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ENHANCED DNA EXTRACTION FROM TRIGONA HONEY: A LOW-VOLUME OF SAMPLE, HIGH-PURITY APPROACH FOR MOLECULAR RESEARCH

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Key words: honey, trigona, DNA extraction method, DNA amplification.

Abstract

Bees synthesize honey from floral nectar, with pollen inadvertently incorporated during the foraging process. Pollen constitutes the primary source of plant DNA in honey; however, its extraction is impeded by the high concentrations of sugars, phenolic compounds, and carbohydrates, often resulting in low purity and necessitating substantial sample volumes. This study presents a modified DNA extraction technique specifically optimized for Trigona honey, aimed at enhancing both efficiency and practicality. The protocol involves a pretreatment step where honey is diluted in a 1:4 ratio with distilled water, incubated at 60 °C for 25 minutes, followed by extraction without sample destruction. The quality of the extracted DNA was assessed using a nano spectrophotometer and PCR, demonstrating a high concentration of 1,790 ng/μL with distinct, smear-free bands. This method is straightforward, time-efficient, and resource-conserving, rendering it highly applicable for molecular research and honey authentication.

Introduction

Honey, a natural sweet substance with nutritional and therapeutic properties, has played a central role in traditional medicine for several centuries. It has long been used to treat burns, respiratory diseases, digestive infections, and wounds, thus underscoring its bioactive potential (NORDIN et al. 2018). In addition to its cultural and medicinal importance,

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honey is a complex biological matrix comprising of carbohydrates, amino acids, minerals, enzymes, vitamins, phenolic compounds, and water. This biochemical richness contributes to both its health-promoting qualities and its significance as a product of its ecological and agricultural value.

While Apis mellifera accounts for the majority of commercial honey, stingless bees (*Trigona* spp.) produce a distinct type of honey known as meliponine honey, pot honey, sugar bag honey, or kelulut honey in Malaysia. Stingless bee honey has been reported to have higher antioxidant activity, approximately 45% greater than that of A. mellifera honey, along with antidiabetic, antimicrobial, and immunomodulatory benefits (AZIZ et al. 2017, KRISHNASREE and UKKURU 2016, ÁVILA et al. 2019). Its unique flavor, medicinal attributes, and cultural heritage emphasize the growing need for the robust authentication and molecular characterization of stingless bee honey.

The quality and identity of honey are primarily determined by the floral sources visited by bees. Honey can be categorized as monofloral or multifloral depending on its botanical origin. Monofloral honeys are derived predominantly from a single plant species and are valued for their distinctive aroma, taste, and biological properties, whereas multifloral honeys are produced from diverse floral sources (SCHIEVANO et al. 2016). The composition of floral sources directly influences the phenolic and flavonoid content, shaping both the nutritional and therapeutic properties of honey. For example, antibacterial honey is often derived from *Corymbia calophylla, Eucalyptus marginata,* and *Leptospermum polygalifolium* (IRISH et al. 2011). Geographic location, seasonal variation, and nectar sources further contribute to differences in honey phytochemistry (VALDÉS-SILVERIO et al. 2018, ZAWAWI et al. 2021, LARSEN and AHMED 2022).

Accurate identification of floral origins is therefore essential not only to ensure quality and prevent adulteration, but also to explore honey as a natural archive of environmental DNA (eDNA). DNA-based molecular techniques have become preferred tools for species identification because of their speed, accuracy, and reproducibility (GULTOM et al. 2023; GULTOM et al. 2025, HAFZARI et al. 2024). Studies such as those by WIRTA et al. (2021) and SOARES et al. (2017) have demonstrated the ability of DNA metabarcoding to identify pollen sources down to the species level, reconstruct floral diversity, and detect plant taxa such as *Calluna vulgaris, Lavandula* spp., and *Eucalyptus* spp. from honey. Recent advances have highlighted honey-derived DNA as a promising source for environmental monitoring and plant-pollinator interaction studies in different ecosystems (ULLAH et al. 2024).

Despite its promise, DNA extraction from honey remains technically challenging. High concentrations of sugars, phenolics, and polysaccharides frequently inhibit enzymatic reactions and reduce the DNA yield and quality (RIBANI et al. 2022, UTZERI et al. 2018). Established protocols often require large sample volumes (up to 50 mL), specialized glass-bead grinding equipment, and prolonged pretreatment steps, limiting their practicality for large-scale molecular studies (RIBANI et al. 2022). Previous studies WITA et al. (2021) emphasized the necessity of optimizing extraction protocols; however, a gap remains in developing a method that is simple, cost-effective, and efficient, while still producing high-quality DNA from small honey volumes.

This study aimed to address this gap by presenting a modified DNA extraction protocol specifically optimized for stingless bee honey (*Trigona* spp.). Key innovations include reducing the required sample volume, shortening the incubation duration, and optimizing lysis and washing steps. By improving the efficiency without compromising DNA integrity, this method seeks to provide a practical tool for molecular studies, honey authentication, and broader applications in biodiversity monitoring and food traceability.

Materials and Methods

Collection of honey *Trigona* sp. sample

The honey samples used in this study were Trigona and multiflora honey types obtained from forests in Riau.

DNA extraction: improve method

The DNA extraction procedure has been modified, as LOWE et al. (2022) referenced. DNA extraction from honey samples involves several stages: pretreatment of the honey sample, incubation, centrifugation to isolate fractions for sample pellets, DNA extraction utilizing a modified commercial kit, and assessment of DNA quality through electrophoresis and a nano spectrophotometer. The pretreatment of honey samples involves diluting the sample with sterile distilled water. The dilution ratio of honey to distilled water is 1:4 (1 ml of honey to 4 ml of distilled water). Honey diluted with distilled water is incubated at 60 °C for 25 minutes. The subsequent phase involves partitioning the incubated honey sample into four tubes (1,250 µl each), followed by centrifugation at 10,000 rpm for five minutes. The supernatant is discarded, and the particle is retained. The pellet is diluted by adding 25 µl of sterile distilled water. Subsequently, vortex momentarily at moderate velocity. Subsequently, centrifuge and amalgamate the pellets from the four diluted tubes into a single tube. Moreover, the pellets were directly extracted utilizing the Dneasy Plant Mini kit (QIAGEN), omitting the need for glass beads for sample disruption.

The diluted pellets combined in one tube were supplemented with 400 µl of AP1 buffer and 5 µl of RNase A, then vortexed and incubated for 10 minutes at 65 °C. Introduce 130 µl of P3 buffer. Incubate at -20 °C for 3 minutes. Centrifuge for 7 minutes at 13,500 revolutions per minute. Transfer the supernatant to the QIA shredder spin column and centrifuge at 13,500 rpm for 4 minutes. Transfer the supernatant to a fresh tube, then incorporate 1.5 ml of AW1 buffer. Transfer 650 µl of the mixture to a DNeasy Mini spin column and centrifuge for 1 minute at 8000 rpm. Insert the spin column into a fresh collecting tube. Add 500 µl of Buffer AW2 and centrifuge for 1 minute at 8000 rpm. Eliminate the supernatant. Introduce 500 µl of Buffer AW2 and centrifuge at 13,500 rpm for 3 minutes. Relocate the spin column to a fresh tube; add 50 µl of Buffer AE, incubate for 5 minutes at ambient temperature, and centrifuge at 8000 rpm for 1 minute. The concentration of DNA was quantified with a nano spectrophotometer and PCR amplification.

Evaluation of Polymerase Chain Reaction (PCR) amplification

DNA was amplified using universal primers for the target genes *ITS2* and *rbcl*. The primers used refer to (Urumarudappa et al. 2020). The primer sequences used can be seen in Table 1. PCR was performed with a final reaction volume of 25 µl. A total of 2.5 µl of DNA template was mixed with 12.5 µl of HotStart Taq Polymerase master mix (QIAGEN), 0.5 µl of each primer (5 µM), 1.0 µl of BSA (10 µM) and eight µl of nuclease-free water. The PCR cycle was carried out by following the program as follows: predenaturation at 95 °C for 5 minutes, followed by denaturation at 95 °C for 40 seconds, annealing at 48 °C for 60 seconds, extension at 72 °C for 30 seconds for 35 cycles, and a final extension at 72 °C for 5 minutes and 30. Visualization of PCR results was carried out using electrophoresis. This process was carried out using 1.5% agarose gel in TAE buffer 1X at 70 volts for 70 minutes. After completion, the gel was stained with Gel Red and visualized under

Table 1

List of primer sequences to be used for amplification and metabarcoding

Target gene	Name of primer	Primer sequence 5’-3’
ITS2	SF	5’-ATGCGATACTTGGTGTGAAT-3’
	SR	5’-GACGCTTCTCCAGACTACAAT-3
rbcl	RBF	5’-ATGTCACCACAAACAGAAAC-3’
	RBV	5’-TCGCATGTACCTGCAGTAGC-3’

a UV transilluminator to see the amplified DNA bands. The Thermo Scientific GeneRuler 100 bp DNA Ladder was used to determine the size of the DNA bands. The electrophoresis results were then photographed using gel documentation (BALKANSKA et al. 2020).

Results

DNA concentration and purity

The extraction of genomic DNA is a crucial step in molecular analysis, particularly for species identification and phylogenetic studies. The findings of this study demonstrate that DNA extraction from honey samples can be effectively performed using a modified protocol with reduced sample volume and simplified processing. The DNA concentration varied among the tested honey samples, with the highest concentration obtained from multiflora honey (1,790 ng/µl), followed by Trigona honey with red coloration (965 ng/µl) and Trigona honey with yellow coloration (580 ng/µl). The DNA purity, measured by the A260/A280 ratio, ranged from 1.43 to 1.57, which is slightly below the optimal range of 1.8–2.0 required for high-purity DNA (LUCENA-AGUILAR et al. 2016).

Despite achieving high DNA concentrations, the suboptimal purity suggests the presence of residual contaminants such as proteins, phenols, or carbohydrates, which may interfere with downstream molecular applications. Previous studies have reported that insufficient washing steps during DNA precipitation could contribute to lower purity values (BABADI et al. 2022). Increasing the number of washing steps or incorporating additional purification techniques may enhance DNA purity. However, the high DNA yield obtained in this study highlights the effectiveness of the modified protocol in extracting sufficient DNA for molecular applications, especially when sample availability is limited. Elevating the temperature during sample pre-treatment is recognized to enhance the efficacy of cell lysis (MENCHHOFF et al. 2020)

Polymerase Chain Reaction (PCR) amplification

To further validate the applicability of the extracted DNA, PCR amplification was performed using *ITS2* and *rbcl* gene-specific primers. The PCR results demonstrated clear and well-defined DNA bands with no visible smearing, confirming the suitability of the extracted DNA for molecular analysis (Figure 1). The absence of smear indicates that the extracted DNA was of adequate quality for enzymatic reactions, despite its relatively low purity. This finding is consistent with previous research suggesting that PCR performance can remain unaffected when DNA purity is slightly below the ideal range, provided that contamination levels do not inhibit polymerase activity (Rodríguez-Riveiro et al. 2022).

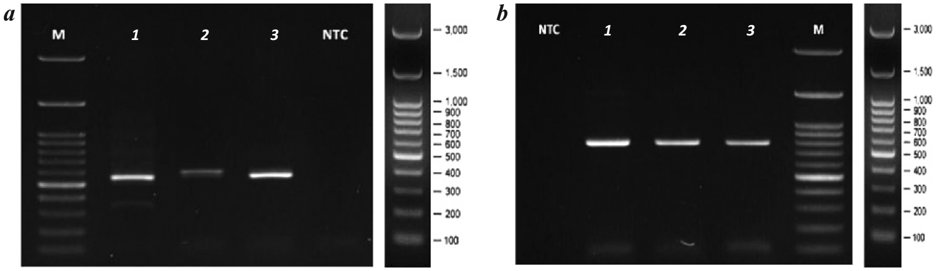

Fig. 1. DNA amplification results using a template from a modified extraction method
Explanation: *a* – *ITS2* gene amplification; *b* – *rbcl* gene amplification; *1* – yellow trigona honey; *2* – red trigona honey; *3* – multiflora honey

Several factors likely contributed to the successful PCR amplification observed in this study. First, the low level of contaminants allowed the Taq polymerase enzyme to function efficiently. Second, the high DNA concentration compensated for minor purity deficiencies, ensuring sufficient template availability for amplification. Third, the inclusion of PCR additives such as bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) may have mitigated the effects of potential inhibitors, enhancing amplification efficiency (Sutanta et al. 2022).

Discussion

The results of this study demonstrate that the modified DNA extraction method provides an efficient and reliable approach for extracting DNA from honey samples, particularly Trigona and multiflora honey. This method successfully reduces the sample volume requirement, processing time, and complexity, making it more accessible for laboratories with limited resources. The ability to extract DNA without mechanical disruption (e.g., glass beads grinding) enhances its practicality for routine analysis. However, while the DNA concentration obtained was relatively high (up to 1,790 ng/µl), the purity values (1.43–1.57) were slightly below the ideal range of 1.8–2.0 (Lucena-Aguilar et al. 2016), suggesting the presence of residual contaminants such as proteins, phenols, or polysaccharides that could interfere with downstream applications.

A central focus of this study is the impact of pre-treatment conditions on the efficiency of DNA extraction. The elevated temperature during the incubation phase (60 °C for 25 minutes) likely enhanced cell lysis and DNA release, as corroborated by previous research (MENCHHOFF et al. 2020). Nonetheless, it is plausible that higher temperatures also facilitated the co-extraction of undesirable compounds, thereby reducing DNA purity (BABADI et al. 2022). To mitigate this issue, additional modifications such as enzymatic digestion (e.g., proteinase K treatment) or supplementary ethanol precipitation steps could further enhance purity (RODRÍGUEZ-RIVEIRO et al. 2022).

Despite the slightly lower purity values, PCR amplification of *ITS2* and *rbcl* genes was successfully achieved, indicating that the extracted DNA was of sufficient quality for molecular analysis. The clear, well-defined bands in gel electrophoresis suggest that Taq polymerase activity was not significantly inhibited, even though impurities were present. This is in agreement with previous research showing that DNA purity is not always the limiting factor for PCR success, provided that sufficient DNA concentration and optimized reaction conditions are maintained (SOARES et al. 2017). In this study, the inclusion of PCR additives such as BSA and DMSO may have contributed to overcoming the effects of minor inhibitors, enhancing amplification efficiency (SUTANTA et al. 2022).

Comparisons with existing DNA extraction methods

Traditional honey DNA extraction methods, such as those reported by LALHMANGAIHI et al. (2014), typically require large sample volumes (≥50 ml) and long incubation times (≥1 hour), making them labor-intensive and costly. The method developed in this study significantly improves upon these approaches by:

– reducing sample volume (1 ml honey per reaction instead of ≥50 ml);

– shortening the incubation time (25 minutes instead of ≥1 hour);

– eliminating the need for glass beads or mechanical disruption, making it more practical for routine use;

– using a modified DNeasy Plant Mini Kit approach, optimizing buffer composition to enhance DNA recovery.

Compared to DNA-based authentication techniques used in honey botanical origin studies (WIRTA et al. 2021), this study demonstrates that high DNA concentrations can be obtained even from small sample sizes, making it suitable for large-scale honey authentication, pollination source identification, and biodiversity assessment.

Implications for honey authentication and molecular research

The ability to efficiently extract DNA from honey has important applications in food safety, authenticity testing, and conservation biology. DNA metabarcoding techniques, which rely on high-quality DNA extraction, are increasingly used to detect honey adulteration, verify botanical origin, and monitor ecosystem biodiversity (URUMARUDAPPA et al. 2020). The method developed in this study provides a practical solution for researchers and industry stakeholders seeking to implement genetic traceability methods for honey products, particularly in regions where fraudulent labeling is a concern.

Furthermore, the results of this study underscore the potential of honey-derived DNA as a valuable resource for reconstructing plant biodiversity. Previous research has demonstrated that pollen DNA extracted from honey can effectively reflect floral diversity across different geographical regions (SOARES et al. 2017). More recently, a study on Southeast Asian honey from Apis cerana and Heterotrigona itama in Karangasem, Indonesia, employed pollen DNA metabarcoding (ITS2 amplicon sequencing) to trace the botanical and geographical origins of honey, identifying dominant plant taxa such as Schleichera and Syzygium (ULLAH et al. 2024). These findings suggest that the method described herein can be further adapted for environmental DNA (eDNA) analyses, enabling researchers to investigate pollinator–plant interactions and monitor ecosystem dynamics using honey samples.

Conclusions

This study introduces an innovative method for extracting DNA from Trigona honey. This approach requires a smaller sample size, is time-efficient, and does not necessitate specialized equipment. Although the purity of the extracted DNA is relatively low (1.43–1.57), the yield is substantial (up to 1,790 ng/μL), and it performs effectively in PCR assays, yielding clear results. This demonstrates the method's reliability for subsequent molecular investigations.

The method's simplicity and cost-effectiveness make it accessible to laboratories with limited resources, facilitating large-scale honey studies. It addresses a significant challenge in honey research by simplifying the extraction of DNA from complex samples. This advancement can aid in assessing honey quality, tracing its geographical origin, and exploring biodiversity through environmental DNA.

Future research should aim to enhance DNA purity, evaluate the method across various honey types, and integrate it with advanced sequencing technologies. These enhancements will strengthen the utility of honey DNA in food safety, ecosystem studies, and environmental monitoring.

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QUALITY OF THE *LONGISSIMUS LUMBORUM* MUSCLE IN CROSSBRED FATTENERS FED DIETS SUPPLEMENTED WITH PROBIOTIC, PREBIOTIC, AND SYNBIOTIC*

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Key words: feed additives, nutrition, fatteners, meat, physicochemical traits.

Abstract

This study aimed to evaluate the quality of the *longissimus lumborum* muscle in PLW×PL pigs fed diets supplemented with a probiotic, a prebiotic and a synbiotic. The study material comprised the *longissimus lumborum* (*LL*) muscle sampled from fattening pigs. The animals were fed complete feed rations according to the following scheme: control group (I) – no feed additives; group II – 0.3% EM Bokashi; group III – 3% inulin; and group IV – 0.3% EM Bokashi + 3% inulin. The pigs were fattened until they reached an average body weight of approximately 112 kg. The inclusion of feed additives in the diets did no affect carcass muscularity or fatness ($p > 0.05$). Supplementation with inulin and EM Bokashi (group IV) significantly reduced muscle pH at 45 min and 2 h *post-mortem* compared to group II. No significant effect of the feeding strategy was observed on the electrical conductivity, water holding capacity or the *LL* muscle tenderness. However, the highest drip loss and muscle tenderness were recorded in pigs fed diets supplemented with inulin. The muscles from pigs in groups I and II were darker in colour (L*), but less saturated in red and yellow hues than the *LL* muscle from pigs in groups III and IV ($p \leq 0.05$). A significantly higher intramuscular fat (IMF) content (1.73% and 1.67%) was recorded in the muscles of pigs

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fed diets with EM Bokashi or inulin, respectively, compared to the control group (1.28%). Simultaneously, the *LL* muscle from pigs receiving the prebiotic-supplemented diet contained significantly less cholesterol than that from groups II and IV. The higher IMF levels in the *LL* muscle of pigs fed inulin or EM Bokashi contributed to a significantly ($p \leq 0.05$) increased content of oleic, saturated, and monounsaturated fatty acids compared to that of control pigs. Based on these findings, the inclusion of 3% inulin in complete feed rations for fattening pigs is recommended, as it resulted in the lowest cholesterol level and most favourable fatty acid profile in the *longissimus lumborum* muscle.

Introduction

Meat is a source of high-quality proteins, minerals (zinc, selenium, iron, and phosphorus), B-group vitamins, and fatty acids, and plays an important role in the human diet (KUNACHOWICZ et al. 2020, MILCZAREK 2021, RYBARCZYK et al. 2021, ZDUŃCZYK et al. 2024). Despite the high nutritional value of red meat, studies (HERFORTH et al. 2019, DI et al. 2023, SHI et al. 2023) indicate that the consumption of meat products high in fat and cholesterol may be associated with an increased risk of cardiovascular, metabolic, and oncological diseases. Global pork consumption in 2021 was 32.5%, whereas poultry and beef consumption were 39.5% and 21.8%, respectively (OECD/FAO 2023). The quality and safety of pork are closely related to the health of humans. Therefore, improving pork quality and ensuring the safety of meat for consumers is crucial.

Research (SOBOLEWSKA and GRELA 2014, PEREIRA PINTO et al. 2019, WANG et al. 2019, GRELA et al. 2021, ZHOU et al. 2025) has shown that modifying the composition of pigs' diets by introducing bioactive components such as probiotics, prebiotics, or synbiotics may affect not only growth performance, but also carcass composition and the physicochemical properties of meat. Probiotics are a live beneficial microorganisms which, confer health benefits to the host, when administered in adequate amounts (FAO/WHO 2001). CHEN et al. (2005) and TUFARELLI et al. (2017) demonstrated that supplementation of pig diets with additives containing microorganisms improves digestion, enhances animal health status, and boosts weight gain. However, the effects of probiotics on the physicochemical properties of pork meat remain inconclusive. Some researchers have confirmed the positive impact of probiotics on meat quality (LIU et al. 2013, SUO et al. 2012 BALASUBRAMANIAN et al. 2018), whereas others (RYBARCZYK et al. 2016, CHANG et al. 2018) have not observed beneficial interactions.

Probiotics are commonly combined with prebiotics in livestock feeding (GRELA et al. 2021, LEE et al. 2009, RINGSEIS and EDER 2022, SCOTT et al. 2017). According to the latest consensus of The International Scientific Association for Probiotics and Prebiotics (ISAPP), prebiotics are dietary substrates that are utilised by beneficial microorganisms (*Bifidobacteria* and *Lactobacillus*) in the gastrointestinal tract (GIT), thereby enhancing host health and preventing disease (SCOTT et al. 2017, PATTERSON et al. 2010). Inulin is a feed additive that exhibits prebiotic activity (ROBERFROID 2007, KIERNAN et al. 2023). Inulin is a polymer that contains oligosaccharides and polysaccharides. It is a type of fructan mixture found in a wide variety of plants (chicory roots, Jerusalem artichoke, dandelion, and elecampane). However, in industrial applications, it is most commonly extracted from chicory roots (ROBERFROID 2007, VAN BEKKUM et al. 2008). The degree of polymerisation of inulin fructans can range from approximately 2 to 60. Fructooligosaccharides (FOS) is obtained via the enzymatic hydrolysis of inulin, which reduces the degree of polymerisation (CHIKKERUR et al. 2020). The degree of polymerisation directly influences the physical properties of compounds. The higher the degree of polymerisation of inulin, the greater its gel-like behaviour, with longer chains having lower solubility (FRANCK 2002). Inulin passes through the upper digestive tract unchanged and reaches the lower gastrointestinal tract, where it undergoes anaerobic fermentation by bacteria. The fermentation products are short-chain fatty acids (SCFAs), including acetic, propionic, and butyric acids. It has been shown that butyrate serves as a primary energy source for colonocytes and provides protection against colorectal cancer and inflammation (FLINT et al. 2012). NOWAK et al. (2012) reported that an increase in the production of volatile fatty acids may help regulate cholesterol levels, as well as support the absorption of calcium, iron, and magnesium. Furthermore, other studies (DELZENNE and KOK 1999, WILLIAMS 1999) have demonstrated that inulin has beneficial effects on lipid metabolism in both humans and animals. ZHOU et al. (2025) suggest that maternal inulin supplementation during gestation mitigates offspring hepatic lipid deposition through butyrate-mediated epigenetic regulation, where microbial-derived butyrate from inulin fermentation inhibits HDAC activity, enhances histone acetylation levels, and upregulates fatty acid β-oxidation gene expression. The inclusion of inulin in animal diets may contribute to improved growth performance, increased villi length, and reduced levels of skatole, indole, and cresol (JENSEN and HANSEN 2006, HANSEN et al. 2006, GRELA et al. 2013, WANG et al. 2019) as well as affect the carcass composition and pork meat quality (SOBOLEWSKA and GRELA 2014, WANG et al. 2019, GRELA et al. 2021).

Probiotics may enhance the beneficial effects of prebiotics on biological systems (ALLOUI et al. 2013, MARKOWIAK and ŚLIŻEWSKA 2017), and their simultaneous inclusion in the diets of monogastric animals may result in a synergistic effect on growth rate (SHIM et al. 2005, LEE et al.2009) and meat quality.

Therefore, this study aimed to evaluate the quality of the *longissimus lumborum* muscle in PLW×PL pigs fed diets supplemented with EM Bokashi, inulin, or a combination of EM Bokashi and inulin.

Materials and Methods

Animals, slaughter and carcass treatment

The experiment was performed according to the recommended EU Directive 2010/63/EU for animal experimentation. The investigation was carried out on 80 barrows (20 animals in each group) derived from Polish Large White × Polish Landrace crossbred fatteners. The animals of the each group (I, II, III and IV) were fattened (for different number days) from starting an average body weight of 30.5 kg (±1.5 kg) to 112 kg (±5 kg). The fatteners were kept on the same farm (Mazovia district, Poland) in a non-bedding system with unrestricted access to water and were fed *ad libitum* with Grower 1 up to 60 kg, Grower 2 from 60 kg to 90 kg, and then up to 112 kg with Finisher. The rations were isoprotein, isoenergetic, and balanced in accordance with nutritional recommendations (NRC 2021). The animals were divided into four equinumerous feeding groups as follows: group I (control) receiving complete feed mixtures without probiotic or prebiotic, group II – complete feed + 0.3% EM Bokashi, group III – complete feed + 3% inulin, and group IV – complete feed + 0.3% EM Bokashi + 3% inulin (Table 1).

EM Bokashi probiotic contained a complex of effective microorganisms, such as *Saccharomyces cerevisiae* ($3.3 \cdot 10^5$ CFU/ml IFO 0203), *Lactobacillus casei* (1 k 2 $1.95 \cdot 10^7$ CFU/ml ATCC 7469), and *Lactobacillus plantarum* (1 k 2 1.95×10^7 CFU/ml ATCC 8014). Chicory inulin contained approximately 92% inulin with $DP \geq 10$ and 8% other carbohydrates (glucose, fructose, and sucrose).

After the fattening period, all pigs were loaded in small groups by qualified personnel into transport vehicles. Fatteners were transported 30 km to the slaughterhouse at night and rested for 2 h in lairage pens, following density standards and with constant access to water. At the slaughter line, lean meat content, backfat, and muscle thickness were measured using a Sydel CGM optic-needle apparatus, and hot carcase weight (HCW) was measured immediately afterwards (accuracy up to 0.1 kg). The carcasses were then chilled in a blast-cooling tunnel and stored at 4 °C for up to 24 h after slaughter.

Table 1

Ingredients and chemical composition of the pig diets

Item	Grower 1				Grower 2				Finisher			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
Ingredients:												
Barley	23.9				21.5				20.5			
Triticale	24.78				29.71				29.71			
Wheat	12.6				8.6				5.8			
Corn	7.0				10.0				14.9			
wheat bran	1.0				2.5				3.5			
non-GMO soybean meal	11.68				8.16				5.18			
Sunflower meal	1.0				1.0				1.0			
DDGS	5.0				5.0				5.0			
Rapeseed meal	5.0				6.0				7.0			
Sunflower oil	2.1				1.5				1.3			
Mineral-vitamin supplements (e.g., limestone, 1-Ca-pfosphate, salt, etc.), amino acids, premix	2.94	2.91	2.94	2.91	3.03	3.0	3.03	3.0	3.11	3.08	3.11	3.08
Corn starch	3.0	3.0	-	-	3.0	3.0	-	-	3.0	3.0	-	-
EM Bokashi	-	0.3	-	0.3	-	0.3	-	0.3	-	0.3	-	0.3
Inulin	-	-	3.0	3.0	-	-	3.0	3.0	-	-	3.0	3.0
Total	100	100	100	100	100	100	100	100	100	100	100	100
Nutritive value per 1 kg of diet:												
Crude protein [g]	170				160				150			
Lysine [g]	11.6				10.7				9.80			
Methionine [g]	4.0				3.6				3.4			
Crude fibre [g]	41				46				50			
Ca [g]	7.6				6.8				6.0			
P [g]	5.5				4.7				4.1			
Na [g]	1.3				1.1				1.0			
Metabolic energy [MJ]	13.1				13.0				12.9			

Explanations: DDGS – dry distillers grains with solubles

Meat quality attributes

Both pH and EC measurements were performed directly in hanging half-carcasses in the *longissimus lumborum* muscle (*LL*) behind the last rib (from 35 min. to 24 h *post-mortem*) while remaining meat quality attributes were measured in meat samples taken at the last rib and 1^{st}–4^{th} lumbar vertebra (after 24 h of chilling). Each muscle sample was separated from the bone, external fat, and epimysium, packed in polyethylene bags, placed in cooling boxes (below 4 °C for approximately 1 h), transported to the laboratory, and stored at 4 °C in refrigerators. Four slices were cut from each carcase: three slices (2-cm thick) were used to determine pork quality attributes, while the remaining slice was used to evaluate the proximate composition, fatty acid profile, and cholesterol content.

Muscle pH and electrical conductivity (EC) were measured after 45 min of storage (pH_{45}), 2 (pH_2), and 24 hours *post-mortem* (pH_{24}) directly in meat plants using a temperature-compensating pH meter pH-Star and conductometer LF-star with a frequency of 1.2 kHz, respectively. Both apparatuses were calibrated prior to measurement (the pH meter was standardised using pH 7 and 4.6 buffer solutions. The electrodes were placed crosswise on the muscle fibres.

Meat colour was measured at 24h *post-mortem* after 10 min. blooming period in the CIE $L^*a^*b^*$ system: L^* – lightness, a^* – redness, and b^* – yellowness (CIE 2007) with a Minolta portable chroma meter with a 50 mm aperture. Two illuminant/observer combinations were applied: illuminant C (average daylight) and standard observer 2°, and illuminant D65 (daylight) and standard observer 10°, as recommended by HONIKEL (1998) for the measurement of meat colour. The instrument was standardised using a white calibration plate with the following coordinates: $Y = 92.80$, $x = 0.3175$, and $y = 0.3333$. In the measuring system used, L^* denotes psychometric colour saturation, which is a spatial vector, and a *and b *are trichromatic coordinates (a *is a positive value that corresponds to red, its negative corresponds to green, positive b^* corresponds to yellow, and negative b^* corresponds to blue). The colour parameters a^* and b^* were used to calculate chroma (C^*) and hue (h°) using the formulas used by MILCZAREK and OSEK (2019).

Drip loss (DL) was determined according to the method described by PRANGE et al. (1997). Meat samples (approximately 100 g) were cut from carcasses 24 h *post-mortem*, weighed, and placed in plastic bags. After storage at 4 °C, the samples were weighed again at 24 and 48 h. Drip loss [%] was calculated as the difference in sample weight before and after storage with respect to the initial weight.

Water absorption, expressed as water holding capacity (WHC), was determined using GRAU and HAMM'S (1952) method, as modified by POHJA and NINIVARRA (1957), based on the amount of free water (expressed in %) lost by the meat sample placed on the filter paper pressed between two glass plates. The infiltration area (cm^2) was measured using a mechanical planimeter HAFF-Planimeter No. 313.

Cooking loss was assessed as follows: samples of known weights (approximately 100 g) were placed in hot water for approximately 30 min. Upon reaching a temperature of 72 °C, all samples were cooled to room temperature (21 °C), dried, and weighed. Cooking loss was calculated as the difference between the sample weights before and after thermal treatment with respect to the initial weight. After 24 h of storage at 4 °C, the shear force was measured using a Stable Micro Systems TA.XT Express Enhanced with a Warner–Bratzler knife according to PN-ISO NORM 11036:1999 (1999). Three cuboids (1.0 cm in diameter) were cut from each meat sample along the muscle fibres, measured, and expressed as mean values.

Proximate chemical composition, fatty acid profile, cholesterol, and energy value of muscle

The following proximate chemical compositions were measured in the raw and ground meat samples according to the official methods of analysis of the AOAC (2003): moisture content by oven-drying samples to a constant weight (950.46), crude protein content by the classical Kjeldahl method (981.10), and intramuscular fat content by petroleum ether extraction (960.39) using a Soxhlet apparatus. Based on proximate composition and energetic value of protein ($5.75 \text{ kcal} \cdot g^{-1}$) and fat content ($9.46 \text{ kcal} \cdot g^{-1}$), the energetic value of *longissimus lumborum* muscle was estimated according to Atwater energy equivalents (MILCZAREK and OSEK 2016).

Sample preparation for the determination of total cholesterol content (extraction, separation of unsaponifiable fraction, preparation of trimethylsilyl sterol ethers) and chromatographic analysis with mass spectrometry (GCMS) were performed according to the Polish Standard PN-EN 12228:2002 (2002) using an Agilent 8890 GC apparatus.

The fatty acid profile of the lipid fraction was determined according to FOLCH et al. (1957) by gas chromatography (GC-FID) of methyl esters using a Perkin Elmer Clarus 580 gas chromatograph with a flame ionisation detector (air-hydrogen). A CP-Sil 88 capillary column (60 m × 0.25 mm × 0.20 µm) was used for the analysis. The injector temperature was 260 °C, the detector temperature was 260°C, and the column temperature was 140 °C (initial) and 240 °C (final). Helium was used as the carrier gas at a flow rate of 0.5 ml per minute. The fatty acids were calculated using chromatogram peak areas. Although the total fatty acid profile was determined, only fatty acids with values ≥0.1% were included, reflecting the apparatus' detection limit.

Statistical analysis

The obtained results were statistically analysed using STATISTICA SOFTWARE VER. 13.1 (2019). The normality of the data distribution was tested using the Shapiro–Wilk test. The calculated parameters included measures of location (arithmetic mean) and dispersion (standard error of the mean), and data were analysed using one-way analysis of variance. The model is expressed as follows:

$$y_i = \mu + a_i + e_i$$

where:

y_i – the measured ith trait
μ – the overall population mean
a_i – the analysed factor effect of the i^{th} trait
e_i – the random error.

The significance of the differences between the means was evaluated using Tukey's post hoc multiple range test. Statistical significance was assumed to exist when the probability was less than 0.05.

Results and Discussion

The introduction of EM Bokashi, inulin, or both additives combined into the diets for PLW×PL crossbred pigs had no significant effect ($p > 0.05$) on warm carcase weight, muscularity, longissimus lumborum muscle height, and backfat thickness (Table 2).

Table 2

Slaughter carcass value

Traits	Experimental groups				*p*-value	SEM
	I	II	III	IV		
Hot carcass weight [kg]	81.04	81.81	82.22	82.02	0.257	0.887
Lean meat content [%]	56.84	57.17	57.99	58.45	0.341	0.348
Backfat thickness [mm]	56.70	57.40	58.25	59.75	0.373	0.640
Muscle thickness [mm]	14.55	15.30	15.75	15.55	0.118	0.533

Explanations: group I – control (complete feed mixtures without additives); group II – fed complete mixtures with 0.3% EM Bokashi; group III – fed complete mixtures with 3% inulin; group IV – fed complete mixtures with 0.3% EM Bokashi + 3% inulin
SEM – standard error of the mean

Several researchers (CHANG et al. 2018, RYBARCZYK et al. 2020, 2021, ZHOU et al. 2025) have assessed the slaughter value of pigs fed diets with probiotics, prebiotics, and synbiotics. According to CHANG et al. (2018) and RYBARCZYK et al. (2020), no statistical differences in the lean meat content were noted between the experimental groups. In addition, ZHOU et al. (2025) found that 1.5% inulin inclusion resulted in no statistical differences in lean meat content; however, the authors observed a slight increase in backfat thickness. However, in the study by RYBARCZYK et al. (2021), the addition of 0.3% EM Bokashi to the Naïma x P-76 diet resulted in a statistically higher lean meat content (57.01% vs. 54.75%), higher *longissimus lumborum* muscle thickness (57.88 vs. 57.01%), and a decrease in backfat thickness (19.64% vs. 16.54%).

Supplementation of the fatteners' diet with inulin and EM Bokashi (group IV) significantly ($p \leq 0.05$) reduced muscle pH at 45 min and 24 h *post-mortem* compared with pigs fed diets supplemented with EM Bokashi alone (Table 3).

Table 3

Physicochemical traits of *longissimus lumborum* muscle ($N = 80$)

Traits	Experimental groups				*p*-value	SEM
	I	II	III	IV		
Acidity of the muscles						
pH_{45}	6.21*ab*	6.28*a*	6.16*b*	6.15*b*	< 0.05	0.023
pH_{24}	5.64*a*	5.56*ab*	5.53*b*	5.58*ab*	< 0.05	0.013
Electrical conductivity						
EC_2	3.61	3.14	3.28	3.89	0.101	0.117
EC_{24}	3.91	3.49	3.43	3.75	0.508	0.126
Colour						
L^*_{24}	51.62*b*	51.45*b*	53.91*ab*	54.36*a*	< 0.05	0.386
a^*_{24}	8.77*b*	8.51*b*	12.17*a*	12.46*a*	< 0.05	0.378
b^*_{24}	-0.63*b*	-0.77*b*	1.81*a*	2.48*a*	< 0.05	0.280
$C^*_{24} = [(a^*)^2 + (b^*)^2]^{0.5}$	8.83*b*	8.58*b*	12.53*a*	12.97*a*	< 0.05	0.410
$h°_{24} = \log(b^*/a^*)$	-0.08*b*	-0.10*b*	0.09*a*	0.15*a*	< 0.05	0.020
Drip loss	3.56	3.06	3.54	3.07	0.283	0.123
Cooking loss [%]	34.09	33.81	31.46	33.90	0.084	0.420
Water holding capacity WHC [cm^2]	5.68	6.42	6.89	6.43	0.121	0.178
Shear force [N/kg]	18.25	17.01	19.67	18.30	0.325	0.502

Explanations: group I – control (complete feed mixtures without additives); group II – fed complete mixtures with 0.3% EM Bokashi; group III – fed complete mixtures with 3% inulin; group IV – fed complete mixtures with 0.3% EM Bokashi + 3% inulin
a, *b* – statistically significant at $p \leq 0.05$, SEM – standard error of the mean

Dietary probiotics and prebiotics have the potential to improve pork quality traits (LIU et al. 2013, RYBARCZYK et al. 2021). However, the mechanisms by which they modify the quality traits are not fully understood. Probiotics and prebiotics may exert beneficial effects on meat quality through the gut-muscle axis by modulating the gut microbiota, producing beneficial metabolites, competitively excluding pathogenic microorganisms, and modulating the immune system of the host (CHEN et al. 2022, WEN et al. 2024). There are several potential mechanisms by which probiotics and prebiotics may influence meat quality via the gut-muscle axis. These include skeletal muscle metabolism, transformation of muscle fibre type, and intramuscular fat deposition (WEN et al. 2024).

Post-mortem pH decline plays a crucial role in determining of pork quality. Normally, in the *longissimus* muscle, pH declines from 7.2–7.4 in living muscle to 5.7–5.5 at 24 h after slaughter. Muscles with rapid *post-mortem* glycolysis exhibit a pH lower than 5.8 at 1 h after slaughter (SCHEFFLER and GERRARD 2007). In this study, the observed pH values in all experimental groups were within the range typical for normal meat across all measurements; however, pigs supplemented with EM Bokashi (II) produced pork with a higher pH at 45 min and 2 h in comparison with group III i IV. At 24 h *post-mortem,* the lowest pH value was noted in group III (pigs fed with 3% inulin). However, it should be stated that all experimental treatments (II, III, and IV) showed a tendency to lower muscle acidity compared to the control group (I).

SUO et al. (2012) noted that the addition of *Lactobacillus plantarum* ZJ316 increased pH measured at 45 min. after slaughter and lowered shear force and texture. Additionally, RYBARCZYK et al. (2016) showed that 0.3% EM Bokashi addition increased pH measured 24 hours after slaughter (5.59 vs. 5.66); however, it also increased drip loss (3.7% vs 2.5%) and cooking loss (31.02% vs. 28.57%). CHANG et al. (2018) found that *Lactobacillus plantarum* ($2.5 \cdot 10^7$ CFU/mL) statistically lower pH value; however, no adverse effect on WHC was found by the cited authors, which was also conferment in this study. RESZKA et al. (2020) showed that pigs fed a standard diet with soybean meal and probiotic had lower WHC than those from the control group and reported no effect of EM Carbon Bokashi on ultimate pH (pH_{48}), although both control and probiotic supplemented pigs displayed low pH_{48} (5.1–5.2).

No statistically significant effect ($p > 0.05$) of the feeding strategy was observed on the electrical conductivity, drip loss, cooking loss, and water holding capacity (WHC) of the *longissimus lumborum* muscle (Table 3). However, the highest drip loss was recorded in pigs fed diets supplemented with inulin (group III). Simultaneously, the muscles of pigs fed diets containing inulin exhibited the greatest hardness ($p > 0.05$).

According to the aforementioned studies, the effect of prebiotics, such as inulin supplementation, on water distribution in pork remains inconclusive. ROSENVOLD and ANDERSEN (2003) reported that the loins of pigs supplemented with high doses of inulin (25%) had lower drip loss than those in the control group. In contrast, ALUWE et al. (2013) showed that supplementation with 7% inulin increased the drip loss from pork loins. PRZYBYLSKI et al. (2019) showed that supplementation of inulin (7% in diet) had no significant effect on drip loss, although loins from inulin fed pigs for 40 days displayed higher drip loss (by 1.4 pp.). Other studies have shown no effect of inulin supplementation on drip loss (HANSEN et al. 2008, WANG et al. 2019, GRELA et al. 2021). In this study, low drip loss values (< 4% according to BERTRAM et al. 2000) measured 48 h after slaughter were observed, regardless of the experimental group. However, a slight decrease in its value (c.a. 0.5%) was noted for the experimental groups in which 0.3% EM Bokashi was used (II – 0.3% EM Bokashi and IV – 0.3% EM Bokashi and 3% inulin). Moreover, no statistical differences were observed in the WHC among the experimental groups. However, the addition of probiotics and synbiotics (II, III, and IV) tended to increase their values. This finding is contradictory to that of JUKNA et al. (2005), JIANG (2011), LIU et al. (2013), and GRELA et al. (2021) showed that the addition of *Saccharomyces cerevisiae, Lactobacillus casei, Lactobacillus acidophilus, Streptococcus faecium*, and *Bacillus subtilis* to pig diets statistically improved the water holding capacity. However, RYBARCZYK et al. (2020) showed that 0.5% EM® probiotic (*Saccharomyces cerevisiae*, *Lactobacillus casei,* and *Lactobacillus plantarum*) increased drip loss by 2-3 percentage points, cooking loss, and electrical conductivity at 2 and 24 h *post-mortem*. Additionally, the authors noted that the addition of probiotics at 0.3% did not influence the aforementioned traits. The tendency to decrease shear force in group II (0,3% EM Bokashi) was complementary to the results noted by GRELA et al. (2021). The authors found lower shear force (by 17%, $p \leq 0.05$) in pork fed with the addition of prebiotics. In addition, PEREIRA PINTO et al. (2019) reported that the addition of 6% inulin significantly lowered the shear force and chewiness. However, ZHOU et al. (2025) noted that inulin did not influence shear force. In conclusion, drip loss is an important quality cue for consumers, although it is less important in the decision-making process than fat cover and colour (NGAPO et al. 2007, VERBEKE et al. 2010, FONT-I-FURNOLS and GUERRERO 2014, NGAPO et al. 2017). In general, consumers prefer pork with minimal or no drip, which is perceived as a sign of higher meat quality (FONT-I-FURNOLS and GUERRERO 2014, NGAPO et al. 2017). Thus, pork from pigs supplemented with inulin or synbiotic fits well within the preferences of consumers.

Muscle from pigs fed diets without additives or supplemented with EM Bokashi were darker (L*) but less saturated ($p \leq 0.05$) in red (a*) and yellow (b*) hues than those from pigs receiving diets supplemented with inulin or inulin + EM Bokashi (Table 3). The *longissimus lumborum* muscles of pigs in groups III and IV featured significantly ($p \leq 0.05$) higher colour saturation (C*) than those of pigs in groups I and II. Similarly, hue angle (h°) values were significantly higher in muscles from groups II and IV in comparison with muscles from pigs fed diets supplemented solely with the probiotic (group II).

The lack of effect of EM Bokashi supplementation in the diets of PLW×PL fatteners on the colour parameters (L*,a*,b*) of the *longissimus lumborum* muscle is consistent with the findings of RYBARCZYK et al. (2016), who demonstrated that 0.3% EM Bokashi included in the diet of Naïma×P-76 crossbreds did not result in differences in lightness or colour saturation in the red and yellow directions. In a subsequent study, RYBARCZYK et al. (2020) reported that the meat of pigs supplemented with 0.5% EM® probiotic (containing *Saccharomyces cerevisiae*, *Lactobacillus casei*, and *Lactobacillus plantarum*) was characterised by higher red colour saturation (a*) than that of both the control group and the group supplemented with 0.3% probiotic. In a study by JIANG (2011), the addition of a probiotic preparation containing *Phaffia rhodozyma* significantly increased the redness (a*) of the meat colour of fatteners. Other studies have shown that the administration of probiotics (*L. plantarum*) to pigs enhanced the antioxidant activity in meat, which was due to an increase in the concentration of vitamin C (CHANG et al. 2018). It should be mentioned that vitamin C is characterised by very good antioxidant properties and increasing its concentration in meat improves meat colour and persistence (WHEELER et al. 1996). RYBARCZYK et al. (2020) claimed that the lactic acid bacteria (LAB) dosage had a significant effect on the gut microbiota through a significant increase in LAB count and a decrease in the number of *Enterobacteria*, which might be relating to the changes in *LL* muscle quality, especially in the traits associated with water holding capacity and meat colour-chromatic characteristics (a*, b*, C*, h°).

The inclusion of inulin or inulin + EM Bokashi in pig diets resulted in meat with a lighter colour (L*) and more intense red (a*) and yellow (b*) hues than the meat of pigs receiving control diets or diets with an added probiotic (Table 3). The colour of fresh meat has a crucial effect on consumers' purchasing decisions. However, consumers' expectations for pork colour vary widely by region and culture (ALTMANN et al. 2023). In Europe, most consumers prefer lighter but redder meat (NGAPO et al. 2010, JAWORSKA et al. 2009). Thus, pork from pigs supplemented with inulin or synbiotics may be more preferable to consumers. The results partially support the findings of GRELA et al. (2021), who also observed a lighter colour and increased yellow hue in the meat of pigs fed a diet containing inulin, although with a decrease in redness. In turn, SOBOLEWSKA and GRELA (2014) demonstrated that the type of inulin used in pig diets affects meat colour parameters, as raw loin from pigs fed diets with inulin obtained by water-alcohol extraction, compared to other pig groups (inulin obtained by water extraction, powdered Jerusalem artichoke, or powdered chicory), was characterised by the lightest colour (L*) and the highest saturation in the yellow direction (b*). ZHOU et al. [13] did not confirm the effect of a 1.5% inulin addition to pig diets on meat colour parameters.

The addition of probiotics (EM Bokashi), prebiotics (inulin), or synbiotics (EM Bokashi + inulin) to the diets of fattening pigs did not affect ($p > 0.05$) the proximate composition (dry matter, crude ash, and crude protein) of the muscle, except for crude fat (Figure 1).

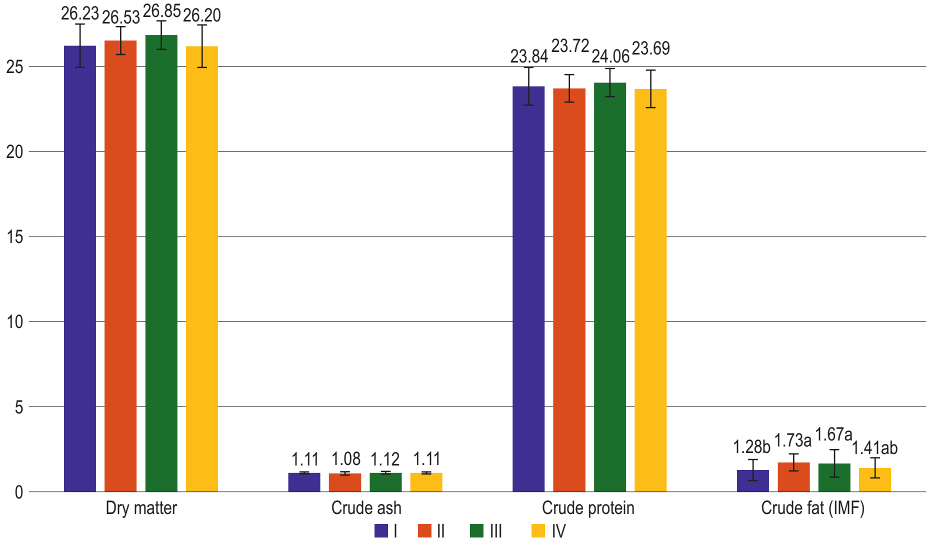

Fig. 1. Proximate chemical composition [%]

The crude fat content was significantly ($p \leq 0.05$) higher in the muscles of pigs fed diets with EM Bokashi (group II) and inulin (group III) than that in the control pigs.

From a human nutrition perspective, fat is a carrier of flavour and a source of saturated fatty acids (MILCZAREK 2021, ZDUŃCZYK et al. 2024). Crude fat also determines the culinary usefulness of meat; for the *longissimus dorsi* muscle, the optimal IMF content ranges from 1.5 to 3.5% (WOOD et al. 1994). In this study, the analysis of the effect of the applied pig feeding regime on the nutritional value of the *longissimus dorsi* muscle confirmed that the crude fat content was the most variable (1.28–1.73%). BREWER (1998) found that meat with IMF content between 1.5 and 3.5% was characterised by higher flavour intensity and juiciness than meat with low (<1.5%) IMF content. Only the muscles of fatteners receiving diets supplemented with EM Bokashi and inulin showed fat levels within the aforementioned range, which may be preferred by most consumers. In Europe, fat content is the most important cue in consumers' choice, preferably with slightly visible fat (including subcutaneous fat cover and intramuscular fat), thus increasing their purchase intention (VERBEKE et al. 2010, FONT-I-FURNOLS and GUERRERO 2014, FONT-I-FURNOLS et al. 2012, DE ARUJO et al. 2022). However, BREWER et al. (2001) reported that chops with less than 2.5% IMF had higher overall acceptability and purchase intention than those with higher IMF content.

RYBARCZYK et al. (2016, 2019, 2020) found that the inclusion of EM Bokashi probiotics (0.3%) or BioPlusYC (0.4%) in diets fed to fatteners did not significantly affect the fat content of meat. However, 0.5% share reduced intramuscular fat compared to the control and 0.3% probiotic-supplemented groups [55]. GRELA et al. (2021) demonstrated that supplementation with a probiotic (*Lactococcus lactis*, *Carnobacterium divergens*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Saccharomyces cerevisiae*) reduced (2.09% vs. 2.22%) intramuscular fat content in the *longissimus lumborum* muscle compared to the control group.

Likewise, in the present study, GRELA et al. (2021) noted a similar content of basic components in the muscle tissue of fatteners fed diets supplemented with inulin, except for the IMF level. Inulin addition increased its content, whereas probiotic reduced.

The feed additives used in the diets of fattening pigs did not affect the energy value of the evaluated *longissimus lumborum* muscle (Table 4).

The amount of energy in the muscle ranged from 149.21 to 154.14 kcal/100 g and was attributed to the nutrient content, especially intramuscular fat (IMF). Due to the muscle's low IMF level, its energy value was lower than that reported by MILCZAREK and OSEK (2016), MILCZAREK et al. (2019) and KUNACHOWICZ et al. (2020).

Animal-origin products, including pork meat, are a source of cholesterol in the human diet (KUNACHOWICZ et al. 2020). KUNACHOWICZ et al. (2020) state that the cholesterol content in pork ranges from 60 to 72 mg/100 g. Lower cholesterol content (52.48–59.48 mg/100 g) was found in the *longissimus dorsi* muscle of PLW × PL crossbreds. The inclusion of probiotics, prebiotics, or synbiotics in the diets of fatteners did not affect the cholesterol content of the *longissimus lumborum* muscle. However, significantly lower amounts (52.48 mg/100 g) of this component were found in the muscle of pigs receiving diets with inulin compared to those fed with EM Bokashi

Table 4

Energy value, cholesterol and fatty acids profile of muscles (*N* = 80)

Traits	Experimental groups				p-value	SEM
	I	II	III	IV		
Energy value [kcal/100 g]	149.21	152.76	154.14	149.53	0.219	0.878
Cholesterol [mg/100 g]	53.72*ab*	59.26*a*	52.48*b*	59.48*a*	<0.05	0.884
Fatty acids [g/100 g]						
Palmitic acid (C16:0)	0.30	0.40	0.36	0.33	0.983	0.015
Stearic acid (C18:0)	0.19	0.25	0.22	0.22	0.788	0.009
Oleic acid (C18:1n9c)	0.46*b*	0.65*a*	0.62*a*	0.49*b*	<0.05	0.127
Linoleic acid, LA (C18:2n6c)	0.19	0.20	0.23	0.20	0.367	0.009
SFA	0.48*c*	0.67*a*	0.63*a*	0.55*b*	<0.05	0.027
UFA	0.81*b*	1.06*a*	1.04*a*	0.86*ab*	<0.05	0.040
MUFA	0.52*b*	0.79*a*	0.76*a*	0.61*ab*	<0.05	0.034
PUFA	0.26	0.26	0.28	0.24	0.399	0.012
Omega-3	<0.10	<0.10	<0.10	<0.10	1.000	1.000
Omega-6	0.23	0.26	0.27	0.23	0.526	0.011
Omega-7	0.14	0.13	0.15	0.13	0.356	0.005
Omega-9	0.46	0.66	0.63	0.51	0.580	0.028

Explanations: group I – control (complete feed mixtures without additives); group II – fed complete mixtures with 0.3% EM Bokashi; group III – fed complete mixtures with 3% inulin; group IV – fed complete mixtures with 0.3% EM Bokashi + 3% inulin

IMF – intramuscular fat; SFA – saturated fatty acids; UFA – unsaturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids

a, *b* – statistically significant at $p \leq 0.05$, SEM – standard error of the mean

(59.26 mg/100 g) or EM Bokashi + inulin (59.48 mg/100 g). RYBARCZYK et al. (2016) found a significantly lower cholesterol level (71.91 mg/100 g) in the muscle of Naïma × P-76 pigs fed a diet with 0.3% EM Bokashi compared to the muscle of pigs from the control group (74.50 mg/100 g). In contrast, GRELA et al. (2013) demonstrated that supplementing pig diets with inulin and garlic water extract significantly reduced the cholesterol content in the *longissimus dorsi* muscle.

The muscles of pigs fed diets supplemented with inulin or EM Bokashi contained significantly ($p \leq 0.05$) higher amounts of oleic acid, saturated fatty acids (SFA), and unsaturated fatty acids (UFA), including monounsaturated fatty acids (MUFA), than those of the control pigs.

From a human nutrition perspective, a beneficial increase in the share of unsaturated fatty acids (UFA) and a decrease in saturated fatty acids (SFA) were observed in the muscles of pigs fed control diets or diets with inulin compared to those fed rations supplemented with EM Bokashi or EM Bokashi

+ inulin. Moreover, the evaluated bioactive additives had no effect on omega-3 and omega-6 fatty acid levels. However, GRELA et al. (2013) found higher levels of omega-3 and omega-6 fatty acids in the *longissimus dorsi* muscle of pigs fed diets supplemented with inulin and garlic extract. Additionally, GRELA et al. (2021) reported a more favourable n6/n3 polyunsaturated fatty acid ratio in the muscle of pigs receiving diets supplemented with a probiotic (*Lactococcus lactis, Carnobacterium divergens, Lactobacillus casei, Lactobacillus plantarum,* and *Saccharomyces cerevisiae*) than that in the meat of fattening pigs from the control group.

Conclusions

In conclusion, it must be stated that the inclusion of 0.3% EM Bokashi, 3% inulin, and 0.3% EM Bokashi + 3% inulin in the diets of PLW × PL crossbreds did not affect carcass value and allowed for the production of meat with desirable physicochemical properties.

From the consumer nutrition perspective, the inclusion of 3% inulin in complete feed rations for fattening pigs is recommended, as it resulted in the lowest the cholesterol level (52.48 mg/100 g) and the most favourable fatty acid profile in the *longissimus lumborum* muscle. Both of the above-mentioned impacts, that is, on the physicochemical and dietary value, can be considered factors influencing consumers' willingness to purchase, due to increased awareness of a healthy diet and a potential decrease in diseases of affluence, which may also attract greater interest from processors.

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TRANSITION BIAS AND NEUTRAL SELECTION DRIVE THE EVOLUTION OF THE POLYKETIDE SYNTHASE GENE IN *ASPERGILLUS* SECTION *NIGRI*

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Key words: transition, neutral selection, evolution, polyketide synthase gene, *Aspergillus* section *Nigri*.

Abstract

The significance of the polyketide synthase (*pks*) gene in the biosynthesis of ochratoxin A in *Aspergillus* section *Nigri* has been well reported. However, factors driving the evolution of this gene in black aspergilli are seldom studied. This study, was therefore, aimed at investigating these factors as a means to understanding how to circumvent their wide epidemiological coverage in the nearest future. To achieve this, a total of one thousand cassava powders (*Lafun*) were collected from the four geopolitical zones of Ogun State, Nigeria and processed for black aspergilli using standard mycological procedures. The isolated organisms were subsequently characterised phenotypically, followed by screening for ochratoxin A production and sequencing of the *pks* gene associated with its biosynthesis. The sequenced *pks* genes were used for mutation discovery, estimation of selection, substitution pattern and phylogenetic analyses. The results revealed a total of 279 black aspergilli, distributed as follows: *Aspergillus niger* – 88 (8.8%), *A. welwitschiae* – 82 (8.2%), *A. carbonarius* – 56 (5.6%), and *A. aculeatus* – 47 (4.7%). Among these, 18 strains, comprising 6 *A. niger,* 3 *A. aculeatus,* 7 *A. carbonarius,* and 2 *A. welwitschiae* were identified as ochratoxigenic based on both phenotypic characterization and molecular amplification of the *pks* gene. The quantitative measurements of their ochratoxins ranged from 9.12–11.08 for *Aspergillus aculeatus* through 10.52–12.74 and 19.39–23.61 for *Aspergillus carbonarius* and *Aspergillus niger* to 23.88–27.02 for *Aspergillus welwitschiae.* All the ochratoxigenic *Aspergillus welwitschiae, Aspergillus aculeatus* and *Aspergillus niger* were clustered together on the phylogenetic tree. The maximum likelihood estimate of the transition-transversion bias of the *pks* genes of black aspergilli depicts that the number of proteins in the data set

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of the *pks* genes, the transition-transversion bias ratio and the maximum likelihood estimate were 2215 bp, 1.01 and –13279.996 respectively. The maximum composite likelihood estimates of the pattern of nucleotide substitution revealed frequent transitions than transversions in the polyketide synthase genes of *Aspergillus* section *Nigri*. The results showed that A/G and T/C transition were more frequent than C/T and G/A while the codon-based Fisher's exact test analysis of selection, the Codon-based Z-test of neutral evolution and the results from Tajima's Neutrality Test connotes significant bias for neutral evolution ($p < 0.05$). The above results suggest that transition and neutral selection drive the evolution of the *pks* gene of *Aspergillus* section *Nigri*.

Introduction

The importance of *Aspergillus* section *Nigri* in the contamination of several agricultural products has been well documented (Thomas et al. 2014, Cabanes and Bragulat 2018, Gil-Serna et al. 2019). This is because an estimated 30 to 50% of food commodities are lost due to contamination by filamentous fungi during pre-and post-harvest food processes which in turns threaten global food security in addition to wasting 1.47–1.96 Billion hectares of arable land, 0.75–1.25 trillion cubic meters of water and 1 to 1.5% of global energy (Fox and Fimeche 2013). What is even more deleterious about the consumption of these organisms and their secondary metabolites is their documented harmful consequences on human and animal health (Tanaka, Thomas et al. 2021). Consequently, the Food and Agriculture Organization (FAO) has estimated that these secondary metabolites contaminate about 25% of global food and feed production, entailing losses of about 1 billion tons of food and food products (Altomare et al. 2021).

The growing awareness of the reverberations of mycotoxins on livestocks has affected the marketability of food commodities and raise global food safety concerns (Mateus) because of the recorded literature of the vulnerability of more than five billion people through various unknown pathways (Khodaei). Of these mycotoxigenic moulds, the black aspergilli are important as ochratoxin-producing organisms which contaminate several agricultural products including grape-derived products (Cabañes et al. 2002), coffee and cocoa (Samson et al. 2004) and even dried cassava powder (*garri*) (Thomas et al. 2014). Taking in ochratoxin through food can lead to intoxication, known as ochratoxicosis (Battacone et al. 2010). This ochratoxicosis, mainly from ochratoxin A, cause diverse toxic effects including hepatic, neural, teratogenic, mutagenic, and carcinogenic outcomes. At cellular levels, they inhibit DNA and RNA synthesis by interacting with nucleic acids (Thomas et al. 2014, Samuel et al. 2021).

Generally, ochratoxin A (OTA) production has been strongly associated with the prevalence of black aspergilli (Bellí et al. 2005, Kapetanakou et al. 2009, Thomas et al. 2014). The capacity to synthesize OTA is encoded by a polyketide synthase (*pks*) gene, which belongs to the large fungal polyketide synthase family responsible for producing diverse mycotoxins and secondary metabolites (VARGA et al. 2003). The *pks* gene encodes a multifunctional enzyme containing distinct domains, including *β*-ketoacylsynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP). This enzyme is central to OTA biosynthesis (O'CALLAGHAN et al. 2003). In *Aspergillus carbonarius*, five *pks* fragments have been identified (ATOUI et al. 2006). Given the diversity of these genes and their roles in metabolite production across *Aspergillus* species, understanding how they are regulated, whether at the transcriptional, translational, or epigenetic level, is essential. This study therefore aimed to explore the evolutionary dynamics of the *pks* genes and identify potential strategies to limit their spread and the associated contamination of food substrates.

Materials and Methods

Sample Collection

Cassava flour (*Lafun*) samples were obtained from 24 localities belonging to four different political zones in Ogun state, South-Western Nigeria. These locations were chosen because of their known association with the production of cassava flour. The climate of Ogun state is tropical wet and dry with average annual rainfall of 1,340 mm across a total crop land area of 693.21k ha. Briefly, a total of 1000 *lafun* samples (250 each from 4 different markets in each zone) were collected in pre-sterilized aluminum pan from the four geopolitical zones of Ogun state, Nigeria between March 2013 to February 2014. The samples were collected at intervals and spread over the study period. The collection of samples was done as described by International Commission for Microbiological Specification for foods (ICMSF 2002). Briefly, aseptic techniques were used during sampling with sterile tools and containers, ensuring representative portions were taken from different parts of each batch. Samples were sealed immediately to prevent contamination or moisture uptake and stored at ambient temperature under dry conditions until analysis.

Fungal Isolation and Characterisation

Fungal isolation was carried out as described by VINCENTE et al. (2008) but with little modifications. First, 10 g from each sample was inoculated at room temperature for 30 min in 100 mL of sterile saline solution containing

200 µg/L penicillin, 200 µg/L streptomycin, 200 µg/L chloramphenicol and 500 µg/L cycloheximide. These antibiotics (penicillin, streptomycin, and chloramphenicol) were included to suppress bacterial contaminants, while cycloheximide was used to inhibit rapidly growing saprophytic fungi, thereby facilitating selective recovery of the target *Aspergillus* species. After the initial incubation, 20 mL of sterile mineral oil was added to the solution, followed by vigorous shaking for 5 min. The flasks were then left undisturbed for 20 min to allow phase separation. The oil-water interphase, where fungal spores and conidia typically accumulate due to their hydrophobic properties, was carefully collected using a sterile pipette. This fraction was chosen to enhance recovery of *Aspergillus* species while minimizing bacterial carryover. The collected interphase was inoculated onto Potato Dextrose Agar (Oxoid, United Kingdom) and incubated at room temperature for 72 h. The grown dark colonies were then isolated and stored on Mycosel agar. The fungal isolates were characterized both macro- and micro-morphologically prior to molecular identification. The macroscopic characterization was done by observing the colony features (color, shape, size and hyphae), and microscopically by a compound microscope with a digital camera using a lactophenol cotton blue stained slide mounted with a small portion of the mycelium. This procedure, adapted from GADDEYYA et al. (2012), which provides a standardized approach for fungal morphological identification.

Screening of the black aspergilli for ochratoxin A production

The screening of the black aspergilli for ochratoxin A production was carried out biphasically. First, preliminary screening of potential OTA-producing isolates was done following the method of HEENAN et al. (1998). Briefly, the black aspergilli (*Aspergillus niger* and *Aspergillus carbonarius*) were sub-cultured on Coconut Cream Agar medium following two approaches. First, Commercial coconut cream (Kara, 24% fat, Singapore) was used as the primary source to ensure reproducibility of nutrient composition across batches. Additionally, fresh coconut milk was extracted from mature coconuts (*Cocos nucifera*) obtained from local markets in Nigeria, prepared by blending grated endosperm with warm distilled water (1 : 2, w/v) and filtering through sterile muslin cloth. Both preparations were incorporated into the agar formulation (200 mL coconut milk, 20 g glucose, 15 g agar, distilled water to 1 L) and sterilized by autoclaving at 121 °C for 15 min. The use of commercial cream provided consistency, while the fresh extract demonstrated the feasibility of employing locally available raw materials. The inoculated organisms were incubated at room temperature 27 ±2 °C for 7 days after which the reverse of the inoculated Coconut Cream Agar medium was checked for characteristic green fluorescence on exposure to long wavelength UV light (365 nm) in the dark environment to depict potential OTA-producing strains. These potential OTA-producing strains were fumigated with 26.8% ammonia and examined under long-wave UV light (365 nm) in the dark. Ammonia vapour was used because it increases the local alkalinity, thereby ionizing OTA's phenolic and carboxylic groups, which enhances its natural fluorescence. Colonies showing intensified fluorescence were recorded as positive OTA producers. Secondly, the positive OTA-producing strains were subjected to ochratoxin A quantitation following the method described by THOMAS and OGUNKANMI (2014). To achieve this, three agar plugs (the diameter was 4 mm) were removed from the inner, middle and outer area of each of the positive isolate and extracted with 25 mL of 50% methanol for 1 hour in darkness. The extracts were vortexed and filtered with whatman no 1 filter paper containing pre-sterilised cotton wool. Ochratoxin A (OTA) levels were quantified using a competitive direct ELISA kit (Veratox® OTA, Neogen Corporation, USA) following the manufacturer's protocol. Briefly, fungal extracts and OTA standards were incubated with enzyme conjugate and transferred into anti-OTA antibody-coated wells. After washing, substrate and stop solutions were applied, and absorbance was measured at 650 nm using a microplate reader (Bioline Technologies, India). All assays were performed in triplicate, with both positive and negative controls included. OTA concentrations were determined from a standard curve and expressed in ng/g.

Molecular characterisation of ochratoxigenic black aspergilli

The molecular identification was carried out polyphasically viz, DNA extraction, amplification and sequencing. The DNA extraction from the black aspergilli was conducted on a week-old fungal culture using DNeasy Plant Mini Kit (Qiagen, The Netherlands). Primers AcPKS-F1 (AGCATC-TATGCTGGCCAATC) and AcPKS-R 1 (AATG – TACTCTCGCGGGCTAA) were used to amplify the ketosynthase domain of *pks* gene of the black aspergilli. The PCR reactions included 100–200 ng DNA, 50 mM KCl, 10 mM Tris-HCl, 80 µM each dNTP, 1 µM each primer, 2 mM $MgCl_2$ and 1 U of Taq DNA polymerase (Thermo Scientific DreamTaq, USA). The thermal cycler was programmed as follows: an initial step at 94 °C for 4 min, followed by 35 cycles of 94 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s, and a final elongation step of 72 °C for 10 minutes. PCR products were purified using the QIA quick PCR purification kit (BAO et al. 2012) and sent for sequencing at the International Institute of Tropical Agriculture, Ibadan Nigeria. Sequence quality was assessed by inspecting chromatograms for peak resolution and background noise, and low-quality reads were trimmed prior to other downstream analyses. The obtained sequences were compared with related sequences using nucleotide BLAST (BLASTn) against the NCBI GenBank database (nt). Searches were performed in (accessed July 2024), and the closest matches were retrieved for taxonomic identification.

Mutation discovery, estimation of selection, substitution pattern and phylogenetic analysis

Mutations in the pks gene of the isolated black aspergilli were identified by aligning sequences generated in this study using MEGA software (TAMURA et al. 2021). Recurrent mutations occurring at the same position were counted once. Substitution types were analyzed separately, with transitions (purine ↔ purine or pyrimidine ↔ pyrimidine) distinguished from transversions (purine ↔ pyrimidine), and single-base versus multiple-base substitutions examined independently. Selection pressure on the pks gene was assessed through codon-based analyses. We estimated the ratio of non-synonymous to synonymous substitutions ($dN/dS = \omega$), evaluated changes in amino acid properties, and calculated the ratio of non-synonymous to synonymous polymorphisms. Neutrality tests were also performed to detect deviations from neutral evolution. Different nucleotide substitution models were applied at different steps to account for model fit and accuracy. The Kimura 2-parameter model (KIMURA 1981) was used to estimate substitution patterns and distinguish between transitions and transversions. The JUKES-CANTOR model (1969) was applied for rate variation across sites under a gamma distribution, as it assumes equal base frequencies and simplifies correction for multiple substitutions. For phylogenetic inference, the Hasegawa-Kishino--Yano model (HKY 1985) was employed because it accommodates unequal base frequencies and transition/transversion bias. The TAMURA-NEI model (TAMURA et al. 2011) was also tested, as it accounts for both GC content bias and differences in substitution rates, providing robustness in tree reconstruction. Molecular phylogenetic analyses were performed in MEGA X and MEGA 11 using the maximum likelihood method. Initial heuristic searches were conducted with Neighbor-Joining and BioNJ algorithms, and the best-scoring topology was selected based on log-likelihood values. Branch support was estimated with bootstrap analysis. Final trees were drawn to scale, with branch lengths representing substitutions per site. The dataset included 18 nucleotide sequences and 993 aligned positions.

Results

Table 1 above represents the presence and distribution rate of black aspergilli in the sampled cassava powder circulating in Ogun state, Nigeria. As shown in Table 1, the highest organism isolated from the samples was *Aspergillus niger* 88 (8.8%), closely followed by *Aspergillus welwitschiae* 82 (8.2), and then *Aspergillus carbonarius* 56 (5.6) and finally *Aspergillus aculeatus* 47 (4.7). The increasing order of the isolated organisms per geopolitical zones was *Aspergillus niger, Aspergillus welwitschiae, Aspergillus carbonarius* and *Aspergillus niger* for Egba, Ijebu, Remo and Yewa respectively. In general, a total of 279 organisms representing 27.9% of black aspergilli were isolated from the sampled cassava powders. The cassava powders collected from the Remo zone were the most contaminated followed by the samples from the Yewa region. Even though, the Egba samples were the least contaminated, there was no statistical significant difference between the Egba samples and the samples from Ijebu.

Table 1

Occurrence of black aspergilli in the sampled *Lafun* in Ogun State (*n* = 1000)

GPZO	*n*	Black aspergilli				
		AN	AA	AC	AW	*N* [%]
Remo	(250)	18	12	29	19	78 (31.20)
Egba	(250)	22	6	14	21	63 (25.20)
Ijebu	(250)	14	11	13	26	64 (25.60)
Yewa	(250)	34	18	6	16	74 (29.60)
Total	(1000)	88 (8.8)	47 (4.7)	56 (5.6)	82 (8.2)	279 (27.9)

Explanation: AN – *Aspergillus niger*; AA – *Aspergillus aculeatus*; AC – *Aspergillus carbonarius*; AW – *Aspergillus welwitschiae*; *N* – total number of isolates; GPZO – geopolitical zones; *n* – sample size; % – percentages

The mean ochratoxin A (OTA) concentration of the eighteen positive strains of the black aspergilli is depicted in Table 2 above. The quantitative measurements of the OTA in parts per billion (ppb) shows the two positive *Aspergillus welwitschiae* as the highest producers of this toxin with an average value of 25.3 ±1.72 ppb. This observation was closely followed by the six positive strains of *Aspergillus niger* which had a mean ochratoxin A content of 21.5 ±2.11 ppb. *Aspergillus carbonarius* and *Aspergillus aculeatus* also produced mean OTA contents of 11.63 ±1.11 and 10.10 ±0.98 respectively.

Table 2
Mean Ochratoxin A (OTA) concentration in the positive strains of the black aspergilli ($n = 24$)

Isolates	OTA content [ppb]	Range [ppb]
AN ($n = 6$)	21.5 ±2.11	19.39–23.61
AA ($n = 3$)	10.10 ±0.98	9.12–11.08
AC ($n = 7$)	11.63 ±1.11	10.52–12.74
AW ($n = 2$)	25.3 ±1.72	23.88–27.02

Explanation: n – positive strains of the individual species; ppb – part per billion

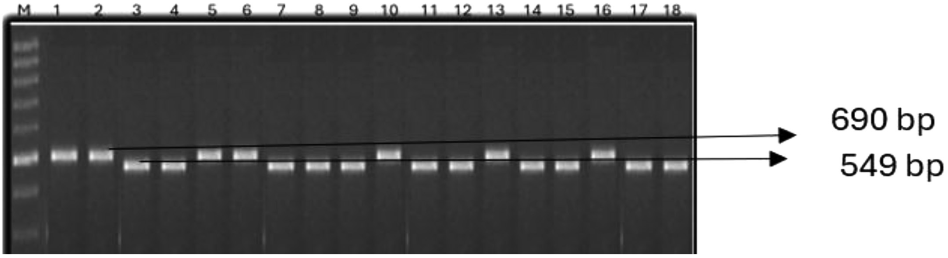

Fig. 1. Characterization of the polyketide synthase gene of black aspergilli. Explanation: *Aspergillus carbonarius* – 1–2, 5–6, 10, 13 and 16; *Aspergillus niger* – 7–9, 11–12, 14; *Aspergillus welwitschiae* – 3–4; *Aspergillus aculeatus* – 12, 17–18

Figure 2 represents the phylogenetic relationships of the different black aspergilli. As shown in this Table 3, all the *Aspergillus niger* aggregates (*Aspergillus welwitschiae* and *Aspergillus aculeatus*) clustered together with two other strains of *Aspergillus niger* at 75% percentage level of similarity except for two strains of *Aspergillus aculeatus that clustered with both Aspergillus carbonarius* and *Aspergillus niger* with a percentage similarity of 91%. The strains of *Aspergillus carbonarius* studied were found to share between 84–98% relatedness with themselves and 52% and 56% with *Aspergillus aculeatus* and *Aspergillus niger* respectively (Figure 2).

The maximum likelihood estimate of the transition-transversion bias of the *pks* genes of black aspergilli is depicted in Table 3. The frequency of the DNA building blocks (A, T, C and G) was found to be at equilibrium with a percentage substitution of 25% for each nucleotide of the *pks* genes. The number of protein in the data set of the *pks* genes, the transition-transversion bias ratio and the Maximum likelihood estimate were estimated to be 2215 bp, 1.01 and –13279.996 respectively.

The maximum composite likelihood estimate of the pattern of nucleotide substitution reveals that transitions were more frequent than transversions at the polyketide synthase genes of *Aspergillus* section *Nigri*. The results

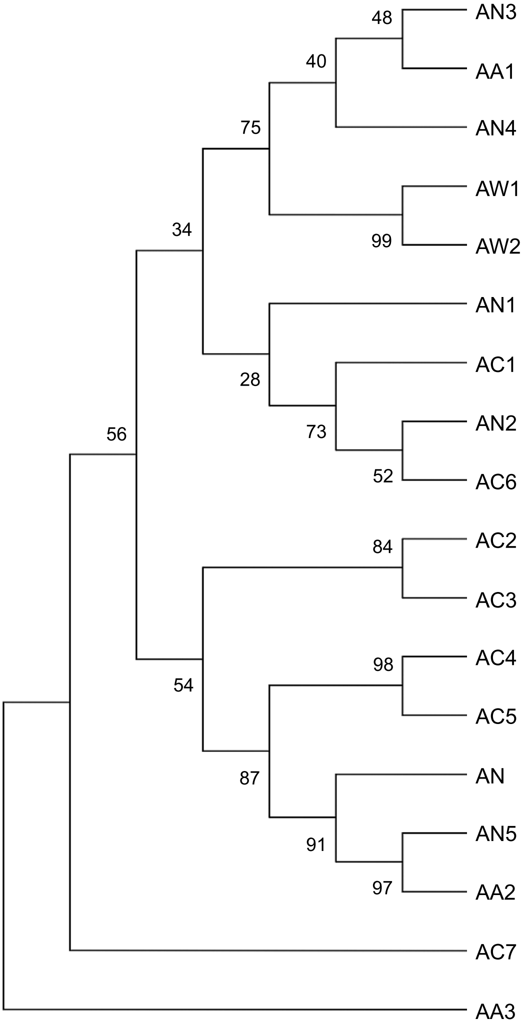

Fig. 2. Phylogenetic relationship of the polyketide synthase genes of isolated black aspergilli
AN – *Aspergillus niger*; AA – *Aspergillus aculeatus*; AC – *Aspergillus carbonarius*;
AW – *Aspergillus welwitschiae*

also showed that A/G and T/C transition were more frequent than C/T and G/A as evident in Table 4.

The codon-based Fisher's exact test analysis of selection of the *pks* gene of *Aspergillus* section *Nigri* provides a test of selection based on the comparison of the numbers of synonymous and non-synonymous substitutions between the different sequences of the *pks* gene of *Aspergillus* section *Nigri*. As shown in Table 5, eighteen *pks* genes of different black aspergilli

Table 3

Maximum likelihood estimates of transition-transversion bias of the *pks* genes of black aspergilli

Nucleotide	NF [%]	ML	NOP	R
A	25	–13279.996	2215	1.01
T	25	–	–	–
C	25	–	–	–
G	25	–	–	–

Explanation: A/T/C/G – adenine/thymine/cytosine/guanine; nt – nucleotide; NF [%] – nucleotide frequency [%]; ML – maximum likelihood estimate; NOP – number of positions in the final dataset; R – transition – transversion bias ratio

Table 4

Maximum composite likelihood estimate of the pattern of nucleotide substitution

Nucleotide	A	T	C	G
A	–	5.61	6.42	**13.73**
T	5.45	–	**14.27**	6.72
C	5.45	**12.46**	–	6.72
G	**11.14**	5.61	6.42	–

were compared in terms of numbers of synonymous and non-synonymous substitutions and none has a probability value of less than 0.05 and so the null hypothesis of neutral evolution is accepted.

The codon-based Z-test of neutral evolution connotes significant association between some (18) *pks* gene sequence combinations (AN-5 and AN-1, AA2 and AN-1, AC4 and AN-1, AN-5 and AN-2, AA2 and AN-2, AA2 and AN-5, AA2 and AN-6, AA3 and AA1, AC1 and AN-5, AC1 and AN-6, AC4 and AN-1, AC4 and AN-2, AC5 and AN-5, AC5 and AC4, AC6 and AN-3, AW1 and AA3, AW2 and AN-5, AC7 and AC3, AC7 and AW2) and neutral evolution. Consequently, larger combinations of the compared pks genes (133 compared sequences) have probability value above 0.05 and so the hypothesis upholding neutral evolution was accepted.

As denoted in Table 7, eighteen *pks* gene sequences were used for the Tajima's neutrality test analysis and the number of segregating sites was estimated to be 960 with a nucleotide diversity of 0.136116. The Tajima's neutrality test value was calculated to be 0.343161.

Table 5

Codon-based Fisher's exact test of selection analysis of the *pks* gene of *Aspergillus* section *Nigri*

	AN-1	AN-2	AN-3	AN-4	AN-5	AN-6	AA1	AA2	AA3	AC1	AC2	AC3	AC4	AC5	AC6	AW1	AW2	AC7
AN-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-2	0.5699	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-3	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-4	1.0000	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-5	1.0000	1.0000	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-6	1.0000	1.0000	1.0000	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-	-	-	-
AA1	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-	-	-
AA2	1.0000	1.0000	0.4978	1.0000	1.0000	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-	-
AA3	0.0916	0.0765	0.3934	1.0000	0.3334	0.3585	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-
AC1	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.5427	0.3511	-	-	-	-	-	-	-	-	-
AC2	1.0000	1.0000	0.4835	0.4939	0.3409	0.1806	1.0000	0.3016	1.0000	1.0000	-	-	-	-	-	-	-	-
AC3	0.3082	0.3082	1.0000	0.2990	0.3130	0.4282	1.0000	1.0000	0.0969	1.0000	0.3039	-	-	-	-	-	-	-
AC4	1.0000	1.0000	0.4059	0.4376	1.0000	1.0000	1.0000	0.3017	1.0000	0.0691	1.0000	1.0000	-	-	-	-	-	-
AC5	0.4299	0.7536	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.0765	1.0000	1.0000	0.2769	1.0000	-	-	-	-	-
AC6	1.0000	1.0000	1.0000	1.0000	0.4140	0.5537	0.1263	0.5318	1.0000	0.1675	0.3353	1.0000	0.4341	1.0000	-	-	-	-
AW1	0.5160	0.5160	0.5617	0.5035	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.5160	0.1119	-	-	-
AW2	1.0000	1.0000	1.0000	0.4725	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.5529	1.0000	1.0000	1.0000	1.0000	-	-
AC7	1.0000	1.0000	0.4068	0.3281	0.1786	0.2634	0.4218	1.0000	0.1981	0.3833	1.0000	1.0000	1.0000	1.0000	0.3275	0.5495	1.0000	-

Table 6

Codon-based Z-test of neutral evolution

	AN-1	AN-2	AN-3	AN-4	AN-5	AN-6	AA1	AA2	AA3	AC1	AC2	AC3	AC4	AC5	AC6	AW1	AW2	AC7
AN-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-2	0.15909	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-3	0.95258	0.95258	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-4	0.81312	0.76647	0.78959	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-5	0.04969	0.03379	0.21231	0.32868	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-6	0.08189	0.05723	0.68136	0.27448	0.26507	-	-	-	-	-	-	-	-	-	-	-	-	-
AA1	0.88668	0.88668	0.79289	0.91495	0.13838	0.22067	-	-	-	-	-	-	-	-	-	-	-	-
AA2	0.03680	0.04689	0.74956	0.66034	0.00308	0.01963	0.26376	-	-	-	-	-	-	-	-	-	-	-
AA3	0.15112	0.11714	0.80903	0.13547	0.56306	0.50131	0.00163	0.61756	-	-	-	-	-	-	-	-	-	-
AC1	0.20376	0.20376	0.28737	0.48497	0.00275	0.01515	0.38261	0.97770	0.47602	-	-	-	-	-	-	-	-	-
AC2	0.62513	0.62513	0.66884	0.91360	0.43145	0.25601	0.62846	0.57635	0.39325	0.85779	-	-	-	-	-	-	-	-
AC3	0.58510	0.55329	0.45772	0.51898	0.49520	0.64930	0.16700	0.08101	0.19669	0.55988	0.54052	-	-	-	-	-	-	-
AC4	0.00989	0.01425	0.63329	0.77937	0.30383	0.25739	0.61149	0.50292	0.27942	0.09582	0.24279	0.55616	-	-	-	-	-	-
AC5	0.08494	0.31886	0.95258	0.76647	0.03379	0.05723	0.88668	0.05922	0.11714	0.20376	0.62513	0.55607	0.01425	-	-	-	-	-
AC6	0.47302	0.51015	0.04895	0.99756	0.67162	0.91803	0.14739	0.98629	0.15108	0.30313	0.45709	0.40229	0.76890	0.51015	-	-	-	-
AW1	0.82840	0.82840	0.86637	0.80788	0.19985	0.30666	0.93931	0.50472	0.00238	0.60737	0.52485	0.07296	0.71451	0.82840	0.17288	-	-	-
AW2	0.86702	0.86702	0.50512	0.80952	0.02046	0.06823	0.12559	0.26784	0.62405	0.09398	0.92236	0.96817	0.24293	0.86702	0.19428	0.23000	-	-
AC7	0.84043	0.79427	0.78483	0.69284	0.32365	0.56448	0.81746	0.09029	0.30573	0.50258	0.42202	0.03023	0.46021	0.79427	0.69143	0.95436	0.00160	-

Table 7

Results from Tajima's Neutrality Test

m	S	Ps	Θ	π	D
18	960	0.433409	0.126007	0.136116	0.343161

Explanation: m – number of sequences; n – total number of sites; S – number of segregating sites; p_s –S/n; Θ – p_s/a_1; π – nucleotide diversity; D – Tajima test statistic

Discussion and Conclusion

The importance of polyketide synthases (PKSs) in the biosynthesis of polyketides such as OTA and the subsequent association of the polyketide synthase (*pks*) genes in OTA production has been well documented (GEISEN 2004, GALLO et al. 2009, KAROLEWIEZ and GEISEN 2005). In this study, four different black aspergilli namely, *Aspergillus niger, Aspergillus aculeatus, Aspergillus carbonarius* and *Aspergillus welwitschiae* were isolated from different cassava powders sampled in Ogun state, Nigeria. These black aspergilli besides their economic importance, also play important role in ochratoxin A production that contaminates several agricultural products including grape-derived products like coffee and cocoa (CABAÑES et al. 2002, SAMSON et al. 2004), dried cassava powders (THOMAS et al. 2014), among other foods. The *Aspergillus niger* isolated in this study has been regarded to as one of the most important pollutant species of foods in the world, especially in postharvest fruit products (fresh or dried), some vegetables, and several crops and is by far the most common *Aspergillus* species responsible for postharvest decay of fresh foods (PLASCENCIA-JATOMEA et al. 2014). *Aspergillus aculeatus* on the other hand causes post-harvest dry rot of tomatoes (FAJOLA 1979) and, with other members of Section *Nigri*, is involved in *Aspergillus* bunch rot of grapes (JARVIS and TRAQUAIR 1984, LEONG et al. 2004). *Aspergillus carbonarius* which is one of the three most ochratoxigenic species among the aspergilli, together with *Aspergillus niger* and *Aspergillus ochraceus* (TANIWAKI et al. 2003) was also found in this study. *Aspergillus welwitschiae* have also been found as food-contaminating species (PERRONE and GALLO 2017). The significant contamination of the analyzed cassava powders with different species of black aspergilli may not be unconnected to the practices associated with the production, processing and post processing handling of these cassava products which includes spreading on the floor, mats, displaying in open bowl in the markets as well as the use of various packaging materials to haul finished products from rural to urban areas (OGIEHOR and IKENEBOMEH 2005).

Of the twenty-four toxigenic strains, six *Aspergillus niger*, three *Aspergillus aculeatus,* seven *Aspergillus carbonarius* and two *Aspergillus* *welwitschiae* were delineated. This observation is not completely unexpected because *Aspergillus* species have been labelled as a major OTA-producing species in the tropics (ZHANG et al. 2016), of which this study sites belong to. Ogun State which is one of the thirty-six states in Nigeria has a tropical monsoon climate that supports the distribution characteristics of OTA-producing strains in different climates (HOCKING 2006). The fact that all of the black aspergilli screened were phenotypically positive also exhibits discrete amplification of the polyketide synthase gene further reinforces the involvement of this gene in the biosynthesis of ochratoxin A (GALLO et al. 2014, WANG et al. 2015, ZHANG et al. 2016).

The maximum likelihood estimate of the transition-transversion bias of the *pks* genes which was estimated to be 99% contradicts the general observation that transitions are more common than transversions (STOLTZFUS and NORRIS 2016, LYONS and LAURING 2017). The reason for this observation may be linked to the molecular mechanisms generating this type of mutational substitution (THOMAS et al. 2019, POPOOLA et al. 2024). However, it is believed that if all of the possible pair-wise nucleotide substitutions occur at the same rate, the transition-transversion ratio is expected to be 50%, because there are twice as many possible transversions as transitions (SZCZEPANOWSKA and TRIFUNOVIC 2020). For example, the transition-transversion ratio for *Drosophila* nuclear genome, humans and primate mitochondrial DNA are 2, 4 and 15 respectively (MEYER et al. 1999) and these therefore make transitions highly sensitive to mutational saturation because transitions will saturate more rapidly than transversions (PURVIS and BROMHAM 1997).

The maximum composite likelihood estimate of the pattern of nucleotide substitution reveals that transitions were more frequent than transversions at the polyketide synthase genes of *Aspergillus* section *Nigri*. The results showed that A/G and T/C transition were more frequent than C/T and G/A. This observation further emphasizes the widely reported significant bias of nucleotide base substitution toward transition than transversion (LUO et al. 2016). The reason for this may be due to differences in the conformation of purines and pyrimidines because purines have a bicyclic structure while pyrimidines have a single ring structure and these therefore make the process of transversion probably more complicated than the process of transition (SMITH and SIMMONDS 1997, ZHANG and GERSTEIN 2003). Our findings are however, contrary to, those observed in grasshopper pseudogenes where no significant difference was observed between transition and transversion rates (KELLER et al. 2007). The explanation for this may be that transition–transversion bias differs according to the region of the genome as well as the type of organism. The codon-based Fisher's exact test analysis of selection of the *pks* gene of *Aspergillus* section *Nigri* provided a test of selection based on the comparison of the numbers of synonymous and non-synonymous substitutions between the different sequences of the *pks* gene of *Aspergillus* section *Nigri* to depict a probability value of less than 0.05 and so the null hypothesis of neutral evolution was accepted. This finding was further supported by the results of Tajima's Neutrality Test ($D = 0.343161$), which is very close to zero. A value near zero indicates that the observed nucleotide variation does not significantly deviate from the expectations under neutral evolution, suggesting the absence of strong selection pressures (either purifying or positive). Since the p-value was greater than 0.05, the null hypothesis of neutral evolution could not be rejected, implying that the mutations in the pks gene are likely evolving neutrally (KORNELIUSSEN et al. 2013). Our results therefore showed population evolving at drift–mutation equilibrium with no evidence of selection (SHRINER et al. 2004). This observation was also strongly supported by the codon-based Z-test of neutral evolution which rejects the alternative hypothesis (dN > dS) that states that positive selection is the major driving force of evolution of the *pks* genes in favor of the null hypothesis of strict-neutrality (dN = dS). These results therefore suggest that transition and neutral selection drives the evolution of the *pks* gene of *Aspergillus* section *Nigri*.

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COMPARISON OF RAPID UREASE TEST WITH IMMUNOHISTOCHEMICAL EXAMINATION OF GASTRIC MUCOSA SPECIMEN IN THE DIAGNOSTICS OF *HELICOBACTER PYLORI* INFECTION AMONG OUTPATIENTS

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Key words: *Helicobacter pylori*, rapid urease test.

Abstract

Helicobacter pylori infection affects half of the human population. It is the most common infection in the world. Its presence is associated with numerous complications, including gastric cancer. More than 30 years ago, this bacterium was included by the World Health Organisation in the list of class 1 carcinogens, precisely because of its link to gastric cancer (CHO et al. 2021, LEE et al. 2024, TOBI et al. 2023). Its prevalence varies depending on geographical location, and even within a single country, different incidence rates have been reported (CHO et al. 2021, CODOLO et al. 2022). Infection most often occurs around the age of 10 and is usually asymptomatic. Therefore, it slowly leads to numerous complications. Relatively few patients experience symptoms associated with this condition and seek medical attention. The diagnostic pathway includes non-invasive and invasive tests, depending on the patient's age and the presence of worrying symptoms.

Introduction

Although the human digestive tract is colonized by many species of bacteria, mainly Firmicutes, as in other mammals, it seems that *Helicobacter pylori* is the most well-known pathogen (BURAIMOH 2023).

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Helicobacter pylori is a Gram-negative microaerophilic bacterium with numerous features that allow it to survive in the harsh milieu of the stomach. It is characterised by high geographical variability (Figure 1). Its prevalence depends on many socio-economic and demographic factors, antibiotic resistance, and genetic factors (CHO et al. 2021). It is reported that the highest number of infections takes place in Africa, where it affects about 70–80% of the population. However, these data seem to be underestimated, which is also connected to the highest risk of a dangerous, chronic complication of *H. pylori* infection, namely gastric cancer (KOLLI et al. 2021, MACHAJ et al. 2025). The main risk factors for infection are poor hygiene and contaminated water (XIE et al. 2024).

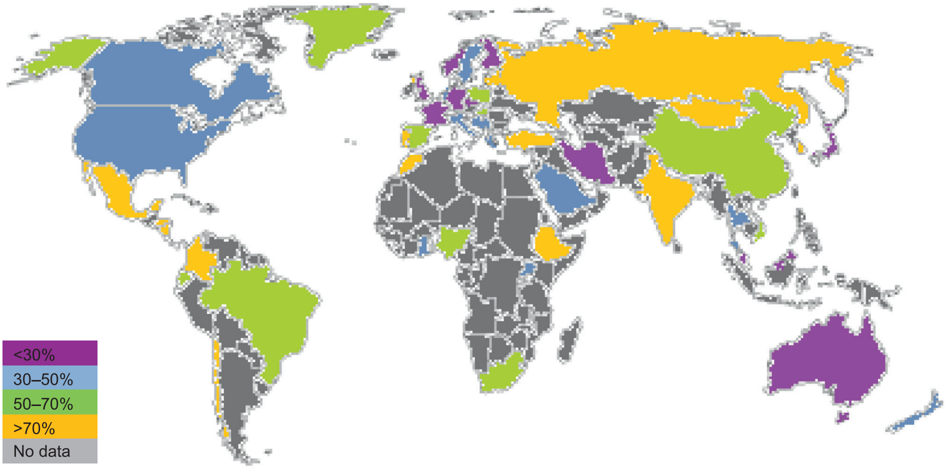

Fig. 1. Global prevalence of *Helicobacter pylori*
Source: based on HU et al. (2017), CC BY 4.0, modified

This bacterium exhibits many mechanisms that allow it to survive in the stomach and dominate its flora (SOUSA et al. 2022). Its arsenal includes motility, adhesion ability, enzymes that help it create favourable conditions for its existence in the stomach as well as virulence factors. The agents responsible for its virulence are proteins such as CagA, VacA, OpiA, and DupA, which provide its survival, as well. Some of the proteins affect the cytoskeleton of gastric epithelial cells and the host's immune response. They cause chronic inflammation of the gastrointestinal mucosa. According to the Correa pathway, chronic inflammation can lead to ulcers of the gastric or duodenal mucosa. It can also lead to atrophy or metaplasia of the gastric mucosa, i.e. precancerous conditions, and ultimately to carcinogenesis and the development of adenocarcinoma or MALT (marginal zone lymphoma)

(Cho et al. 2021, de Brito et al. 2019, Fujimori 2021, GUO et al. 2023, Iino et al. 2021). The frequency of individual gastric complications is as follows: chronic gastritis occurs in 100% of infected patients, peptic ulcers occur in 20%, dyspepsia affects 10% and gastric cancer affects 1% of patients (Liang et al. 2022, Shukla et al. 2024). The literature increasingly discusses the extra-gastric consequences of *H. pylori* infection, yet among the complications proven to date are unexplained anaemia due to iron or vitamin B12 deficiency, and immune thrombocytopaenia (Cho et al. 2021, Hurkała et al. 2023). An inevitable difficulty in taking on diagnostics is that only 1–2 out of 10 infected people experience symptoms of infection. This means they present no need to seek medical attention (Bartnik et al. 2014). According to the majority of gastroenterological societies, *H. pylori* infection is an indication for eradication. Treatment of this infection contributes to the treatment of peptic ulcers and protects against their recurrence. It significantly reduces the risk of gastric cancer and can cure MALT lymphoma (de Brito et al. 2019). Therapies used to treat *H. pylori* are multi-drug and complex, but they reduce the risk of gastric cancer by about 40% (Choi et al. 2025).

Diagnostic methods for *Helicobacter pylori* infection

Methods for detecting *H. pylori* infection include invasive and non-invasive tests. Invasive tests, i.e. those performed during gastroscopy and gastric mucosa biopsy, include the rapid urease test (RUT), culture with 80% sensitivity and 100% specificity, and PCR, for which samples can also be taken from faeces or saliva. PCR tests currently seem to be the field of greatest development in the diagnostics of *H.* pylori, offering enormous potential with high sensitivity (Yu et al. 2025).

The rapid urease test possesses high sensitivity and specificity, reaching 90–95%. It uses the enzyme urease, which is produced by *H. pylori*. Urease breaks down urea into ammonia. In turn, ammonia, by binding with hydrogen, causes a pink colouration of the test plate (Cho et al. 2021). Histopathological examination also has high sensitivity and specificity, 95% and 99%, respectively, depending on the bacterial density and staining method used. However, it is expensive and time-consuming (Lee et al. 2015, LU et al. 2025, Parra-Medina et al. 2025). Its sensitivity can be boosted by new techniques from the field of immunohistochemical methods (Loharamtaweethong et al. 2025).

Non-invasive tests include the urea breath test, which is recommended by a number of societies. It is a highly specific test with a specificity of 95–100% and a sensitivity of 88 to 95%. The patient consumes a solution of urea labelled with carbon C^{13}. Urease breaks down the urea, producing ammonia and carbon dioxide, which when exhaled with the carbon C^{13} incorporated, indicates a positive test result. Another widely used and easily accessible test is the faecal antigen test, which searches for *H. pylori* antigens excreted in the stool. It is also a high-quality test, with sensitivity and specificity reaching 97%. However, it should be noted that these tests may have varying sensitivity depending on the manufacturer (HORSMA-HEIKKINEN et al. 2025, SZCZEKLIK et al. 2022). Serological blood tests also belong to the so-called non-invasive tests, but due to their low sensitivity and specificity (80%) and the problematic phenomenon of antibodies persisting for years after *H. pylori* eradication, they are not recommended. Apart from serological assay, the above tests are burdened with a raised risk of error due to the use of, among other things, proton pump inhibitors, gastric mucosal atrophy, bleeding or the presence of neoplastic cells (IMPERIAL et al. 2024, SZCZEKLIK et al. 2022). New, increasingly advanced diagnostic methods are constantly being sought.

Treatment

Despite effective treatment, the annual risk of recurrence reaches 3%. Among the therapeutic strategies analysed by Sun et al. 209 different therapies were taken into account in terms of treatment duration and drugs used. Their research demonstrated how much geographically diverse the problem is, depending on local antibiotic resistance mechanisms. Quadruple therapy with bismuth is most often recommended (SUN et al. 2025).

Curing *H. pylori* infection prevents the development of gastric cancer, influences the resolution of chronic inflammation, the progression of inflammation to atrophic gastritis or its metaplasia. It may also exert a positive impact on the progression of metaplasia and atrophy of the gastric mucosa, although the effects are less pronounced in this case (SHARARA et al. 2025).

It is particularly in these precancerous conditions that eradication is paramount and recommended for all patients (MORGAN et al. 2025). However, there are voices pointing to the negative aspects of the global eradication of *H. pylori*, which are also worthy of considerable attention, namely the growing antibiotic resistance. This is estimated at 15–50%. This is counterbalanced by the possibility of selecting individualised therapy thanks to genetic sequencing, as *H. pylori* strains differ from one another with regard to virulence as well as sensitivity to treatment (UMAR et al. 2025, WRONECKI et al. 2025).

Material and Methods

88 women (70%) and 38 men (30%) were included in the study, with the mean age of 47 years (the youngest participant was 18, the oldest 82). Gastroscopy was performed with a rapid urease test and biopsy of the gastric mucosa. The biopsy specimen was subjected to immunohistochemical assay, manufactured by Roche, to diagnose the presence of *H. pylori* in the pathomorphological examination. Both diagnostic tests were performed in each patient.

Approval was obtained from the Bioethics Committee at the Faculty of Medicine of the University of Warmia and Mazury in Olsztyn on 21st February 2019, resolution No. 9/2019.

Results

Among 126 subjects, a positive rapid urease test was found in 51 subjects (40.5%), and a negative test in 75 subjects (59.5%). *H. pylori* infection was confirmed in histopathological examination in 55 patients (43.6%), and not confirmed in 71 patients (56.4%). Our findings are presented in Table 1. A total number of 64 people (50.8%) were confirmed to have *H. pylori* infection by one or both methods.

Table 1

Results of diagnostic tests for *Helicobacter pylori* (HP)

Test *n* [%]	HP positive	HP negative
Urease test	51 (40.5%)	75 (59.5%)
Biopsy	55 (43.6%)	71 (56.4%)

The concordance of positive results of the rapid urease test and the pathomorphological examination occurred in 42 subjects, i.e. 65.6% of those infected. A positive histopathological examination result was found in 85.9% of the infected population, and a positive rapid urease test result was found in 79.7% of the infected population (Table 2).

Table 2

Distribution of positive results for *Helicobacter pylori*

Test *n* [%]	Infected *n* = 64 (50.8%)	All *n* = 126 (100%)
Positive urease test	51 (79.7%)	51 (40.5%)
Positive biopsy	55 (85.9%)	55 (43.6%)
Positive urease test + positive biopsy	42 (65.6%)	42 (33.3%)

On the grounds of that, the sensitivity of the rapid urease test (13 false negative results) can be determined at 83%, while that of the pathomorphological examination (9 false negative results) at 87.7%.

Discussion

SUN et al. analysed nine online databases containing guidelines on *H. pylori*. Among the 25 guidelines, the urea breath test is the main recommended tool for the diagnostics of infection. Seven guidelines clearly do not recommend serological blood tests due to the inability to distinguish between past and active infection. The indications for eradication included long-term therapy with aspirin or non-steroidal anti-inflammatory drugs, or a history of peptic ulcer disease. A minority of the guidelines, 40%, recommended getting rid of *H. pylori* as long as it is detected, which is also confirmed by the Kyoto Consensus treating the presence of *H. pylori* as an infectious disease (SUN et al. 2025, SUGANO et al. 2022).

When it comes to verifying the efficacy of eradication, the guidelines mainly recommend the urea breath test, followed by the stool antigen test, while advising against the rapid urease test as a method of confirming effective eradication (SUN et al. 2025). An invasive method of *H. pylori* detection, such as histopathology, is commonly used in Indonesia as the *gold standard*. In their study, MIFTAHUSSURUR et al. (2021) conducted gastroscopy, taking histologic specimens in accordance with the Sydney protocol, then placing the specimens in 10% formalin, paraffin and staining them using the Giemsa method. They compared the effectiveness of histopathological examination in achieving positive results with the urea breath test. Both methods were equally effective, achieving 23.6% positive results. This is considerably less than in our study, where we have demonstrated positive results using histopathology in 43.6% of cases. This is even more striking given the higher prevalence of *H. pylori* in Indonesia, reaching 80–90%, compared to Poland (MIFTAHUSSURUR et al. 2021). On the other hand, MAJALIWA (2024) and his team compared three diagnostic methods: rapid urease test, histopathology and PCR. The rapid urease test was positive in all patients (100%), histopathology in 35% of patients and PCR in 65%. However, when the authors combined all three methods, they achieved 75% positive results for the three tests. In our study, we compared two methods, yet having achieved markedly lower results, with two tests agreeing in 33.3% of our patients. The group studied by MAJALIWA was smaller, consisting of 80 residents of Mozambique, where the expected incidence of *H. pylori* is higher than in the Polish population (MAJALIWA et al. 2024). In other studies, the concordance of three tests was combined: rapid urease test, Gram staining and serology, which resulted in 94% efficacy in detecting infection, or the rapid urease test was combined with serology, which detected 85% of infections (MUJTABA et al. 2025). The combination of different diagnostic methods is becoming increasingly common. Slightly worse results were reported in Bangladesh, where 32.1% of patients were tested positive by PCR and 32.6% were tested positive by rapid urease test. This is comparable to our results regarding the compatibility of rapid urease test and histopathology (FERDAUS et al. 2025). Similarly, in another study, two diagnostic methods were combined: rapid urease test and histopathology, which allowed *H. pylori* infection to be detected in 43.7% subjects (YADAV et al. 2025). In a different study comparing the rapid urease test with histopathology, 58% of patients presented confirmed *H. pylori* infection in the rapid urease test and 52% in histopathology. That is comparable to our results, although the study only involved 50 patients (EL-NASR et al. 2003). The researchers assume that the varying sensitivity of the tests depends on a number of factors, including the presence of precancerous or cancerous conditions in the stomach (KIM et al. 2025).

Conclusions

There is an increasing talk of combining two or three diagnostic methods for detecting *H. pylori*, as each of the methods mentioned in the introduction has its weaknesses, which can generate false negative results. This mainly concerns the use of proton pump inhibitors, antibiotics, bleeding, gastric mucosal atrophy, gastric mucosal neoplastic changes, the history of partial gastrectomy, the pathologist's experience, bacterial density in the biopsy specimen, and staining technique. Given the very high prevalence of *H. pylori* infection worldwide and the serious complications that this infection can lead to, combining several methods of diagnosing infection may be a new reasonable diagnostic trend.

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