFU/g kg^{-1} [46,48]. The enhanced growth performance could be attributed to the influence of *B. subtilis* on digestive processes, contributing to overall health benefits in terms of growth, nutrition, and host protection [50,51]. This result provides further evidence of *B. sbutilis natto* NTU-18 benefits in eel aquaculture. Parameters related to intestinal morphology, including villi length and muscular layer thickness, indicate gut health in fish [52,53]. Although previous studies showed probiotics can promote villus length of *O. niloticus* and *Siniperca cheats* [54,55], the beneficial effects of it on the Japanese glass eel intestinal morphology were not observed in the research. Suggested that adding *B. subtilis natto* NTU-18 benefited Japanese glass eels' growth performance and immunity without inducing changes in intestinal morphology.

IgM plays a specific role in various immune functions, such as bacterial opsonization, neutralizing toxins and viruses, and activating the complement system in fish [56,57]. It is a crucial immune response component, defending against pathogens and promoting immune clearance mechanisms [58]. Additionally, it activates the classical complement pathway upon recognizing pathogens in fish [59]. In this research, the expression of IgM in all groups fed with *B. subtilis natto* NTU-18 was higher than in the control group in liver and head kidney (Fig. 2; Fig. 3). Similar results have been observed in previous studies on *Sparus aurata* [60], indicating that *B. subtilis natto* NTU-18 could serve as an immunomodulator to increase IgM expression. Furthermore, the increased expression of IgM in the liver and head kidney can enhance the ability of Japanese eels to resist pathogens and diseases [61].

Heat shock proteins (HSPs) are classified according to their molecular weight, encompassing HSP110, HSP90, HSP70, HSP60, HSP40, HSP10, and small HSPs [62]. Among these, HSP70 and HSP90 can be induced by stress and are often used as stress indicators for aquaculture creatures, such as *Oncorhynchus mykiss* and *S. aurata* [63,64]. Japanese glass eels fed with paste feed containing over 0.5 % of *B. subtilis natto* NTU-18 increased the expression of HSP70 in the liver and head kidney compared to the control group (Fig. 2; Fig. 3). However, there was no significant difference in the expression of HSP90 among the groups. The results of HSP70 and HSP90 expression (Fig. 2; Fig. 3) were in accordance with previous studies, which showed that adding *B. subtilis* to the diet of Japanese eel elver can increase the expression of HSP70, but there was no significant difference in HSP90 [49]. According to the previous study of probiotics treating feed in Japanese eel, HSP70 was more sensitive to *B. subtilis* and *Lactobacillus pentosus* [13,49], which showed that HSP70 may be a better stress indicator for Japanese eel. Also, the 0.5 % and 1 % groups showed the best survival rate after the *E. tarda* challenge test (Fig. 5), suggesting that *B. subtilis natto* NTU-18 may promote non-specific immunity and *E. tarda* resistance by enhancing the expression of HSP70 in Japanese glass eel as other probiotics in previous studies [13,65,66].

Antioxidant enzymes, such as peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) play a crucial role in preventing the

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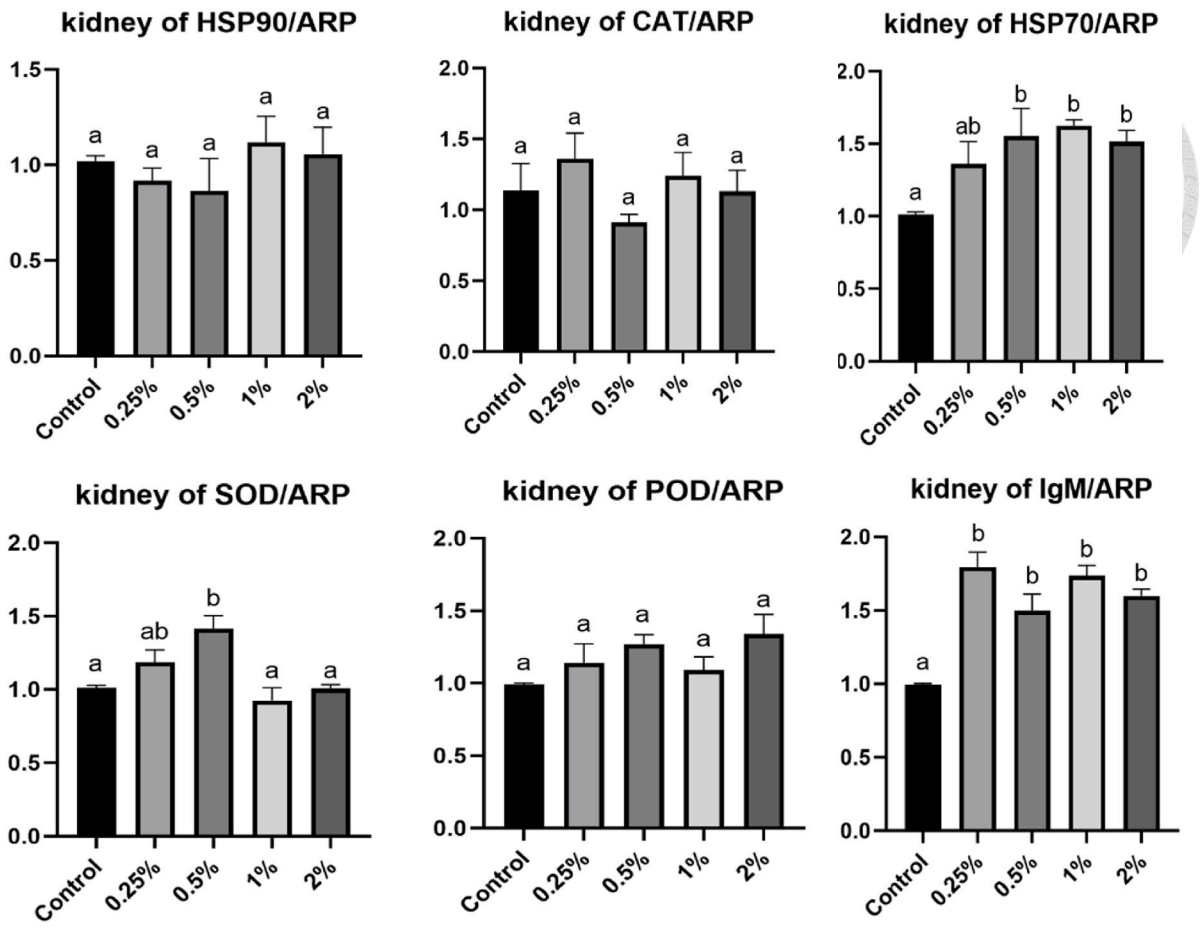

Fig. 3. SOD, POD, CAT, HSP90, HSP70, IgM expression in head kidney of Japanese glass eels treated with different *B. subtilis natto* NTU-18 containing diet.
Different letters indicate significant differences ($p < 0.05$).
ARP: acid ribosomal protein; SOD: superoxide dismutase; POD: peroxidase; HSP90: heat shock protein 70; HSP90: heat shock protein 90; IgM: immunoglobulin M; CAT: catalase.

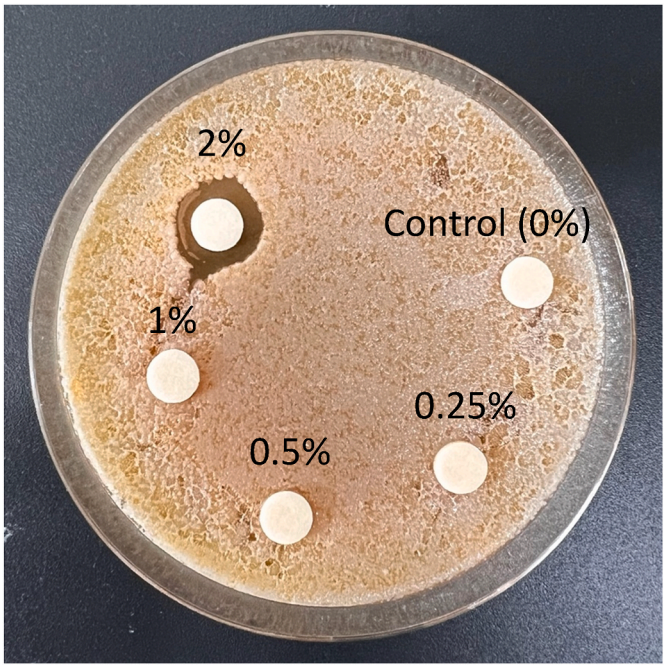

Fig. 4. Bacteriostasis performance of different concentration of *B. subtilis natto* NTU-18 to *E. tarda*.

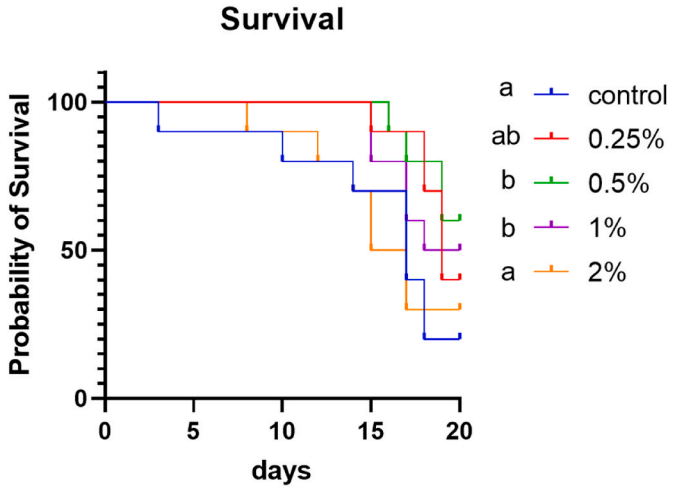

Fig. 5. Survival rate of *A. japonica* fed with different concentration of *B. subtilis natto* NTU-18 after *E. tarda* challenge test.
Different letters indicate significant difference ($p < 0.05$).

harmful effects of oxidative stress within cells [67]. The SOD-CAT system acts against oxygen toxicity stress by inhibiting the formation of reactive oxygen radicals. It is often used as a marker to estimate oxidative stress in teleost fish (Liu, 2011). In this research, only the 0.5

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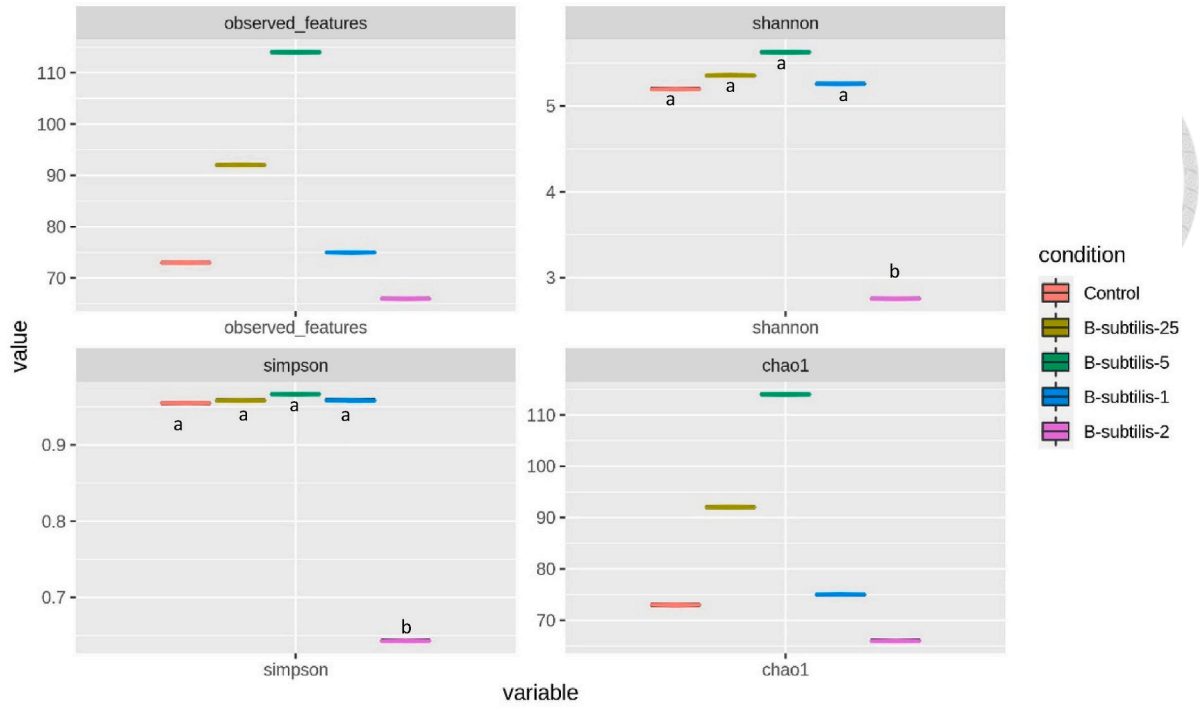

Fig. 6. The number of observed features, estimated OTU richness (showed as Chao1), and diversity index (Shannon and Simpson) for 16S rRNA libraries of gastrointestinal microbiota in Japanese glass eel after *B. subtilis natto* NTU-18 feeding trail. Different letters indicate significant differences between groups ($p < 0.05$).

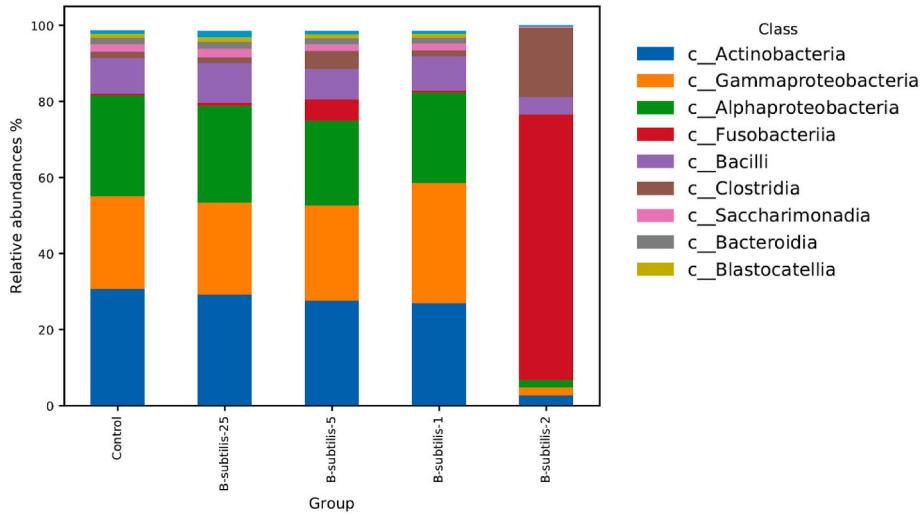

Fig. 7. Relative abundance of gastrointestinal microbiota at the class level in Japanese glass eels after *B. subtilis natto* NTU-18 feeding trail.

% group showed a significant increase in the expression of SOD in the liver (Fig. 2), with no significant difference in the expression of other antioxidant-related enzymes (CAT and POD) in liver or head kidney between groups (Fig. 3). Similar results were also observed in Japanese eels fed with *B. subtilis*, indicating that *B. subtilis* could not stimulate SOD activity in Japanese eels efficiently [49,68]. The fluctuation of the SOD expression peak only found in the liver of the 0.5 % added group may indicate that 0.5 % probably be the most suitable addition concentration for improving the immune response in the liver. Furthermore, the significantly higher SOD expression in the 0.5 % *B. subtilis natto* NTU-18 treated group contributed to a significantly higher survival rate after the *E. tarda* challenge test (Fig. 5). According to the RT-PCR results of CAT and POD, the addition of B. *subtilis natto* NTU-18 did not significantly induce oxidant stress response in eels during the feeding trial.

At the end of the *E. tarda* challenge test, significantly higher survival rates were observed in the 0.5 % and 1 % groups (Fig. 5). According to the results in the immune-related gene, the treatment of *B. subtilis natto* NTU-18 improved the expression of HSP70, SOD, and IgM for Japanese glass eel, which could contribute to a more effective defense against *E. tarda* by previous research [13]. Consistent results were indicated in a previous study of Japanese eel against *Aeromonus hydrophila* and *Vibrio anguillarum* after a *B. subtilis* feeding trial [46,48]. It suggests that *B. subtilis natto* NTU-18 can enhance the immune response of Japanese eels and further control the loss of bacterial infections in RAS.

The metagenomics analysis indicated that the class *Bacilli* constituted the composition of intestinal bacteria in all groups (Fig. 7). And the 2 %

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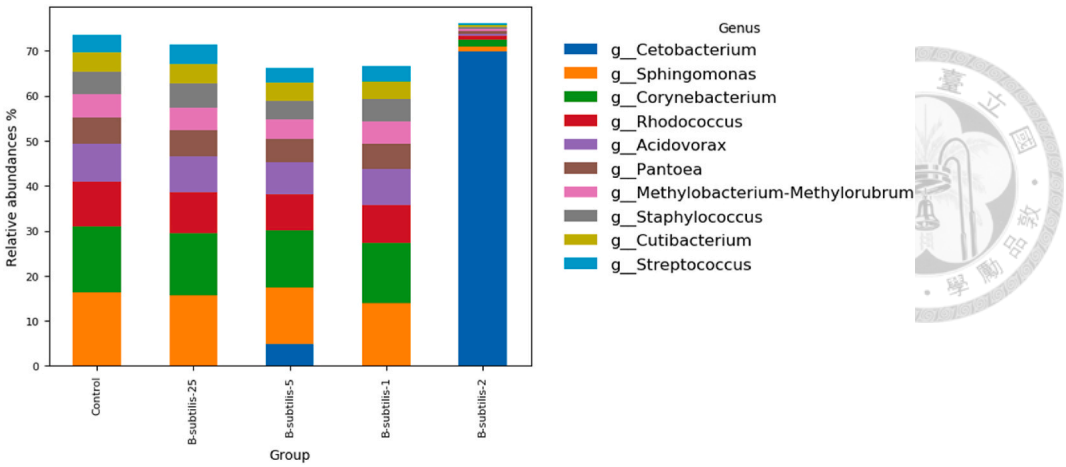

Fig. 8. Relative abundance of gastrointestinal microbiota at the *genus* level in Japanese glass eels after *B. subtilis natto* NTU-18 feeding trail.

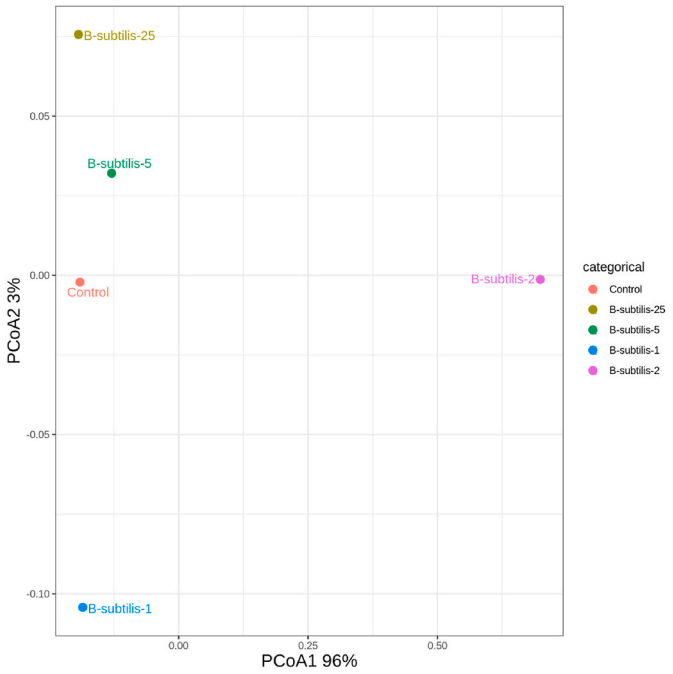

Fig. 9. Principal coordinates analysis (PCoA) of gastrointestinal microbiota in Japanese glass eels after *B. subtilis natto* NTU-18 feeding trail.

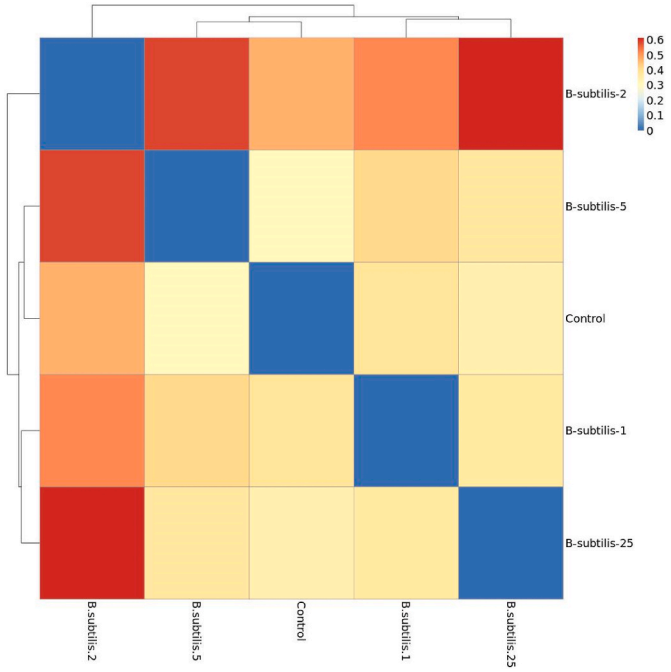

Fig. 10. Unweighted Unifrac heatmap of gastrointestinal microbiota in Japanese glass eels after *B. subtilis natto* NTU-18 feeding trail.

group showed a significantly lower intestine microbial alpha and beta diversity ($p < 0.05$) (Fig. 6; Fig. 9; Fig. 10; Fig. 11). This phenomenon may cause by the excessive *B. subtilis natto* NTU-18 in the eel intestine of the 2%-treated group, which may inhibit other intestinal bacteria. The results in the antibacterial circle (Fig. 4) were consistent with the metagenomic analysis, indicating that only 2 % of *B. subtilis natto* NTU-18 exhibited an inhibition zone against *E. tarda*. This observation may be attributed to a decrease in intestinal microbiota diversity. Although there were no significant differences, a relatively lower growth enhancement was shown in the 2 % group. The substantial increase in the proportions of *Fusobacteriia* and *Clostridia* colonies in the 2 % group (Fig. 7), which these two colonies indicated gastric cancer [69], may cause growth retardation in the excessive addition group. However, the 2 % group still exhibited a significantly higher growth rate than the control group. This difference may be because of the changes in intestinal microbiota, particularly with *Cetobacterium* (Fig. 8), which positively correlated with the eel growth rate in a previous study [70]. *Cetobacterium* has been pointed out to produce high concentrations of vitamin B12 [71], which can promote the metabolic rate of eels and reduce the feed conversion rate. In chicken, it stated that the addition of *B. subtilis* (yb-114 & yb-246) and *B. licheniformis* (yb-214 & yb-245) improved the activities of chymotrypsin, lipase, and amylase in the digestion process [72]. Although the digestive systems of chicken and eel differ a lot, it would still be possible that the intestinal enzyme activity of eel is enhanced by feeding *B. subtilis natto* NTU-18 containing feed. Generally, *B. subtilis natto* NTU-18 in the 2%-treated group may overdosed, showing no further improvement in growth performance, immune-related gene expression, and disease resistance in Japanese glass eel. The excessive concentration of *B. subtilis natto* NTU-18 significantly reduced the diversity of gastrointestinal microbiota (Fig. 5), negatively impacting the immune response to *E. tarda* and a lower growth rate compared to the optimal addition groups (0.5 % and 1 %).

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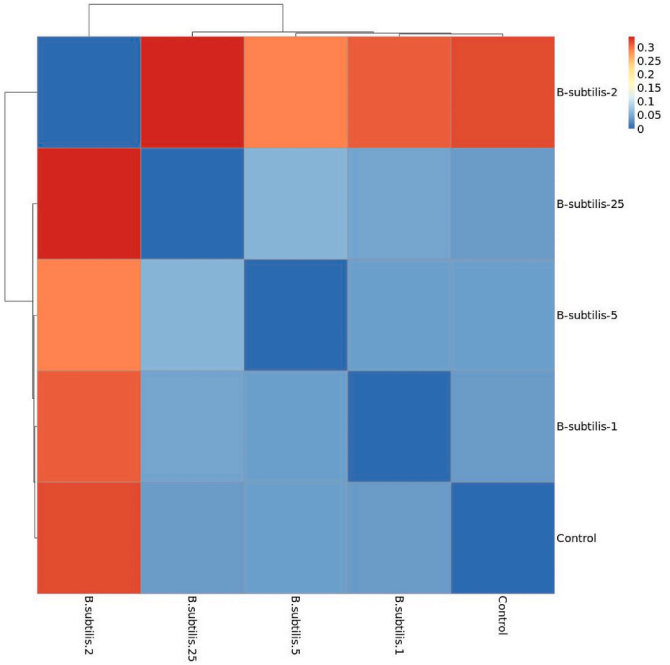

Fig. 11. Weighted Unifrac heatmap of gastrointestinal microbiota in Japanese glass eels after *B. subtilis natto* NTU-18 feeding trail.

5. Conclusion

This research demonstrated a significant improvement in the growth performance of Japanese glass eels fed with *B. subtilis natto* NTU-18 supplemented paste feed. However, the exceeded concentration of *B. subtilis natto* NTU-18 at 2 % group reduced the intestinal microbiota diversity of Japanese glass eel, resulting in a worse growth performance than the optimal addition groups. According to this research, paste feed with *B. subtilis natto* NTU-18 concentrations higher than 1 % did not further improve growth performance, and a 0.5–1.0 % concentration was the most optimal for Japanese glass eels in RAS to achieve best growth performance, immune response, and disease resistance.

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Conflicts of interest

The authors declare that they have no conflicts of interests.

Ethics approval

The experiment was performed in accordance with the recommendations from the Institutional Animal Care and Use Committee for the care of animals used for experimental or other scientific purposes (approval number 'NTU110-00152').

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Code availability

Not applicable.

Author contributions

Authors' contributions, Yen-Ting Lin and Yu-Chen Hung mainly conducted the experiments, analyzed the results. Yen-Ting Lin wrote this manuscript. Kung-Ta Lee and Li-Han Chen supplied the Bacillus Subtilis natto NTU-18. Yu-San Han designed and supervised the experiments. All authors participated in manuscript writing and interpretation of results. All authors read and approved the final manuscript.

Data availability

Data will be made available on request.

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Effects of adding spermidine carbon quantum dots in feed on growth, intestinal morphology, immunity and disease resistance of *Anguilla japonica* and *Anguilla marmorata*

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ABSTRACT

Japanese eel (*Anguilla japonica*) and giant mottled eel (*Anguilla marmorata*) are the main eel species reared in recirculating aquaculture system (RAS) in Taiwan. RAS increases the rearing density of eel. On the other hand, it spreads pathogens and diseases more efficiently and uncontrollably. Currently, eel farmers use antibiotics to control disease in RAS, but their misuse can lead to resistance and food-safety issues. Spermidine carbon quantum dots (CQDSpds), a newly developed carbon nanomaterial with positive charges on the surface, can bind to the negatively charged bacterial cell membrane to destroy bacteria and not cause drug resistance. This research investigated the effects of different concentrations of CQDSpds as paste feed additives on the growth performance, intestinal morphology, immune-related gene expression, and disease resistance of Japanese and giant marble eels. The results showed the highest final body weight, weight gain, and specific growth rate of Japanese eel and giant mottled eel fed with 1 ppm and 0.5 ppm CQDSpds added. Compared with the control group, 1 ppm CQDSpds group showed higher lysozyme expression in Japanese eels. In the part of intestinal morphology, there was not a significant difference in intestinal villus height and intestinal morphology between the CQDSpds groups and the control group in both eel species. The survival rate of each group supplemented with CQDSpds was higher than the control group after the *E. tarda* challenge test, among which the highest were found at 1 ppm group in Japanese eel and 0.5 ppm group in giant mottled eel. By this research, the additive of CQDSpds in eel culturing could improve the growth and immune performance with not caused an impact on the intestine of both eels, which can be a potential feed additive in intensive eel farming.

1. Introduction

Aquaculture production has expanded rapidly in Taiwan in recent years. *Anguilla japonica* (Japanese eel) is one of the essential culturing species in eastern Asia (Shahkar et al., 2015) due to its high market demand and retail price. Since the catch of Japanese glass eel is far below the demand of the aquaculture industry, the resource of Japanese eel is declining numerously (Tsukamoto et al., 2009). The discussion about whether Japanese eel should be listed in the CITES Appendix II has been taken since 2016, so Japanese eel may not be able to have international trade of glass eel in the future (Han, 2019). In order to prevent the impact of the potential restriction of the eel culturing industry in Taiwan, developing rearing skills in other eel species may be essential. *Anguilla marmorata* (giant marbled eel) is the most widely distributed eel species (Miller et al., 2002). The low growth rate and high mortality caused by disease pose a considerable obstacle to developing giant marble eel farming (Han, 2010). Under the overfished of the Japanese glass eels, the shrinking of giant marbled eel farming techniques to increase growth performance and efficiency is necessary (Luchiari, Pirhonen, 2008). The reduction of Japanese glass eels led to the profitability decline of eel culturing in eastern Asia (Yuan et al., 2022). Since that, how to reduce the mortality and increase the productivity of eel will be necessary for improving the eel farming industry in Taiwan (Han, 2010).

Recirculating aquaculture systems (RAS) have been more popular worldwide due to environmental pollution, lack of water and land, and the increasing frequency of extreme climate (Dalsgaard et al., 2013). RAS can reduce water exchange rates to 1/30–1/50 that of traditional

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outdoor ponds with a running-through system and promote the production capacity by more than ten times (Martins et al., 2011; Deviller et al., 2005). The survival rate of eel rear in RAS can be as high as 95% (Xu, 1997), which also shows an improvement in growth performance (Huang, 2007). However, rearing creatures are more susceptible to disease in RAS because of the weakened immunity caused by stress (Raman et al., 2013; Mota et al., 2014). *Edwardsiella tarda* has been regarded as one of the primary pathogens in intensive aquaculture systems (Katya et al., 2016). The typical symptoms of infection of *E. tarda* in eels are rotting lesions on the liver and kidneys (Kou, 1974) and, in some severe cases, even observing skin perforation (Kou, 1979). Moreover, it has high infectivity and mortality to eels and is one of the main threats to eel farmers (Michael & Abbott, 1993).

Carbon Quantum Dots (CQDs) is a novel type of carbon-containing nanomaterials with excellent antibacterial efficacy, tiny size, easy synthesis and modification, low cytotoxicity, and good water dispersibility (Alavi et al., 2021 ; Zhu et al., 2012). CQDs have received significant attention, promoting their application in biomedical engineering in recent decades. CQDs have great potential in various antibacterial applications, which with specific antibacterial activities, will play a new role in treating bacterial infections (Chou et al., 2021 ; Miller, 2017). Spermidine (spd) is a kind of polyamine that exists in ribosomes and tissues. Also have been found to reduce the minimum inhibitory concentrations of β-lactamide antibiotics in many bacteria (Kwon, Lu, 2007). Spermidine carbon quantum dots (CQDSpds) are carbon quantum dots synthesized by Li et al. using carbon quantum dots and spermidine through a one-step thermal cracking method (Li et al., 2016). Compared with other CQDs, CQDSpds modified with spermidine have an ultra-high positive charge (Jian et al., 2017). The antibacterial mechanism is the solid disintegrating effect of CQDSpds on bacterial membranes (Jian et al., 2020). With an ultra-high positive charge, CQDSpds can bind to phospholipids, peptidoglycans, or porins found on the bacterial membrane, resulting in the synergistic destabilization of the bacterial cell membrane and inhibition of membrane synthesis (Harroun et al., 2017 ; Ma et al., 2020 ; Huang et al., 2020). In aquaculture applications, they had also been found that the WSSV-infected *Litopenaeus vannamei* could significantly reduce their mortality and enhance immune responses after being treated with CQDSpds (Huang et al., 2020). Also, by the toxicity assessments, CQDSpds show high biocompatibility of *Danio rerio* (Chung et al., 2021; Chung, 2022).

The good antimicrobial properties, low toxicity, and exemplary performance in aquaculture applications, CQDSpds has the potential as a feed additive. Commonly, eel farmers use antibiotics during the feeding process to control various diseases in RAS. However, overuse of antibiotics can lead to many adverse side effects, such as antibiotic resistance, decreased fish immunity, water pollution, and food-safety issues (Alderman et al., 1998; Rasul et al., 2017; Chen et al., 2020). Therefore, water environment management, early prevention, and increasing fish immunity are the main methods to increase eel productivity (Martins et al., 2009). However, there is still no research about Japanese eel and giant marbled eel feed with CQDSpds recently, so we study the effects of CQDSpds on Japanese eel and giant marbled eel growth performance, intestinal morphology, innate immunity, and the resistance to *E. tarda*. Moreover, they aim to replace dietary antibiotics using CQDSpds at both eel species in RAS.

2. Materials and methods

2.1. Experiment diet

The composition of the experiment diet is shown in Table 1. Five experiment feeds were formulated above, no CQDSpds added (control), and four concentrations graded of CQDSpds from 0.25, 0.5, 1, and 2 ppm. The experimental diet was based on a paste feed made from fish paste, krill, and some necessary nutrient additives and was checked without any pathogen before feeding. CQDSpds was obtained from Giant Bio-Technology, New Taipei City, Taiwan. Diets were prepared every two weeks to make sure quality. The fresh ingredients were first mixed by an electric mixer, then followed by the dry matter and CQDSpds. After mixing uniformly for 45 min, the paste feed would be packaged and stored at − 20 °C until used for feeding.

Table 1
Composition of the experimental paste feed.

Ingredients	0 ppm	0.25 ppm	0.5 ppm	1 ppm	2 ppm
Shrimp mince (%)	70	70	70	70	70
Fish meal (%)	25	25	25	25	25
Guar gum (%)	4.8	4.8	4.8	4.8	4.8
Vitamins premix (%)	0.1	0.1	0.1	0.1	0.1
Mineral premix (%)	0.1	0.1	0.1	0.1	0.1
CQDSpds (ppm)	0	0.25	0.5	1	2

2.2. Experiment eels and feeding trail

A. japonica and *A. marmorata* were caught from eastern Taiwan (*A. japonica* from Lanyang River, 24.7163°N, 121.8348°E; *A. marmorata* from Xiuguluan River, 23.4612°N, 121.5008°E) at 2020/11–2021/02. The eel was transported to the National Taiwan University (Taipei, Taiwan) lab with a fish bag full of oxygen and a control water temperature of 15 °C. There was a checking for the health of eels right upon arrival at the laboratory. Those in good condition were soaked in a 2.5 ppm potassium permanganate (KMnO4) solution for 10 min to avoid introducing the pathogen into the RAS. After disinfection, 25 fish would be transported to indoor RAS systems with five tanks (30 × 30 × 45 cm) and maintained in freshwater for acclimation during the first week. The eel was fed with control paste feed during the acclimation. The initial body weight and length were measured after acclimation to make sure there was not a significant difference ($p > 0.05$) between each group (77.7 ± 1.82 mm, 0.40 ± 0.01 g for *A. japonica*; 47.67 ± 0.57 mm, 0.09 ± 0.01 g for *A. marmorata*, both triplicates). The photoperiod was set to 12 h light (7:00–19:00) and 12 h dark. RAS was kept in an appropriate state, and the dissolved oxygen was close to saturation through aeration; water temperature was maintained at 24–25 °C, pH at 7.0–7.5, ammonia nitrogen lower than 0.1 ppm, and nitrite lower than 0.05 ppm. The water exchange rate is about 30 L/day, and 10% of new water is replaced in the RAS weekly. Fish was fed twice a day, with a paste feed amount of about 5% of the total wet body weight. The remaining feed was removed from the tank 1 h after feeding by siphon excrement. The Japanese eel was reared for 56 days. However, the feeding trail of the giant marbled eel was carried out for 84 days because the growth rate of the giant marbled eel was slower than Japanese eel. The experiment was conducted by the process and the recommendations of the Institutional Animal Care and Use Committee for the care of laboratory animals (approval number "A201900005").

2.3. Samples collection

The body weight and body length of both eel species would be taken every 2 weeks during the experiment period, and fish would starve for one day before measurement. The percentage weight gain (PWG), condition factor (K), specific growth rate (SGR), feed conversion rate (FCR), and survival rate (SR) were calculated as below:

$$Percentage\ weight\ gain(\%) = \frac{\text{Final body } weight(\text{g}) - \text{Initial bodyw eight(g)}}{\text{Initial } body \text{ weight}} \times 100$$

$$Condition\ factor = \frac{\text{Body } weight(\text{g})}{\text{Body length}^3(\text{cm})} \times 1000$$

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$$Specific\ growth\ rate = \frac{\ln(\text{Final body weight}) - \ln(\text{Initial body weight})}{\text{Rearing day}} \times 100$$

$$Feed\ conversion\ rate = \frac{\text{Final body weight(g)} - \text{Initial body weight(g)}}{\text{Total incidents(g)}} \times 100$$

$$Survival\ rate(\%) = \frac{\text{Final number of } fish}{\text{Initial } number \text{o f } fish} \times 100$$

At the end of the feeding trial, 4 eels were randomly selected from each group and were sacrificed with 700 ppm of 2-phenoxyethanol solution. After decapitation, the intestinal head kidney tissues were sampled for further experiment. The head kidney of 4 eels in the same group was soaked in RNA later solution together and stored at − 80 °C immediately. The contents in the intestinal were removed and placed in a 10% formalin solution for later use.

2.4. Histological analysis

The intest of both kinds of eel were taken to observe the differences in the gut histology in different groups. First, the intestines immersed in 10% formalin solution for 14 days were removed. The middle part of the intestine was taken, dehydrated by gradient alcohol, and immersed in eosin and xylene. Then the guts were embedded in the paraffin and stained with eosin and hematoxylin. Villus height (unit: μm) and villus circumference ratio (unit: %) (Kallakuri et al., 2003) were used as the parameters of eel gut morphology.

$$\text{Villus circumference ratio}(\%) = \frac{The\ \ highest\ \ villi\ \ length(\mu m)}{The\ \ circumference\ \ of\ \ villi(\mu m)} \times 100$$

2.5. Real-time PCR

Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), and purified RNA was quantified using a Nano-300 spectrophotometer (Thermo Scientific, USA) for real-time PCR of immune-related genes. In this research, superoxide dismutase (SOD), peroxidase (POD), lysozyme (LZM), and interleukin-6 (IL-6) four immune-related are selected as the target gene, and acidic ribosomal protein (ARP) was used as the reference gene. The selected genes and primer sequences for Real-time PCR are shown in Table 2. Real-time PCR was run on the Bio-Rad MyIQ real-time PCR system (Bio-Rad, Hercules, CA, USA). Every well would be put in 25 μL of the qPCR reaction mix, which contained 12.5 μL of 2x SYBR green mix (Bionova, Fremont, CA, USA), 1.0 μL forward primer, 1.0 μL reverse primer, 1 μL (10 times dilution) for cDNA samples and 9.5 μL DEPC water. The condition of the real-time PCR reaction was: preincubation (95 °C, 10 min, 1 cycle), denature (95 °C, 15 s, 40 cycles), amplification (60 °C, 15 s, 40 cycles), and melting curve (95 °C, 15 s, 1 cycle). Each sample was tested in triplicate. The calculation of real-time PCR using delta-delta-Ct (Livak and Schmittgn, 2001).

Table 2
List of immune-related gene primers used for qPCR.

Genes	Primer	Sequences
ARP (reference gene)	Forward	5′-GTGCAGCTCATTAAGACCGG-3′
	Reverse	5′-GGCGATATTCCTCACACCCT-3′
SOD	Forward	5′-TAACGTACGACTATGGGGCC-3′
	Reverse	5′-GCCGCCACCATTAAACTTCA-3′
POD	Forward	5′-GACATCACCCGTTTCTGCAA-3′
	Reverse	5′-GTGGATGAAGGAGGGGAACA-3′
LZM	Forward	5′-TGCTGGAATGGATGGATACC-3′
	Reverse	5′-GTAATCGCAGTGCTGATGTC-3′
IL-6	Forward	5′-CCAGATGTCGCTTCACTTCG-3′
	Reverse	5′-ACTTGGATGTCGTCACCCAT-3′

2.6. In vitro antibacterial experiments

The antibacterial activity of CQDSpds test against *E. tarda* was determined by using bacteriostasis circle (Liu et al., 2020). Different concentration of CQDSpds (0, 0.25 0.5, 1.0, & 2.0 ppm) was added 50μl on 8 mm thick paper disk, then cultured in agar medium coated with *E. tarda* in the incubator at 30 °C for 24 h. The antibacterial activity of the CQDSpds was reflected by the size of the inhibition circle show on the agar.

2.7. Challenge test

The challenge test was modified *E. tarda* challenge test from of Japanese eel from Keiichi (Keiichi et al., 1984), and the cultivation method was from Ishihara (Ishihara and Kusuda, 1981). *E. tarda* for cultivation was from the Department of Veterinary Medicine, National Taiwan University (Taipei, Taiwan), and took a single colony of *E. tarda* and dissolved in tryptone soy agar (TSB; Sigma-Aldrich) medium for shaking culture at 37 °C. 6 eels per group were randomly selected to immerse in the *E. tarda* solution at 1 X 10^8 CFU/ ml concentration for 2 h. After immersion, they transported eels to the same culturing RAS without a feeding trial. The mortality of the eels would be recorded for 20 days, and all of the dead fish would be dissected to get the liver and gill for PCR to make sure the death causing by infection of *E. tarda*.

2.8. Statistical analysis

The results were analyzed by SPSS 24.0 for Windows (IBM SPSS Statistics 24.0). All data were expressed as mean ± standard deviation (SD). One-way ANOVA was used for the one-way analysis of variance. If there was a significant difference, Duncan's multi-range test was used to test between groups to confirm whether there was a difference in means between groups. Significantly differences would be considered when $p < 0.05$.

The data of the challenge test were presented by GraphPad Prism 8 survival curve, and the log-rank test Log Rank (Mantel-Cox) was used to compare the survival probability of each group. The level of significant difference was set as $p < 0.05$.

3. Results

3.1. Growth performance

After a feeding trail for *A. japonica* with different doses of CQDSpds for 56 days, the initial body weight (IBW), final body weight (FBW), weight gain rate (WG), specific growth rate (SGR), feed conversion rate (FCR), condition factor (K) and survival rate are shown in Table 3. The IBW of each group ranged from 0.39 ± 0.01 g to 0.40 ± 0.01 g, and no significant difference between groups ($p > 0.05$). FBW in the 1 ppm group was significantly higher than control, 0.25 ppm, and 2 ppm groups ($p < 0.05$) but was not significantly different from the 0.5 ppm group ($p > 0.05$). WG, SGR and FCR in the 0.5 ppm and 1 ppm group showed significantly different in the control, 0.25 ppm, and 2 ppm groups ($p < 0.05$), but there was no significant difference with the 0.5 ppm group ($p > 0.05$). Moreover, there were no significant differences in K and survival rates among each group of Japanese eels ($p > 0.05$).

After 84 days of *A. marmorata* feeding with different doses of CQDSpds in the feed, the IBW, FBW, WG, SGR, FCR, K and survival rate are shown in Table 5. The IBW of each group ranged from 90.73 ± 3.51 mg to 92.28 ± 1.67 mg, and there was no significant difference between the groups ($p > 0.05$). The FBW and FCR in the 0.5 ppm group was significantly higher than the control ($p < 0.05$), but there was no significant difference among the 0.25 ppm, 1 ppm, and 2 ppm groups

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Table 3
Effect of different contant of CQDSpds supplementation on growth and survival rate of A. japonica for 56 days (Mean±SD; n = 50) . Data in the same row with different letters are significantly different ($p < 0.05$) among different treatments.

	0 ppm	0.25 ppm	0.5 ppm	1 ppm	2 ppm
IBW (mg)	395.87 ± 3.85^{a}	400.55 ± 7.99^{a}	393.40 ± 8.94^{a}	389.20 ± 9.90^{a}	398.82 ± 2.29^{a}
FBW (mg)	917.87 ± 33.55^{a}	928.05 ± 15.34^{a}	1058.90 ± 4.63^{b}	1108.70 ± 37.26^{b}	975.82 ± 33.40^{a}
WG (%)	131.82 ± 6.21^{a}	131.77 ± 8.45^{a}	169.37 ± 9.58^{b}	185.56 ± 2.47^{b}	144.65 ± 6.96^{a}
SGR (%/day)	1.00 ± 0.03^{a}	1.00 ± 0.04^{a}	1.17 ± 0.06^{b}	1.24 ± 0.31^{b}	1.06 ± 0.03^{a}
FCR (%)	46.61 ± 9.75^{a}	47.10 ± 7.35^{a}	59.42 ± 7.34^{b}	62.24 ± 5.21^{b}	51.52 ± 1.43^{ab}
K	0.95 ± 0.05^{a}	0.93 ± 0.02^{a}	0.93 ± 0.01^{a}	0.94 ± 0.03^{a}	0.94 ± 0.02^{a}
SR (%)	62.00 ± 2.83^{a}	64.00 ± 5.66^{a}	64.00 ± 5.66^{a}	70.00 ± 2.83^{a}	66.00 ± 2.83^{a}

Table 4
Intestinal morphology of A. japonica fed diet with different concentration of CQDSpds for 56 days (Mean±SD, n = 8) . Data in the same row with different letters are significantly different ($p < 0.05$) among different treatments.

	0 ppm	0.25 ppm	0.5 ppm	1 ppm	2 ppm
villus height (μm)	93.78 ± 5.98	94.75 ± 4.29	94.33 ± 7.14	95.14 ± 5.67	95.02 ± 3.02
villus circumference ratio (%)	13.3 ± 1.71	13.1 ± 3.12	12.9 ± 5.26	13.0 ± 6.40	13.1 ± 1.42

Table 5
Effect of different contant of CQDSpds supplementation on growth and survival rate of A. marmorata for 84 days (Mean±SD, n = 60) . Data in the same row with different letters are significantly different ($p < 0.05$) among different treatments.

	0 ppm	0.25 ppm	0.5 ppm	1 ppm	2 ppm
IBW (mg)	91.15 ± 1.44^{a}	90.73 ± 3.51^{a}	91.76 ± 1.08^{a}	92.28 ± 1.67^{a}	91.20 ± 2.02^{a}
FBW (mg)	222.12 ± 4.82^{a}	231.00 ± 1.68^{ab}	265.62 ± 8.17^{b}	244.67 ± 5.09^{ab}	231.58 ± 1.06^{ab}
WG (%)	144.68 ± 13.95^{a}	153.51 ± 13.17^{a}	183.86 ± 24.16^{a}	167.79 ± 27.12^{a}	153.86 ± 6.49^{a}
SGR (%/day)	1.28 ± 0.08^{a}	1.28 ± 0.05^{a}	1.29 ± 0.12^{a}	1.24 ± 0.08^{a}	1.28 ± 0.08^{a}
FCR (%)	22.74 ± 1.53^{a}	24.35 ± 3.23^{a}	30.18 ± 1.82^{b}	26.46 ± 6.73^{a}	24.37 ± 2.67^{a}
K	1.06 ± 0.07^{a}	1.11 ± 0.06^{a}	1.24 ± 0.10^{a}	1.16 ± 0.12^{a}	1.11 ± 0.03^{a}
SR (%)	61.67 ± 7.07^{a}	60.00 ± 0.01^{a}	68.33 ± 7.07^{a}	70.00 ± 9.42^{a}	65.00 ± 11.78^{a}

($p > 0.05$). Nevertheless, in the part of WG, SGR, K, and survival rate, there was no significant difference between each group of *A. marmorata* ($p > 0.05$).

3.2. Histological analysis

From the results of the villus height and villus circumference ratio of *A. japonica* (Table 4, Fig. 2) and *A. marmaorata* (Table 6, Fig. 5), There was no significant difference in the villi height and villus circumference ratio of Japanese eel and giant marbled eel among the groups ($p > 0.05$). There was no abnormal villi condition (fold breaks, dilation, and villi dwarfs) observed in all intestine samples in this research (Fig. 1), showing that both eels were in good condition.

3.3. Real-time PCR

Figs. 3 and 6 showed the expression of immune-related genes in Japanese eel and giant marbled eel. The expression of SOD in the Japanese eel control group was significantly higher than the 1 ppm group ($p < 0.05$), but there was no significant difference between the other groups ($p > 0.05$). As for the giant marbled eel, a significant difference in SOD expression was observed in the 0.5 ppm and 2 ppm groups ($p < 0.05$). POD expression of Japanese eel and giant marbled eel all showed the highest in the control group. Among Japanese eel, the control was significantly higher than the 0.5 ppm and 1 ppm group ($p < 0.05$); as for giant marbled eel, the control and 0.5 ppm group were significantly higher than 1 ppm and 2 ppm group ($p < 0.05$). The 1 ppm Japanese eel group showed a significant difference from the control and 2 ppm group ($p < 0.05$); the control group of giant marbled eel was significantly different from the 0.5 ppm and 1 ppm group ($p < 0.05$) in terms of LZM expression. IL-6 showed significantly lower expression in the 0.5 ppm group than the control and 0.25 ppm group in Japanese eel ($p < 0.05$). However, in giant marbled eel, the control and 0.25 ppm group presented significantly higher IL-6 expression than other groups ($p < 0.05$). In both eel species, SOD and IL-6 expression showed a rising trend after falling with the increase of CQDSpds gradient.

3.4. In vitro antibacterial experiments

The antibacterial activity of CQDSpds has shown in Fig. 1, the paper disk loaded with CQDSpds from 0.25 ppm had all showed the antibacterial performance to the *E. tarda*. The 1 and 2 ppm group showed the larger inhibition zone than the 0, 0.25, and 0.5 ppm group.

3.5. Challenge test

The survival results of the *E. tarda* challenge test of *A. japonica* and *A. marmorata* are shown in Fig. 4 and Fig. 7. The death of both eel species was observed over 24 h after the challenge test. For the Japanese eel, the survival rate was not significant difference among groups ($p > 0.05$), but the survival rate with 4 groups with CQDSpds was higher than the control group. On the other hand, for giant marbled eel, the 1 ppm and 2 ppm groups showed a significantly higher survival rate than others ($p < 0.05$). All of the dead eels in each group showed swollen livers and confirmed the infection of *E. tarda* by PCR.

4. Discussion

After the feeding trial, the Japanese and giant marbled eels found that all groups treated with CQDSpds had significantly higher weight gain and specific growth rates than the control group ($p < 0.05$). The control group of both eel species showed a significantly worse growth performance ($p < 0.05$), indicating that CQDSpds had a growth-promoting effect for both eel species. At the concentration of 2 ppm, the weight gain and specific growth rate decreased in both Japanese eel and giant marbled eel, presented that the excessive addition of CQDSpds did not help the growth performance, which may be related to the excessive SOD expression in those groups. Eels fed with CQDSpds supplementation at the optimum level in this feeding trial improved FCR, the same phenomenon had been found in previous study conducted on *A. japonica* fed with antibiotics (Lee et al., 2017) and propolis supplementation (Bae et al., 2012). The growth promotion of the eels observed in this research, may due to that CQDSpds in the paste feed enhance the feed ingestion, food absorption, metabolism, and immune related gene expression in both eel specie (Bae et al., 2012). The growth of the giant marbled eel showed a downward trend of growth performance at 1 ppm addition, compared to the Japanese eel, which only showed a lower growth condition in the 2 ppm group. The results also indicate that it was more susceptible to the excess addition of CQDSpds of the Japanese eel than the giant marbled eel. Similar phenomenon also indicated by

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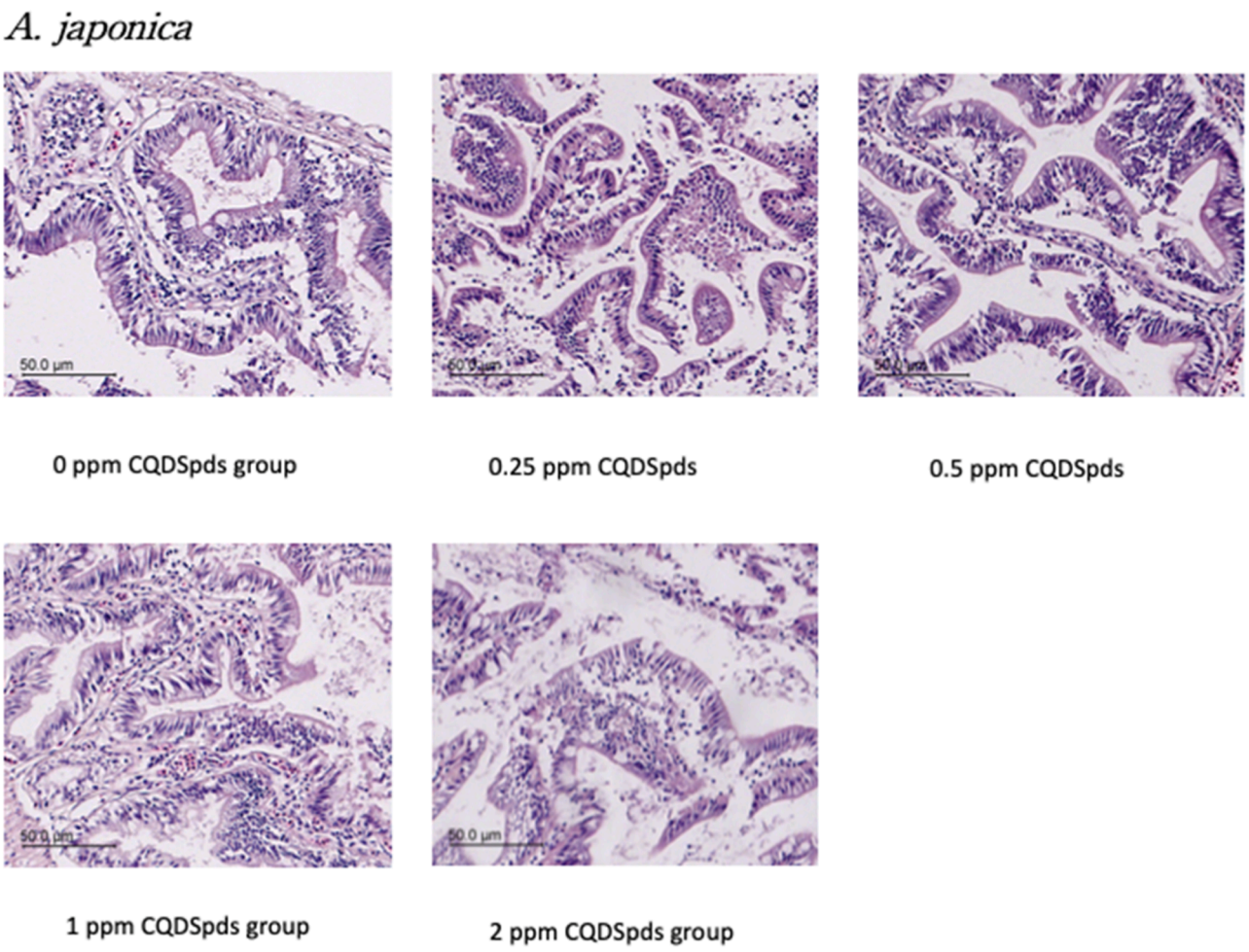

Fig. 2. Details of the intestinal histological examination of *A. japonica* fed diets with different concentration of CQDSpds for 56 days. (Staining; scale bar = 50 μm; Original.
magnification X 200).

previous study about acidifiers supplementation in *A. rostrata* (Zhang et al., 2022), which show the additive exceed optimum concentration may cause negative effect on growth performance of eels.

The intestine is the prominent place for fish digestion, absorption, and osmotic regulation (Gonçalves et al., 2007; Wilson & Castro, 2010). Due to the short intestinal tract of eel compared to other fishes, the effective absorption surface in the gastrointestinal tract plays an essential role in the effect of eel's nutrient absorption. Villi height and villi circumference ratio are two indexes that can show the effective surface in the intestine (Kužir et al., 2012). The histological analysis of intestinal villi of both eel species showed non significantly different between control ($p > 0.05$), which means that adding CQDSpds would not cause damage to intestine function at the concentration. Also, in a previous study by Lee et al. (2003), Lee et al. (2013) although there were significant differences between each group in the case of growth performance, there were no significant differences in the villi histological analysis (Lee et al., 2017).

Typically SOD and POD constitute an antioxidant enzyme system in living organisms. The function of SOD can directly capture free radicals in the body and convert free radicals into less oxidative hydrogen peroxide through disproportionation, thereby avoiding free radical damage to tissues and organs (Fattman et al., 2003; Lin et al., 2008; Liu et al., 2015). Also, SOD and POD were used as an indicator of stress in the previous study (Abele, Puntarulo, 2004). Rearing space constraints in RAS may cause stress for the animal during culturing, threatening their survival (Davidson et al., 2009, 2011, Martins et al., 2009). The relative expression of SOD and POD reached a minimum of 1 ppm in the Japanese eel and 0.5 ppm in the giant marble eel, respectively, but then increased in the higher addition groups. These results suggest that eels in the RAS were under less stress after feeding CQDSpds-containing diets. Less SOD or POD expression may represent fewer free radicals in eels, leading to lower damage to tissues and organs and less stress than the control group. Lower stress in the rearing environment resulted in higher growth rates. It may also represent a lower activation level of the oxidative stress system, reducing the energy consumption of eels and improving their feed conversion ratio to increase their growth rate.

Neutrophils and monocytes mainly secrete LZM. It directly lyses bacteria by hydrolyzing the outermost layer of peptidoglycan (Saurabh and Sahoo, 2008; Smith et al., 2019). LZM is an essential antibacterial and anti-inflammatory enzyme. Like the antioxidant system, LZM may also be affected by stress responses. LZM is essential in maintaining body defenses and is involved in various immune processes, including phagocytosis and complement initiation in fish (Saurabh et al., 2008). In this research, the results of the Japanese eel and giant marbled eel showed a significantly higher LZM expression in the 1 ppm group than the control, indicating that adding CQDSpds could increase the LZM expression of both eels. Previous research indicated that with vitamin E supplementation, the LZM activity of Japanese eels increased significantly (Shahkar et al., 2018). Adding seaweed to the rainbow trout (*Oncorhynchus mykiss*) feed also increased the LZM expression (Vazirzadeh et al., 2020). Compared with the control group, the feed supplemented with herbal extracts for the giant marbled eel had the highest

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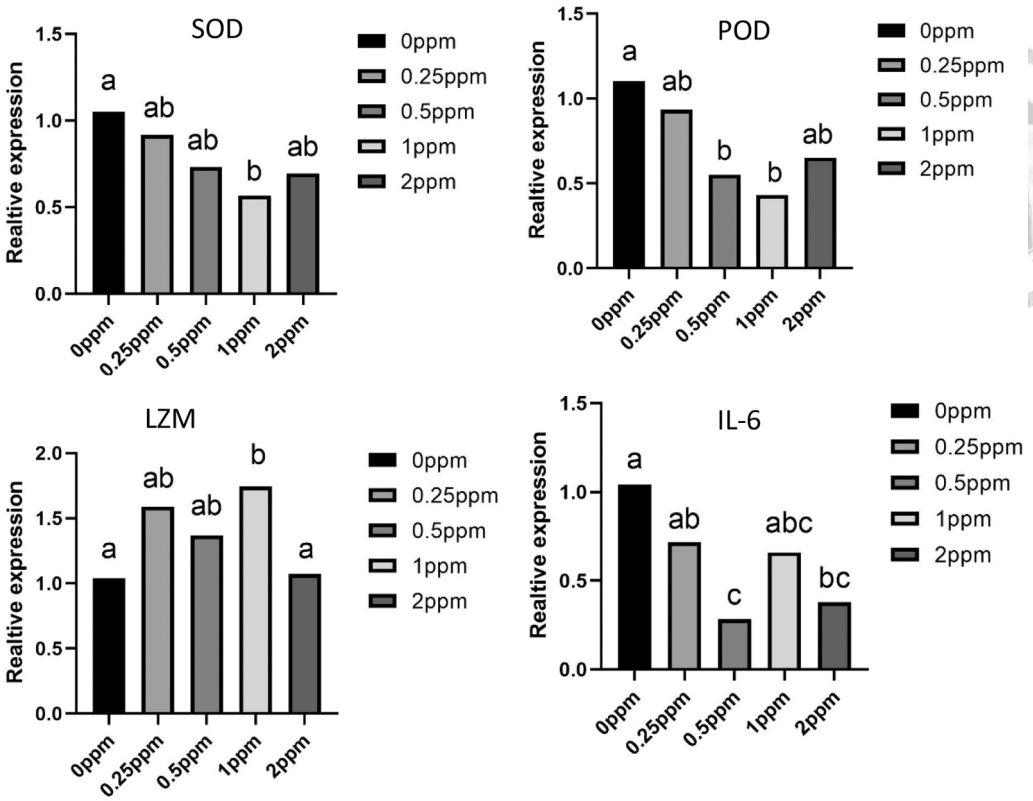

Fig. 3. Immune related gene expression (SOD, POD, LZM, IL-6) in the head kidney of *A. japonica* after feeding trail with different concentration of CQDSpds. Different letters indicate significant difference ($p < 0.05$).

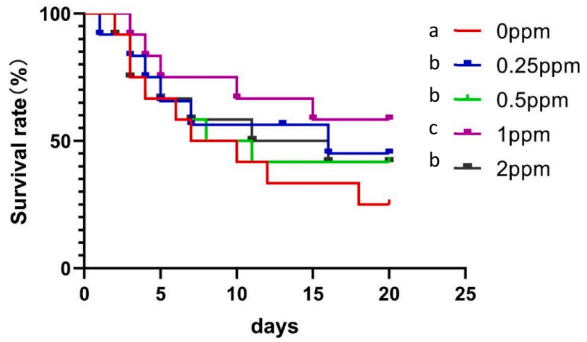

Fig. 4. Survival rate of *A. japonica* fed with different concentration of CQDSpds following challenge with *E. tarda*. Different letters indicate significant difference ($p < 0.05$).

Table 6
Intestinal morphology of A. marmorata fed diet with different concentration of CQDSpds for 84 days (Mean±SD, n = 8) . Data in the same row with different letters are significantly different (p < 0.05) among different treatments.

	0 ppm	0.25 ppm	0.5 ppm	1 ppm	2 ppm
villus height (μm)	61.18 ± 4.98	61.43 ± 6.21	61.79 ± 5.80	60.98 ± 7.37	60.73 ± 4.36
villus circumference ratio (%)	10.7 ± 2.10	10.3 ± 1.41	10.5 ± 3.67	10.8 ± 1.92	10.7 ± 4.35

LZM activity, weight gain rate, and specific growth rate (Xie, 2017). The results of this experiment are consistent with the above results. In this research, we speculated that CQDSpds could increase the non-specific immunity of Japanese eel and giant marbled eel juveniles by increasing the expression of disease resistance-related genes.

IL-6 is secreted by the immune system, which can stimulate tissues to initiate immune mechanisms, help the growth of cells, and promote the activation of immune cells of the adaptive immune system (Tanaka et al., 2014; Stefan et al., 2017). IL-6 plays a vital role in cellular immunity with anti-inflammatory functions (Ana et al., 2020). The mice with IL-6 knockout treatment showed various impairments in the inflammatory response, including increased susceptibility to microbial infection (Spencer et al., 2019) since IL-6 is frequently used as a screening marker for infection. The results of the Japanese eel and giant marbled eel pointed out that the relative expression of IL-6 mRNA in the CQDSpds group was lower than that in the control group, showing that the addition of CQDSpds could reduce the expression of IL-6. IL-6 is a screening marker for inflammation, and IL-6 may be activated in large quantities during bacterial infection to produce an anti-inflammatory response (Ana et al., 2020). The result may indicate that paste feed supplemented with CQDSpds had fewer symptoms of a bacterial infection or inflammation and improved health and immunity compared to the control group.

Edwardsiella tarda causes substantial economic losses in aquaculture industries and is one of the significant eel pathogens culturing globally (Xu and Zhang, 2014). In previous study pointed out that effective antibacterial can be added to the feed to prevent the losses caused by *E. tarda* (Mohanty, Sahoo, 2007). Japanese eels fed a mixed diet composed of *Bacillus phlei* (WB60) and mannooligosaccharide for 8 weeks showed a higher survival rate than the control group against *E. tarda* (S.H. Lee et al., 2018; S. Lee et al., 2018). In the antibacterial circle result in this research, CQDSpds showed an impressive inhibition activity against to the *E. tarda* (Fig. 1), which can observe the bacteriostatic response in the concentration from 0.25 ppm. According to the result, all the eels fed with diets containing CQDSpds also showed more better disease resistance to *E. tarda* than the control group (Fig. 4,

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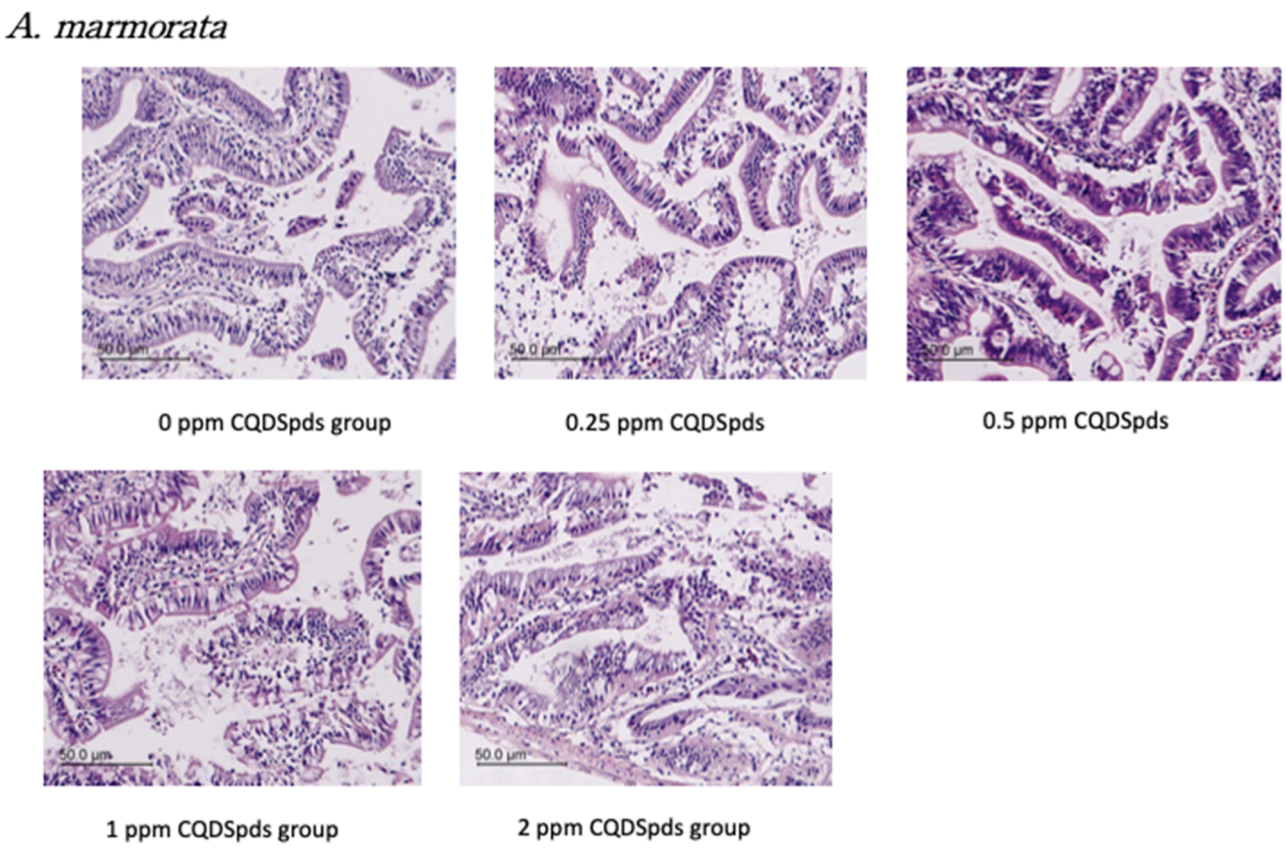

Fig. 5. Details of the intestinal histological examination of the of *A. marmorata* fed diets with different concentration of CQDSpds for 84 days. (Staining; scale bar = 50 μm; Original magnification X 200).

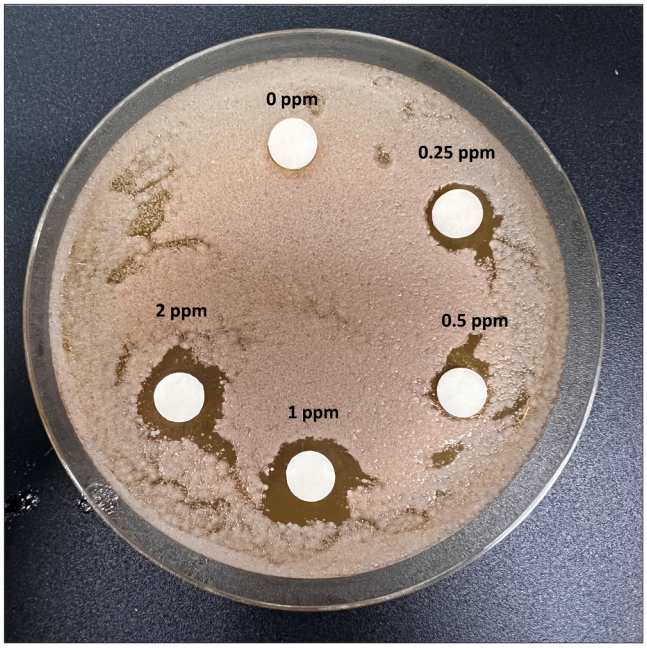

Fig. 1. Bacteriostasis performance of different concentration of CQDSpds.

Fig. 7). However, when the concentration of CQDSpds reaches 2 ppm, the survival rate is lower than at 1 ppm. Although both groups show higher survival rates than the control group, the higher dosage perform worse, the exceed dosage of CQDSpds may weaken the intestine and immune system, which also show the similar trend in the results of Bae et al. (2012) in *A. japonica*. Based on the results, CQDSpds show the potential resistance of *E. tarda*. Also, for eel rearing, appropriate CQDSpds is an excellent feed addition in RAS to reduce the impact when disease outbreaks.

5. Conclusion

After the feeding trial with different contents of CQDSpds in the Japanese eel and the giant marble eel, the Japanese eel at 1 ppm and the giant marbled eel at 0.5 ppm showed the significantly highest weight gain and specific growth rate. The diet supplemented with CQDSpds in an appropriate concentration affected the expression of immune genes in eels. It enhanced the non-specific immunity of eels by increasing LZM activity and the resistance to E. tarda in both eel species without harming intestinal villi. CQDSpds show the potential to be a feed additive for juvenile eels for partially replacing the role of antibiotics by enhancing eel immunity and reducing environmental stress on eels. In the future, it is necessary to conduct further research on the effect of CQDSpds on growth in more aquaculture species for promotion.

Ethics approval

The experiment was performed in accordance with the recommendations from the Institutional Animal Care and Use Committee for the care of animals used for experimental or other scientific purposes (approval number 'A201900005').

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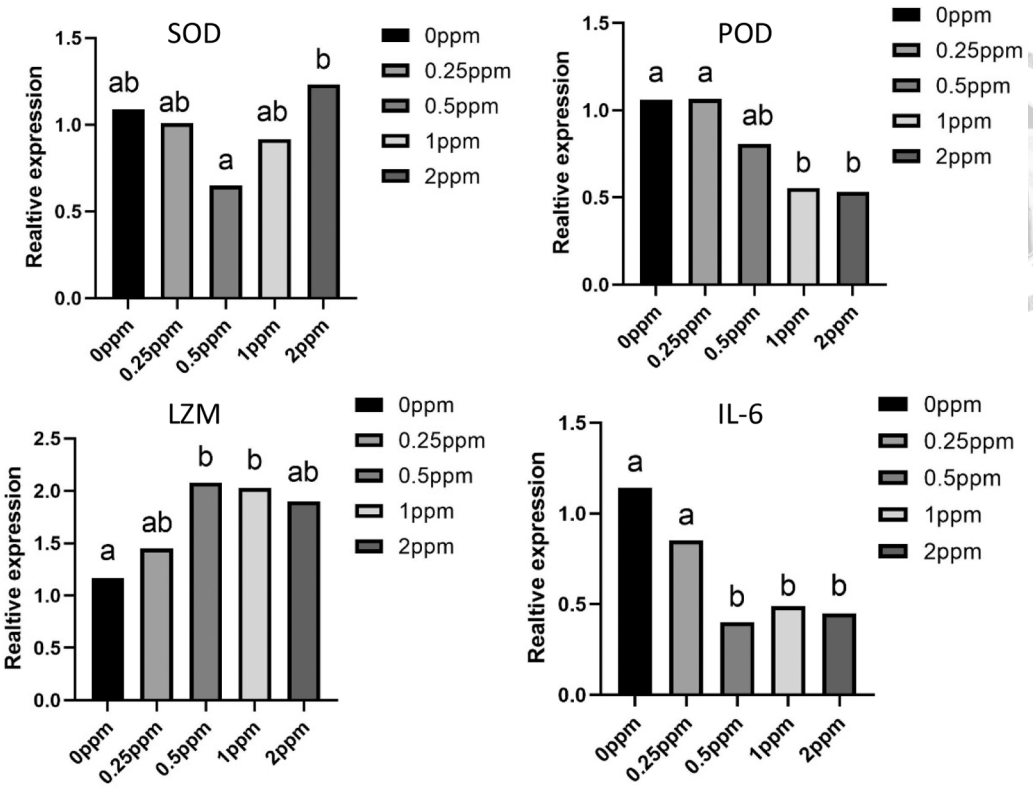

Fig. 6. Immune related gene expression (SOD, POD, LZM, IL-6) in the head kidney of *A. marmorata* after feeding with different concentration of CQDSpds. Different letters indicate significant difference ($p < 0.05$).

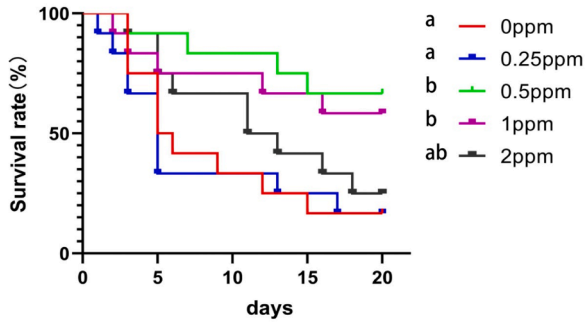

Fig. 7. Survival rate of *A. marmorata* fed with different concentration of CQDSpds following challenge with *E.tarda*. Different letters indicate significant difference ($p < 0.05$).

CRediT authorship contribution statement

Yen-Ting Lin and Yi-Fei Pan mainly conducted the experiments, analyzed the results. Yen-Ting Lin wrote this manuscript. Yu-San Han designed and supervised the experiments. All authors participated in manuscript writing and interpretation of results. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no conflicts of interests.

Data Availability

Data will be made available on request.

Acknowledgement

Authors' contributions, Yen-Ting Lin and Yi-Fei Pan mainly conducted the experiments, analyzed the results. Yen-Ting Lin wrote this manuscript. Yu-San Han designed and supervised the experiments. All authors participated in manuscript writing and interpretation of results. All authors read and approved the final manuscript. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Code availability

Not applicable.

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Effects of Different LED Light Spectra on Growth and Immunity of the Japanese Eel (*Anguilla japonica*) and Giant Mottled Eel (*A. marmorata*)

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Indoor recirculating aquaculture systems make light control possible and enable the usage of specific coloured lights to promote the growth and immunity of aquaculture species. Five different LED wavelengths (white light [460 nm], red light [622 nm], green light [517 nm], blue light [467 nm], and the dark) were used in this study to evaluate growth and immunity in the glass eel stage of two high-valued anguillid species, Japanese eel (*Anguilla japonica*) and giant mottled eel (*A. marmorata*). There were no significant differences in growth of the Japanese eel among the groups after 12 weeks of feeding ($p > 0.05$); the survival rate of each group was over 95%. The giant mottled eel showed better growth in total length and body weight in the red light and dark groups ($p < 0.05$). Expression levels of immune-related genes were not significantly different between each group of the Japanese eel and the giant mottled eel ($p > 0.05$). The growth of the Japanese glass eel was not significantly sensitive to different LED wavelengths, while the giant mottled glass eel showed better growth under red light and dark environments. Neither eel species showed significant differences in innate immunity under different LED wavelengths.

Key words: *Anguilla* eel, Growth, Immunity, LED, Light spectrum.

BACKGROUND

Aquaculture has emerged as the most rapidly growing animal food-producing industry in the last two decades (FAO 2014). Anguillid eels are regarded as an important commercial aquaculture species in East Asia because of their high market demand and nutritional value (Ahn et al. 2015; Shahkar et al. 2015). The Japanese eel *Anguilla japonica* is a traditionally reared, highly valued anguillid species. However, the amount of naturally available *A. japonica* is only 5% of what it used to be in the 1970s because of habitat destruction and overfishing (Chen et al. 2014; Dekker 2004). According to the International Union for Conservation of Nature and Natural Resources (IUCN), *A. japonica* has been classified as "Endangered" in the Red list and needs more attention in the eel aquaculture industry (Jacoby and Gollock 2014). On the other hand, the cultivation of the giant mottled eel (*A. marmorata*), which has abundant glass eels and has low fry prices, has increased in Southeast Asia in recent years (Leander et al. 2013; Luo et al. 2013). However, the low growth rate and high mortality rate caused by disease are still considered the biggest obstacles in the development of giant mottled eel aquaculture (Han 2010).

Indoor recirculating aquaculture systems (RAS)

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have become increasingly popular because of their ability to address concerns related to environmental pollution, lack of water resources and land, and frequent extreme weather events (Martins et al. 2011). Indoor RAS can reduce the water exchange rate to 1/30–1/50 that of traditional outdoor aquaculture ponds, and improve the production capacity by more than ten times (Deviller et al. 2005; Martins et al. 2011). *A. japonica* and *A. marmorata* are usually reared in an enclosed RAS at high density (Hsu et al. 1997; Li et al. 2018). Fish tend to be more easily affected by disease in intensive aquaculture systems due to the stress-induced weakening of their immune systems (Raman et al. 2013). Aquaculturists have traditionally used antibiotics in rearing systems to control various diseases. The overuse of antibiotics causes many negative side effects, such as drug resistance, decline of the immune system, environmental pollution, and food safety issues (Bachère 2003; Pelgrift and Friedman 2013; Cabello et al. 2013). In addition, previous studies have shown that fish in intensive RAS usually show growth retardation and impaired larval development (Davidson et al. 2009; Martins et al. 2009). Therefore, it is imperative to determine an environmentally sustainable method to improve the immunity and growth rate of the cultured species to ensure efficient usage of indoor RAS.

Many studies have indicated that the use of a specific light wavelength (or, light colour) under different light intensities and photoperiods can improve the growth performance of fish and fish embryos and crayfish (Boeuf and Le Bail 1999; Ruchin 2004; Han et al. 2005; Marchesan et al. 2005; Toyota et al. 2022). For example, the juvenile stage of *Candidia barbata* tends to have the best body length growth rate under blue light (460–470 nm) and the worst growth rate under red light (620–630 nm) (Chang 2016). The guppy *Poecilia reticulata* grows better under blue light, while the Chinese sleeper *Perccottus glenii* has a higher growth rate under blue and green light. The crucian carp *Carassius carassius*, the Atlantic halibut *Hippoglossus hippoglossus*, the rainbow trout *Oncorhynchus mykiss*, and the silver carp *Hypophthalmichthys molitrix* showed the best growth under green light and the worst growth under red light (Radenko 1991; Boeuf and Le Bail 1999; Ruchin et al. 2002; Ruchin 2004; Luchiari and Pirhonen 2008). The preleptocephalus stage of the European eel *A. anguilla* showed the best survival rate under low luminosity of red light with a normal photoperiod (Politis et al. 2014). Glass eel-stage *A. marmorata* individuals with a small amount of pigmentation on the skin tend to stay in areas without light, while those without pigmentation tend to stay under red light environments (Mo et al. 2019). Moreover, light of different colours can also affect the innate immune system of the goldfish *Carassius auratus* (Eslamloo et al. 2015). Furthermore, Boeuf and Le Bail (1999) showed that lights of different colours may promote the growth of gonads. For example, the blue damselfish *Chrysiptera cyanea* showed the best gonad development under red light, while *O. niloticus* showed better gonad development under blue light.

Currently, there are no studies comparing the effect of lights of different colours on the growth and immune response of *A. japonica* or *A. marmorata*. Since RAS is mostly an indoor system, it is easy to control light artificially. Therefore, we aimed to determine the effect of different light wavelengths on the growth and immune response in the glass eel stages of the Japanese eel and the giant mottled eel cultured in an indoor RAS system.

MATERIALS AND METHODS

Experimental animals and feeding

Glass eels of *A. japonica* and *A. marmorata* were caught in eastern Taiwan (*A. japonica* from the Yilan River, 24.7163°N, 121.8348°E, and *A. marmorata* from Xiuguluan River, 23.4612°N, 121.5008°E). Eel sampling was approved by the Fishery Agency, Council of Agriculture, Executive Yuan, Taiwan. The specimens were transported at low temperatures through live fish bags filled with oxygen. The health condition of the eels was checked upon arrival at the laboratory located at the Institute of Fisheries Science of National Taiwan University, Taipei. Individuals in good condition were disinfected with 2.5 ppm of potassium permanganate ($KMnO_4$) solution for 10 min to avoid pathogen contamination of the experimental system. After sterilization, the eels were kept in five sets of indoor RAS systems with five tanks (30 × 30 × 45 cm) for each set and maintained in freshwater for three days before feeding. Photoperiods were set at 12 h light (natural light, 7:00–19:00) and 12 h dark during acclimation.

The initial body weight and total length of *A. japonica* and *A. marmorata* (20 *A. japonica* for each tank; 30 *A. marmorata* for each tank in triplicates) were measured before experiment started (56.7 ± 2.0 mm, 0.14 ± 0.01 g for *A. japonica*; 51.04 ± 2.1 mm, 0.15 ± 0.02 g for *A. marmorata*). An LED (EVERLIGHT Electronics Co., Ltd., Taiwan) was used as the light source to control the background spectra for the experiment. Each set of RAS included five tanks (30 L water/tank), each exposed to either white light, red light (622 nm), green light (517 nm), or blue light (467 nm) under 100 Lux (lx) light intensity with photoperiod 12 hours light and 12 hours dark, or the dark (< 5 lx). Each RAS tank was

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covered by a black board to avoid any light influence from neighbouring tanks or the environment (Fig. 1). The water temperature and pH were between 28 ± 1℃ and 7.5 ± 0.5, respectively, with a water exchange rate of 20 L/day for each RAS; oxygen was dissolved to near saturation by aeration. Fish were fed with blood worms (*Chironomus dorsalis* larvae) that about 10 mm × 1.5 mm in size, which are often used as glass eel feed, at an amount of 10% of their body weight twice a day for a total of 12 weeks. The remaining feed was removed from the tanks an hour after feeding. The experiment was performed in accordance with the recommendations from the Institutional Animal Care and Use Committee for the care of animals used for experimental or other scientific purposes (approval number 'NTU-110-EL-00009').

Sample Collection and Analyses

The total length (to the nearest 0.1 mm) and body weight (to the nearest 1 mg) were measured every two weeks. The percentage weight gain, condition factor, specific growth rate, and the survival rate in each group were calculated as follows:

$$\text{Percentage weight gain (\%)} = \frac{\text{Final body weight (g)-Initial body weight (g)}}{\text{(Initial body weight)}} \times 100$$

$$\text{Condition factor (K)} = 1000 \times \frac{\text{Body weight (g)}}{\text{Body length}^3\text{ (cm)}}$$

$$\text{Specific growth rate} = \frac{\text{Exp[ln (Final body weight) - ln (Initial body weight)]}}{84} \times 100$$

$$\text{Survival rate (\%)} = \frac{\text{Final n}}{\text{Initial n}}$$

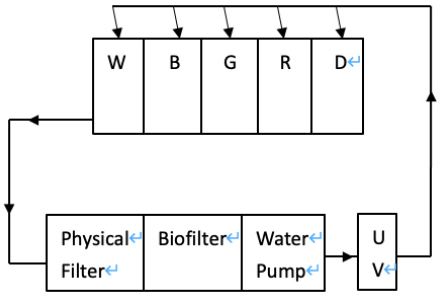

Fig. 1. Graph of a set of recirculating aquaculture systems (RAS) used in this study. The five tanks were each 40 L in volume and covered by a black board. W: white light; R: red light (622 nm); G: green light (517 nm); B: blue light (467 nm).

Three fish from each tank were randomly selected and sacrificed to obtain head kidney tissues. Three head kidney tissues from the same tank were pooled together and stored in an RNA protecting reagent at -80℃ before extracting total RNA using an RNA kit (Bioman Scientific Co. Ltd., Taiwan) for real-time PCR of immune-related genes.

Real-time PCR

Specific candidate genes were selected for real-time PCR based on previous studies about eel immunology (Birhanu et al. 2016; Lee et al. 2017). Four immune-related genes, namely, superoxide dismutase (*SOD*), lysozyme (*LZM*), peroxidase (*POD*), and interleukin-6 (*IL-6*) were selected as the target genes for real-time PCR, and acidic ribosomal protein (*ARP*) was used as the reference gene. The whole genome of *A. japonica* was successfully assembled in our previous study (http://molas.iis.sinica.edu.tw/jpeel/) (Hsu et al. 2015), and was used as a template to annotate the transcriptome data of *A. japonica* and *A. marmorata*. Using the website, all the gene we chose can be annotated in both eels. TRIzol reagent (Bioman Scientific Co. Ltd) was used to extract total RNA, and the purity was quantified by spectrophotometry (Medclub Scientific Co. Ltd). Reverse transcription was performed to synthesize complementary DNA (cDNA) for real-time PCR (Bio-Rad). The primers used for real-time PCR are listed in table 1.

Statistical analysis

All data were analysed by one-way analysis of variance (IBM SPSS Statistics 24.0) to determine the effects of different spectra. Statistical significance was set at $p < 0.05$. A significant effect was followed up with the least significant difference test to compare the means.

RESULTS

Growth rate

The mean initial total length and body weight of the eels from each tank were not significantly different before the start of the experiment (Tables 2, 3). The growth of *A. japonica* showed no significant difference in the total length (Fig. 2) and body weight (Fig. 3) among the different groups ($p > 0.05$) after 12 weeks of feeding (Table 2). The percentage weight gain, condition factor (K), and survival rate also did not show significant differences among the groups ($p > 0.05$)

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(Table 3).

Although *A. marmorata* grew much slower than the Japanese eel, its growth rate was significantly different among each of the treatment groups ($p < 0.05$) (Figs. 4, 5). The mean total length and body weight were significantly higher in the dark and red light groups than in the other groups ($p < 0.05$) (Table 3). The specific growth rate and percentage weight gain in these groups

Table 1. Primers used for qPCR amplification

Genes	Primer	Sequences
ARP (reference gene)	Forward	5'-GTGCAGCTCATTAAGACCGG-3'
	Reverse	5'-GGCGATATTCCTCACACCCT-3'
SOD	Forward	5'-TAACGTACGACTATGGGGCC-3'
	Reverse	5'-GCCGCCACCATTAAACTTCA-3'
LZM	Forward	5'-TGCTGGAATGGATGGATACC-3'
	Reverse	5'-GTAATCGCAGTGCTGATGTC-3'
POD	Forward	5'-GACATCACCCGTTTCTGCAA-3'
	Reverse	5'-GTGGATGAAGGAGGGGAACA-3'
IL-6	Forward	5'-CCAGATGTCGCTTCACTTCG-3'
	Reverse	5'-ACTTGGATGTCGTCACCCAT-3'

Table 2. Growth of *A. japonica* reared in different LED light spectra after 12 weeks

	White	Red	Green	Blue	Dark
Initial TL (mm)	56.8 ± 2.1[a*]	57.0 ± 2.0[a]	56.8 ± 1.9[a]	56.7 ± 1.6[a]	56.3 ± 2.0[a]
Final TL (mm)	95.9 ± 10.2[a]	94.0 ± 10.0[a]	93.9 ± 13.7[a]	93.7 ± 12.5[a]	95.7 ± 7.7[a]
Initial BW (g)	0.14 ± 0.01[a]	0.15 ± 0.01[a]	0.15 ± 0.01[a]	0.14 ± 0.01[a]	0.14 ± 0.01[a]
Final BW (g)	0.78 ± 0.25[a]	0.74 ± 0.28[a]	0.73 ± 0.22[a]	0.75 ± 0.32[a]	0.74 ± 0.28[a]
SGR (%)	1.95 ± 0.43[a]	1.90 ± 0.66[a]	1.88 ± 0.40[a]	1.99 ± 0.65[a]	1.98 ± 0 .65[a]
PWG (%)	446.8[a]	376.4[a]	397.4[a]	409.8[a]	376.9[a]
Initial number	40	40	40	40	40
Final number	37	39	39	39	39
Survival rate (%)	92.5[a]	97.5[a]	97.5[a]	97.5[a]	97.5[a]
Initial K	0.784[a]	0.804[a]	0.791[a]	0.783[a]	0.802[a]
Final K	0.852[a]	0.841[a]	0.867[a]	0.842[a]	0.841[a]

TL: total length; BW: body weight; SGR: specific growth rate; PWG: percentage weight gain; K: condition factor. Different letters indicate significant differences between groups ($p < 0.05$).

Table 3. Growth performance of *A. marmorata* reared in different LED light spectra after 12 weeks

	White	Red	Green	Blue	Dark
Initial TL (mm)	51.0 ± 2.1[a*]	50.7 ± 2.1[a]	51.1 ± 2.2[a]	51.0 ± 1.6[a]	51.4 ± 2.0[a]
Final TL (mm)	62.6 ± 6.1[a]	66.0 ± 6.5[b]	63.3 ± 7.6[a]	62.2 ± 6.1[a]	68.0 ± 7.4[b]
Initial BW (g)	0.15 ± 0.03[a]	0.15 ± 0.03[a]	0.15 ± 0.03[a]	0.15 ± 0.02[a]	0.15 ± 0.02[a]
Final BW (g)	0.32 ± 0.12[a]	0.39 ± 0.18[b]	0.33 ± 0.13[a]	0.31 ± 0.11[a]	0.43 ± 0.18[b]
SGR (%)	0.82 ± 0.46[ab]	1.11 ± 0.47[ac]	0.90 ± 0.39[abc]	0.67 ± 0.43[b]	1.14 ± 0.38[c]
PWG (%)	112.9[ab]	173.4[ac]	123.7[abc]	86.6[b]	174.1[c]
Initial n	90	90	90	90	90
Final n	54	68	56	51	39
Survival rate (%)	60[b]	76[c]	62[b]	57[b]	44[a]
Initial K	1.106[a]	1.080[a]	1.093[a]	1.120[a]	1.078[a]
Final K	1.245[a]	1.262[a]	1.182[a]	1.206[a]	1.384[a]

TL: total length; BW: body weight; SGR: specific growth rate; PWG: percentage weight gain; K: condition factor. Different letters indicate significant differences between groups ($p < 0.05$).

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were also significantly higher than those in the blue light group. The survival rate was significantly higher in the red light group than in the green light and dark groups. However, there was no significant difference in the condition factor (K) among the groups (Table 3). The fastest growing period of the giant mottled eel occurred from the sixth to the eighth week (Figs. 4, 5). On the other hand, the white, green, and blue light groups showed some growth retardation during the eighth to the tenth week. The red light group showed no decrease in growth during the entire experimental period.

Real-time PCR

Real-time PCR was conducted for precise quantification to compare whether the innate immunity of both eel species was affected by different light spectra. The target genes of real-time PCR were *SOD*, *LZM*, *POD*, and *IL-6*, which referred to previous research about eel immunology (B.T. Birhanu et al. 2016; Lee et al. 2017) and the *ARP* was used as the

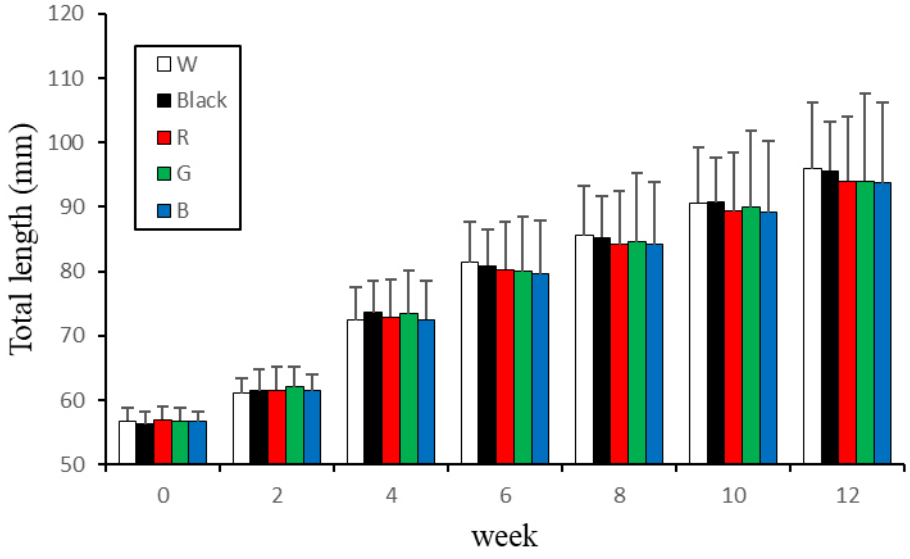

Fig. 2. The total length of Japanese eel reared in different light spectra for 12 weeks. W: white light; Black: dark; B: blue light; G: green light; R: red light.

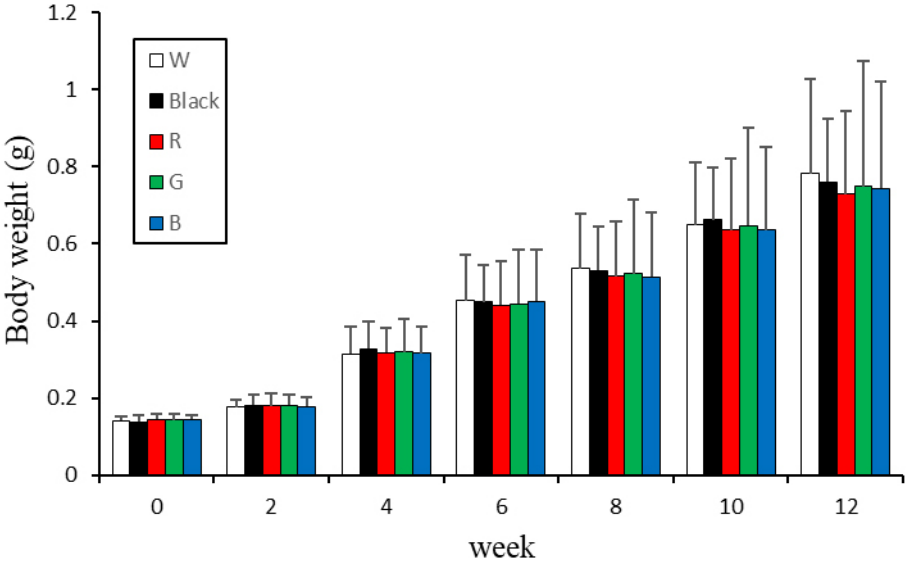

Fig. 3. The body weight of Japanese eel reared in different light spectra for 12 weeks. W: white light; Black: dark; B: blue light; G: green light; R: red light.

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reference gene. The results for *A. japonica* indicated that although the dark group showed higher *SOD* expression, there was no significant difference among the groups ($p > 0.05$) (Fig. 6). The expression levels of *LZM* in the red light and dark treatment groups were higher than in others, but there were no significant differences among groups ($p > 0.05$) (Fig. 7). The white light group showed the highest expression of *IL-6*; however, there was no significant difference among the groups ($p > 0.05$) (Fig. 8). The expression of *POD* was highest in the green light group but was not significantly different from the other groups ($p > 0.05$) (Fig. 9).

The real-time PCR results of the giant mottled eel showed that *SOD* expression was higher in the dark group than in the other groups, but there was no significant difference among the groups ($p > 0.05$) (Fig. 6). The expression level of *LZM* in white light and red light groups was the highest; however, there was no significant difference among the groups ($p > 0.05$) (Fig. 7). The red light and white light groups also showed higher expression levels of *LZM* than the other groups, but without a significant difference ($p > 0.05$) (Fig. 8). The *POD* expression levels in the blue light group were lower than those in others; however, there was no significant difference among the groups ($p < 0.05$) (Fig. 9). Moreover, comparison of the qPCR results between both eel species showed no significant differences ($p > 0.05$) in the expression levels of the four immune-related genes under all light spectra (Figs. 6–9).

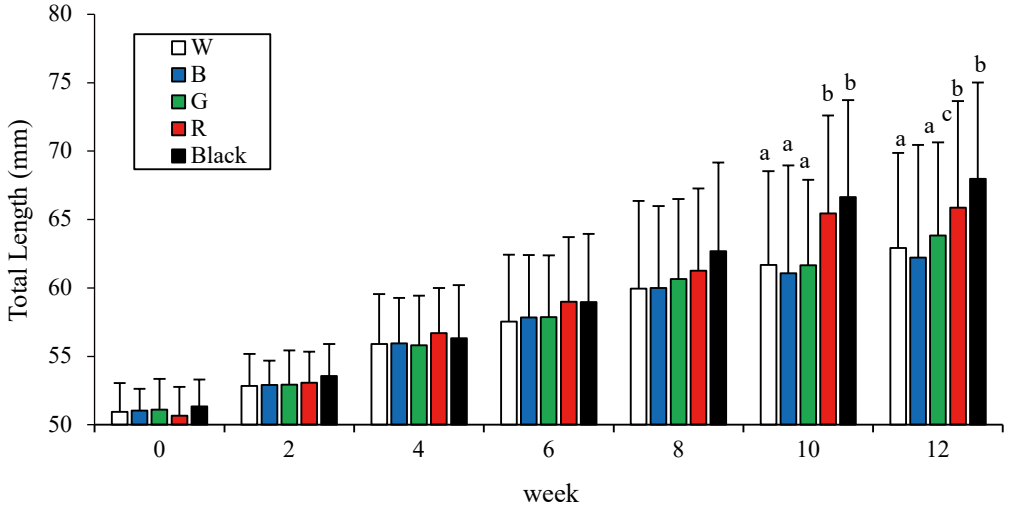

Fig. 4. The total length of giant mottled eel reared in different light spectra for 12 weeks. W: white light; Black: dark; B: blue light; G: green light; R: red light. Different letters indicate significant differences between groups of the same week ($p < 0.05$).

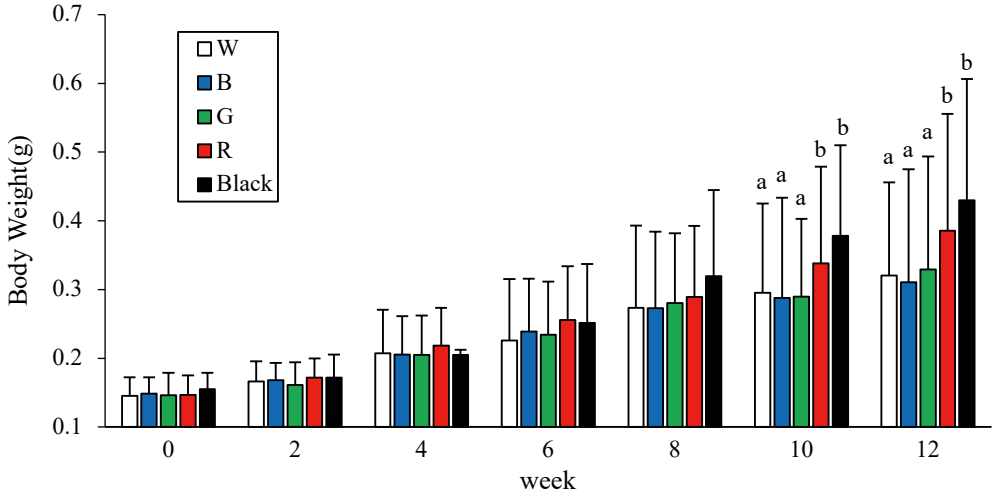

Fig. 5. The body weight of giant mottled eel reared in different light spectra for 12 weeks. W: white light; Black: dark; B: blue light; G: green light; R: red light. Different letters indicate significant differences between groups of the same week ($p < 0.05$).

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DISCUSSION

An earlier study has shown that some fish showed different growth rates under a specific background light spectrum, but the most suitable spectrum differed among species. For example, the pikeperch *Sander lucioperca* exhibits the highest growth rates and cortisol levels under white light and the lowest under blue light due to the enhancement of cone cells for visual sensitivity under longer-wavelength light, (Luchiari et al. 2009). Others, such as the barramundi *Lates calcarifer*, show the best growth rate under red light environments but

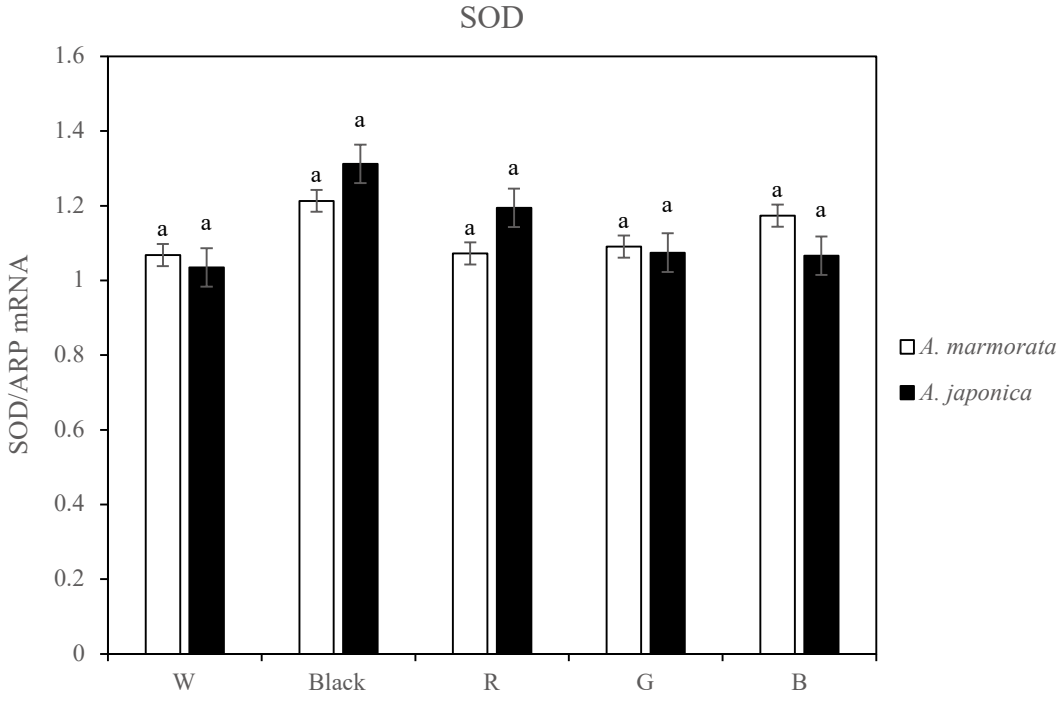

Fig. 6. The *SOD* expression levels of Japanese eel and giant mottled eel reared in different light spectra. W: white light; B: blue light; G: green light; R: red light; black: dark. Different letters indicate significant differences between different spectra groups of the same eel species ($p < 0.05$).

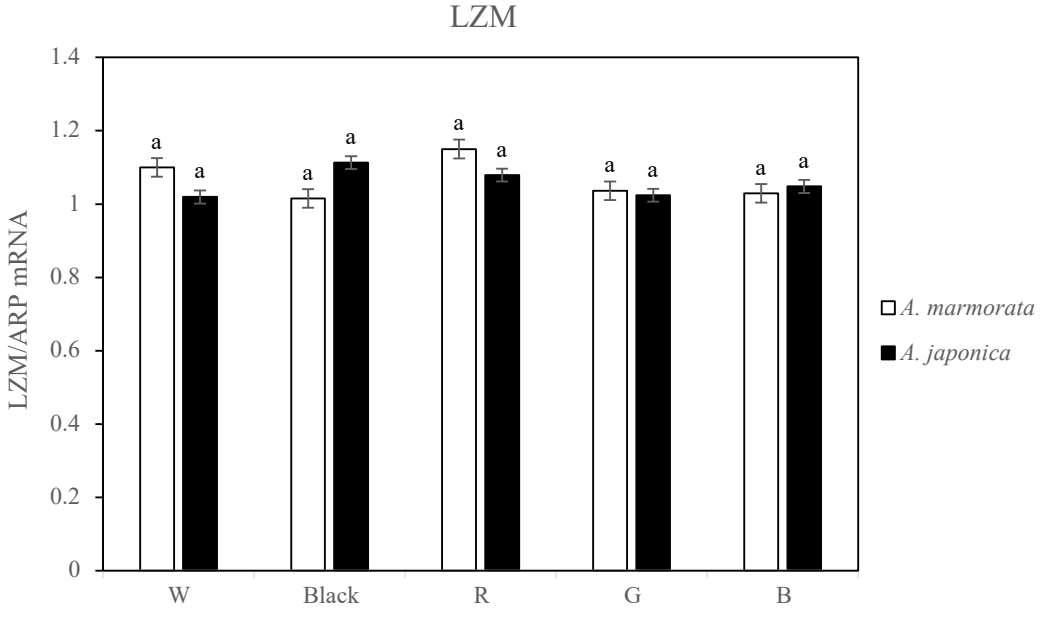

Fig. 7. The *LZM* expression levels of Japanese eel and giant mottled eel reared in different spectra. W: white light; B: blue light; G: green light; R: red light; black: dark. Different letters indicate significant differences between different spectra groups of the same eel species ($p < 0.05$).

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the worst under green light, because the increase in spectral sensitivity under longer wavelength conditions enhances their feeding behaviour (Jeremy et al. 2011). Different spectra may affect fish visual systems and further influence their physiological functions, such as growth, immune response, endocrine system, etc.

There were no significant differences among the groups in the growth experiment of the glass eel stage of the Japanese eel. Interestingly, Japanese eels in the blue light group showed better feeding motivation than the other groups. Red light could stimulate the feeding motivation in Nile tilapia *Oreochromis niloticus* but did not improve its growth (Volpato et al. 2013). McLean et al. (2018) indicated that tank colour did not affect the growth performance of juvenile flounder or tilapia, although fish maintained in red-light tanks showed better percent increases in body weight and lower plasma cortisol levels. Some studies have also pointed out that rearing under different light spectra may not change the growth rate of juvenile fish but may have different effects on other behaviours (Villamizar et al. 2011). Such behavioural effects in Japanese eels cultured under different spectra need to be studied further.

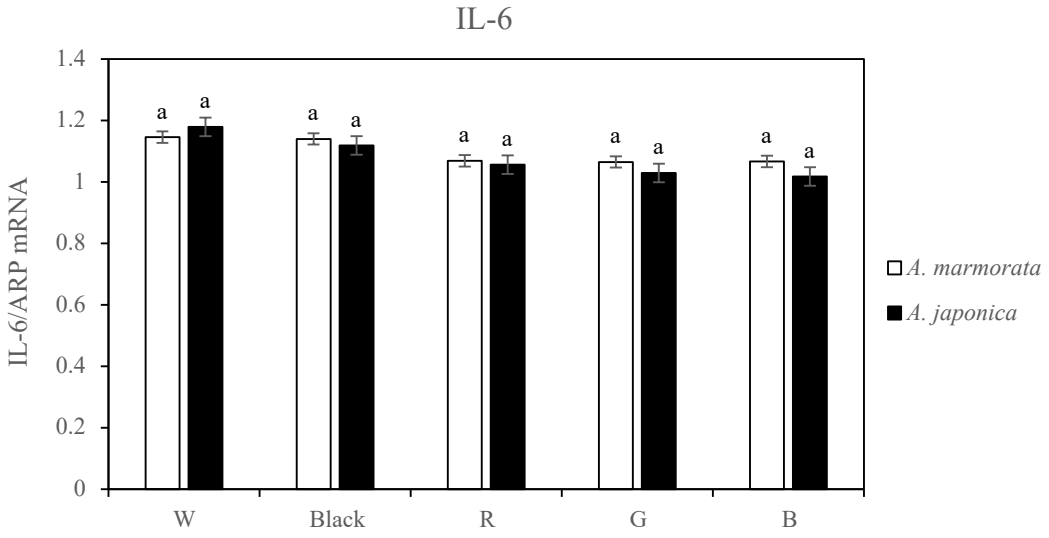

Fig. 8. The *IL-6* expression levels of Japanese eel and giant mottled eel reared in different spectra. W: white light; B: blue light; G: green light; R: red light; black: dark. Different letters indicate significant differences between different spectra groups of the same eel species ($p < 0.05$).

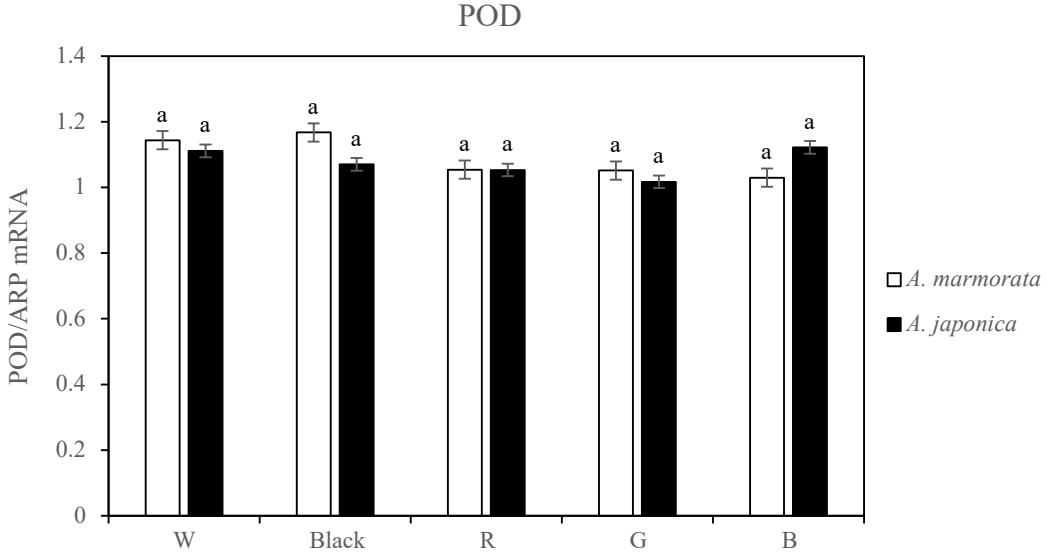

Fig. 9. The *POD* expression levels of Japanese eel and giant mottled eel reared in different spectra. W: white light; B: blue light; G: green light; R: red light; black: dark. Different letters indicate significant differences between different spectra groups of the same eel species ($p < 0.05$).

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On the other hand, the giant mottled eel clearly showed significantly better growth in the dark and red light groups ($p < 0.05$). Aquatic creatures use photoreceptor cells with the highest photosensitivity at a specific wavelength (λ_{max}) to detect underwater objects. λ_{max} can maximize visual acuity, such as in deep-sea fish (Bowmaker 1990), or maximize visual contrast, such as in fish inhabiting shallow water or coastal areas (Lythgoe 1979). Therefore, fish tend to live in environments with the best spectral conditions (Downing and Litvak 2001). Light of specific colours may enhance their growth potential by facilitating food capture or detection (Pérez et al. 2019). In addition, light may have some potential non-visual effects on endocrine secretion in non-mammalian vertebrate brains, including in growth hormone or thyroid hormone (Jonathan et al. 2019). The giant mottled eel shows a blue-shifted rod photoreceptor during its upstream migration stage (Wang et al. 2014), which is insensitive to red light. Moreover, eels are nocturnal animals that prefer to stay away from light. Therefore, it is likely that red light or dark surroundings may reduce stress for the giant mottled eel, resulting in better overall growth. The Japanese eel seems to be more insensitive to the environmental spectrum, and this may have resulted in the lack of significant difference in growth among the treatment groups.

Most of the mortality of the two anguillid species in this study, especially that of the giant mottled eel, resulted from their escape from the tank (Tables 2, 3). This might be because the eels are less adapted to a specific wavelength, increasing their stress levels and eliciting an escape response. The Japanese eel may be more tolerant to lights of different colour, and is thus well-adapted to the environment, resulting in a high survival rate. The escape rate was generally high for the giant mottled eel, especially in the dark group. However, it also had the largest growth rate, and stress did not seem to be an important factor. Alternatively, an earlier study suggested that the escape behaviour may be a natural instinct for the giant mottled eel (Matsuda et al. 2016), considering that it prefers to migrate in its early life stage.

Interestingly, the body colour of the giant mottled eel in the red light and dark groups was slightly lighter than those of the others, which is similar to the results of an earlier study (Shin and Choi 2014). It has been shown that the pigments in fish can respond to the wavelength of the colour of their environmental background (Bayarri et al. 2002). Biofilm attachments were found on the tank wall in some groups in our study. There were attachments with a dark brown muddy biofilm on the bottom of the blue, green, and white light tanks, while the red and dark tanks had no attachments. This may have caused the lighter body colour in red and dark groups to adapt to the environment without dark attachment.

Lysozyme (*LZM*) is an important enzyme that shows antiviral, antibacterial, and anti-inflammatory activities (Saurabh and Sahoo 2008). *LZM* also combines and metabolizes advanced glycosylation end products produced from reactive oxygen species that would otherwise accumulate and cause harm to organisms. Similar to the antioxidant system, *LZM* may also be affected by stress responses (Eslamloo et al. 2015; Zheng et al. 2016; Gao et al. 2017; Li et al. 2018). *LZM* expression levels increase under a red light environment in the pikeperch *Sander lucioperca* (Baekelandt et al. 2019). The results of real-time PCR of *LZM* in both the Japanese eel and giant mottled eel showed no significant difference among groups (Fig. 7), suggesting that different light spectra may not have any significant effect on *LZM* expression.

SOD and *POD* are both important components of the antioxidant system, and catalyse the conversion of superoxide into hydrogen peroxide and oxygen to remove reactive oxygen species; they are also key components of the Nrf2 pathway (Fattman et al. 2003; Lin et al. 2008; Shao et al. 2010; Li 2012; Liu et al. 2015; Deyashi and Chakraborty 2016). The real-time PCR results of both eel species revealed that although the dark group showed the highest *SOD* value, there were no significant differences among the groups ($p > 0.05$) (Figs. 6 and 9). *SOD* and *POD* have been used as biomarkers of stress in previous studies (Abele and Puntarulo 2004; Oliva et al. 2012) due to a dramatic change in the mRNA content and activity of *SOD* and *POD* in response to stress (Shin et al. 2011; Choi et al. 2016; Osman et al. 2019). The stress level of each eel species in each background spectrum may not have differed significantly from each other in our study.

IL-6 is a chemical secreted by the immune system (Tanaka et al. 2014). It can stimulate the body tissues to activate immune mechanisms, help the growth of cells, promote the activation of immune cells of the acquired immune system, and direct blood cells to help macrophages destroy the source of infection (Stefan et al. 2017). An increase in its concentration can lead to a cytokine storm (Ana et al. 2020). The results of real-time PCR for both eel species showed no significant differences among the groups (Fig. 8). This suggests that the expressions of the innate immune genes were not affected by different light spectra in either eel species. The results also showed no significant difference in expression levels between both eel species, which indicates that different spectra only affect the growth of giant mottled eel.

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CONCLUSIONS

Growth is not affected by the wavelength of light in Japanese eels. However, when rearing giant mottled eels, short-wavelength environments should be avoided. Red light environments would be more suitable for their growth and survival. Neither of the eel species showed any significant difference in the expression of four innate immune-related genes (*LZM*, *SOD*, *POD*, and *IL-6*). The regulatory effects of different light spectra on the immune system and endocrine mechanism of fish require further study.

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Competing interests: The authors declare that they have no conflicts of interests.

Availability of data and materials: The data that support the findings of this study are available from the corresponding author, Yu-San Han, upon reasonable request.

Consent to publication: Not applicable.

Ethics approval consent to part: The experiment was performed in accordance with the recommendations from the Institutional Animal Care and Use Committee for the care of animals used for experimental or other scientific purposes (approval number 'NTU-110-EL-00009').

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