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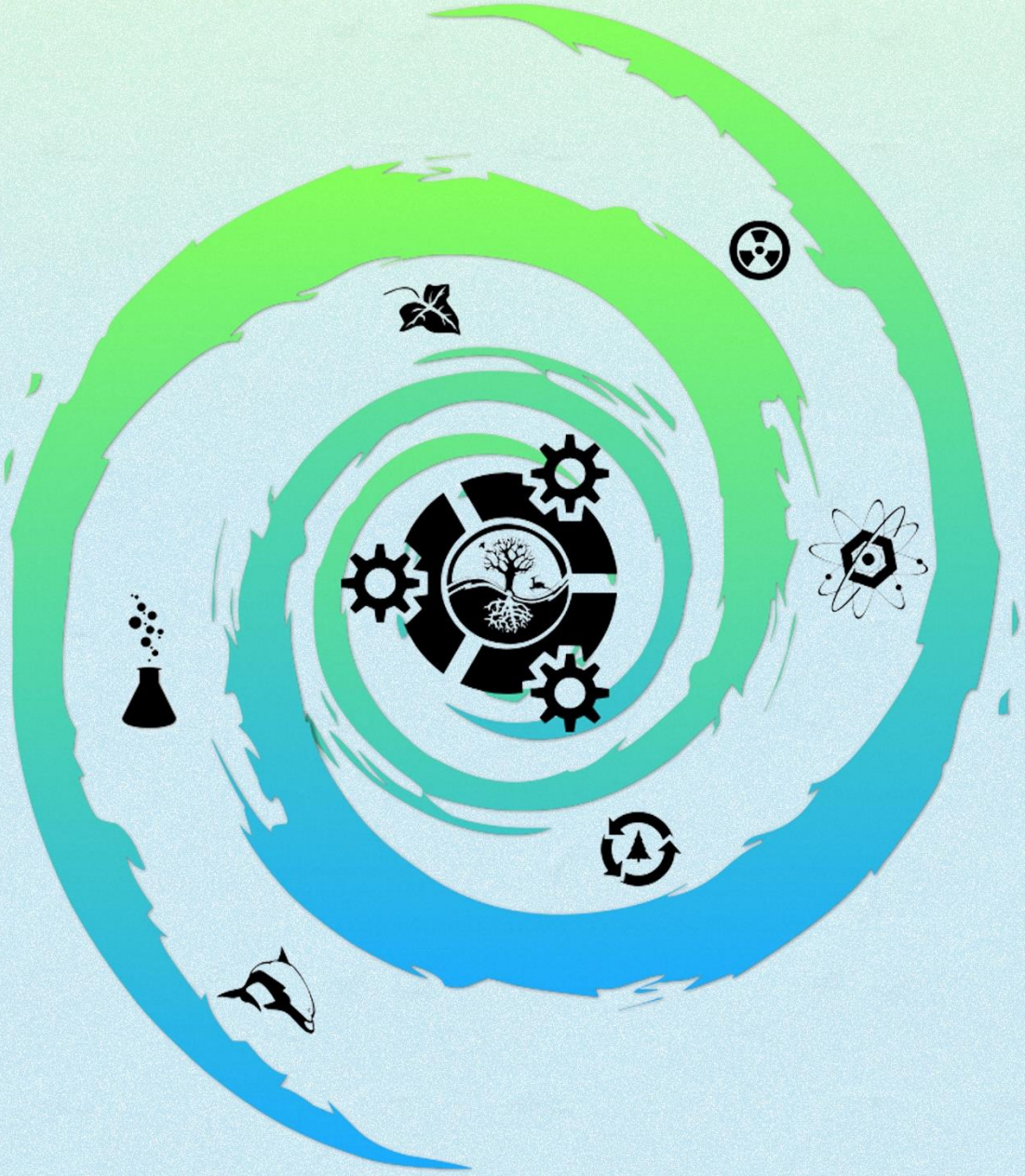

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ACTA NATURA ET SCIENTIA 2023 • VOLUME 4 • ISSUE 1

TABLE OF CONTENTS

CASE REPORTS

RESEARCH ARTICLES

REVIEW ARTICLES

Acta Nat. Sci. (2023) 4(1): 1-9
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Case Report
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ACTA NATURA ET SCIENTIA PRENSİP

Case of Preferential Selection of Attribute over Variable Control Charts in Trend Analysis of Microbiological Count in Water

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ABSTRACT

Monitoring the quality criteria in the healthcare industry and the pharmaceutical field specifically is a crucial mission activity to ensure the delivery of safe and effective treatment to patients with predictable and acceptable medicinal properties. One of the critical ingredients that are found in many activities is water. In the present study, the inspection characteristic trend was monitored by collecting results of the microbial count of Purified Water (PW) at two points in the water treatment station. The dataset was examined for pattern and distribution after processing and stratification and before conducting transformation using Microsoft Excel. Then, control charts were constructed using Statistical Process Control (SPC) software. The results showed that transformation improved data normalization for the Individual-Moving Range (I-MR) chart while the original pattern of the dataset was lost distorted. On the other hand, other advantages could be retained when using the Laney chart where no transformation was implemented on original raw data. The selection should be based on the nature of the process aim and condition.

INTRODUCTION

Consistency in the quality of the materials used in healthcare industry is crucial for delivering products with safe, predictable and effective properties to the final consumers (Mosadeghrad, 2014). These customers are usually health-defective and ill populations with a high risk of vulnerability to microbial infections. Water is one of the vital components in the industry that is involved in many activities (Eissa, 2016). Purified water is used in many non-sterile processes including pharmaceutical and biopharmaceutical manufacturing.

Water is highly dynamic in its inspection characteristics that may change and fluctuate rapidly under various conditions (Sardella et al., 2021). Thus, careful monitoring of its properties is of paramount importance. One of the critical quality items to be examined is the microbiological bioburden level (Eissa et al., 2022). Under these circumstances, the incorporation of the trending concept would be of great value over the reliance on the daily results only.

The use of the process behavior charts is an advantage in this situation as could be proved from earlier works that have involved quality management

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and improvement strategies following Six Sigma principles and rules (*Drinking Water and Health*, 1982). However, the appropriate selection of the trending chart is the dilemma that faces the interpreter, especially, if the primary database might be subjected to modifications, essentially the transformation due to a significant deviation in data distribution from the perquisite distribution (Eissa, 2015).

The crux of the present case is the presentation of two different point of view for analysis of dataset trends from non-normally distributed water samples results with pros and cons that could be drawn from the outcome of this investigation.

CASE STUDY

A small water plant for preparation of Purified Water (PW) from a municipal city source was monitored for quality characteristics, including total viable aerobic microbial count (TVAC) over about 16 months (70 weeks) on an average weekly basis. The number of accessible sampling ports was two: one after processing of water (denoted by PW) and the other at the Sanitization Port (denoted by SP) or specifically the Ultraviolet (UV) compartment. The sampling procedure, transfer and storage were conducted according to earlier research (Essam Eissa, 2018). Handling of samples, analysis, incubation and interpretation were performed using conventional microbiological techniques (Eissa, 2018a). Data were gathered and processed in Microsoft Excel then subjected to further processing using Statistical Process Control (SPC) software (Newton, 2014; Triola, 2014; Held, 2018; Levine et al., 2021).

Time-Series Investigation of the Microbiological Count in Water

Monitoring the mesophilic aerobic microbial count in water throughout the study period is illustrated in Figure 1 using a line graph and surface plot. The graphs showed three distinct levels of microbial count expressed as Colony Forming Unit (CFU)/100 mL. The baseline zone marked the close section to diminishing microbial count which is interrupted by the other two areas. The intermediate count zone showed microbial density between low and high bioburden. The last section demonstrated by few points but with spiking excursions towards the high microbiological population in water. The graph shows that the observed three ranges have visually different ranges from each other.

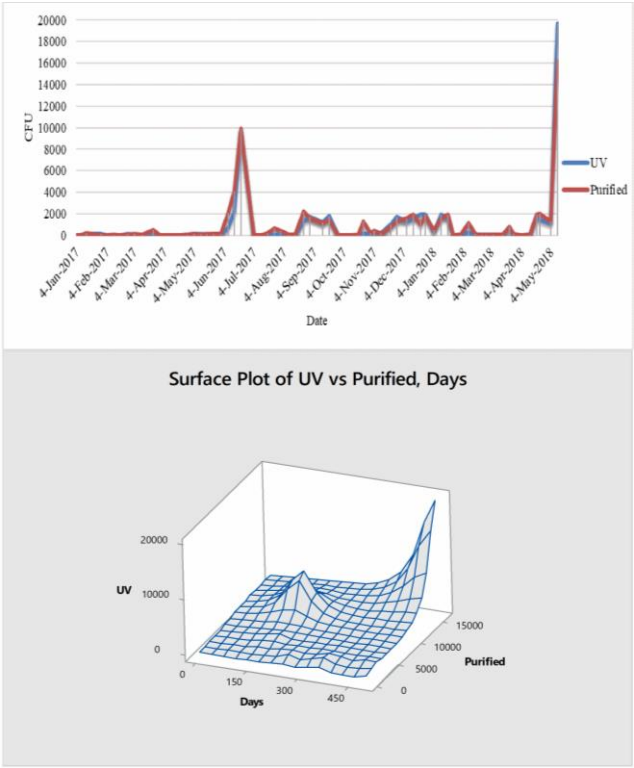

Figure 1. Time-series charts of the microbial contamination count of two monitoring points of the Purified Water plant as line graph (upper chart – generated using Microsoft Office 365) and surface plot (lower graph – generated using Minitab version 17.1.0).

Pareto Analysis of Bioburden Profile in Relation with Time

Pareto charts show the descending order of priority in terms of days based on the microbial density in water samples as could be seen from Figure 2 for the two use points' results. According to Pareto principle of 60/40 most populated water samples were found in April, May, June, August and January results. The wide variation range of the microbial count data distribution pinpoints the necessity of the statistical testing of the nature of the record-spreading pattern.

Examination of Dataset Distribution

The distribution and comparison of data before and after the logarithmic transformation study are demonstrated numerically in Table 1 and illustrated visually in Figures 3 and 4. The core finding from this section is that logarithmic transformation (to base ten) has significantly improved data normalization by reducing data scatter and spreading which is indicated by the absence of outliers after transformations. Accordingly, the use points' results had passed the Kolmogorov–Smirnov (KS) normality test, in addition to the noticeable improvement of skewness and kurtosis of the distribution curve. Also, the spreading between data points was appreciably reduced when variation parameters were reviewed.

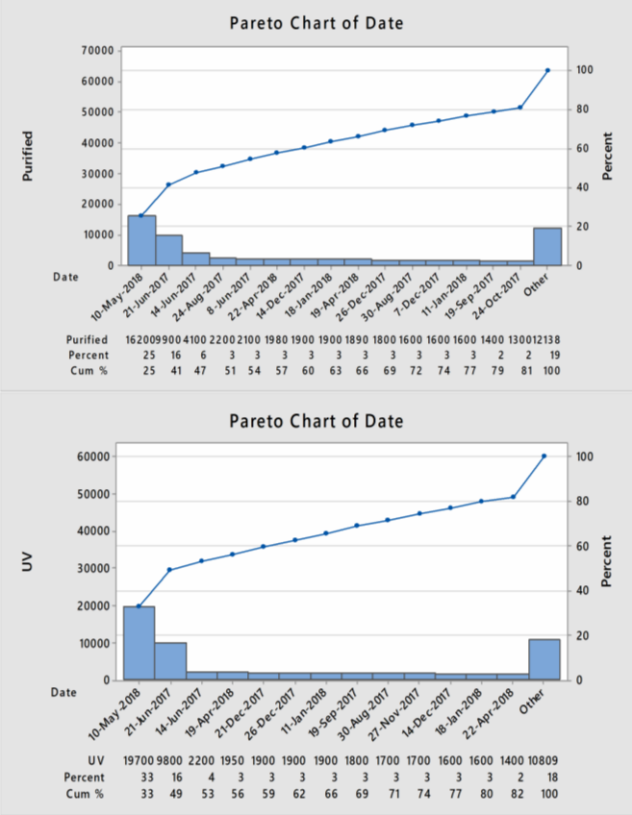

Figure 2. Pareto diagram showing days of the highest microbial count in descending order for two points of PW (generated using Minitab version 17.1.0)

Visual examination of the graphs (histograms, box plots and P-P graphs) would easily resolve the degree of improvement obtained from data logarithmic transformation in the reduction of scattering, removing excursions and normalization enhancement of the datasets for the two points-of-use. This could be viewed as a reasonable outcome for further statistical processing that might require normality assumption in the computation.

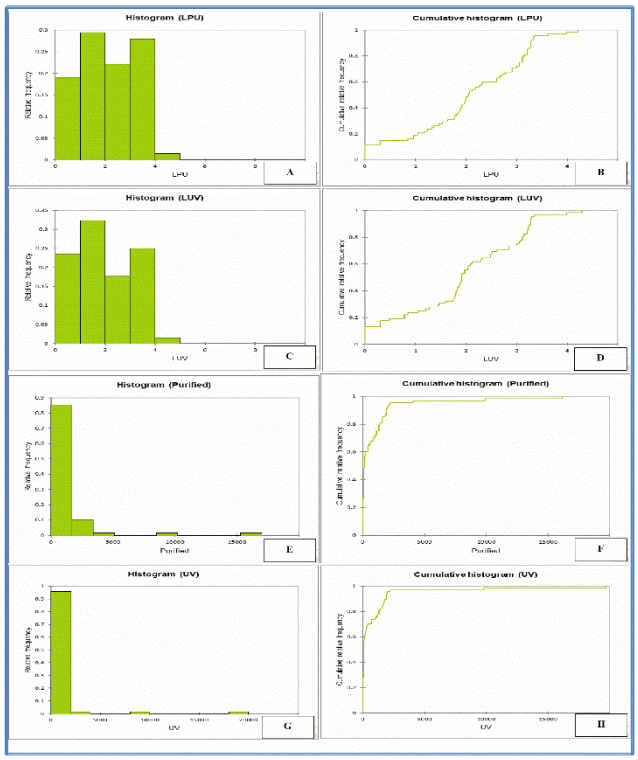

Figure 3. Comparison in distribution of data points between raw results (E, F, G, H) and logarithmically transformed microbial count (A, B, C, D) data [generated using XLSTAT add-in for Microsoft Excel]

Control Charts: Two Approaches Investigation

Two types of trending charts were used herein as an approach to monitor the process pattern and behavior. Figures 5 and 6 show attribute (For counting data, attribute control charts (most often integers, e.g., 4, 5, 6) and variable charts using variable controls for measurable data (most often decimals, e.g., 2.43) types of control charts, respectively. Red dots represent the alarming sample points that embrace assignable causes of variation which could be isolated from the common sources of natural variabilities in the inspection characteristics. The assignable differ from common causes variabilities in that they show special patterns between successive points rather than random variations that occur naturally in stable processes or inspection properties. These variations in turn embrace hidden factors that could lead to out-of-control situations with undesirable outcomes (Hubbard, 2003). Point "1" signals an alarm from abnormal extraneous factors. On the other hand, the

number "2" pinpoints drift in the inspection characteristic means. However, in the Individual-Moving Range (I-MR) charts there are additional out-of-control identification numbers. For instance, "5" and "6" alarms are indicators samples for early warning of a possible process shift. Signal number "3" is a highlight for the improvement or deterioration of the trend of the microbial count. The assessment of the process center (I-chart) and the variation in the process (MR) demonstrated several special causes of variations in microbiological quality of water. It should be noted that transformation – as appeared in the figures – changed the shape of the original data pattern with extreme values retracted minimizing outliers in the dataset.

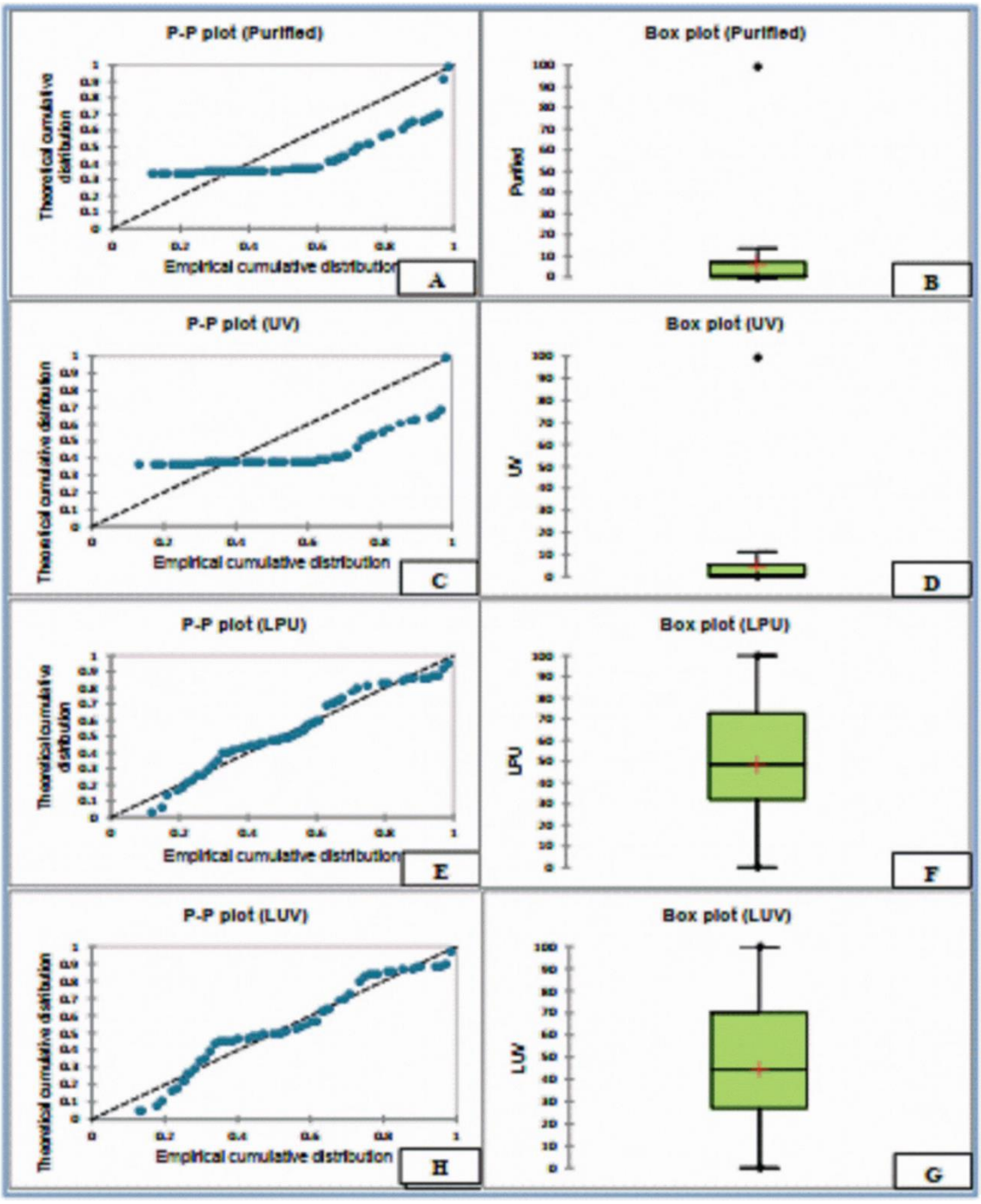

Figure 4. Probability–Probability plot (P value plot) (left column) and Boxplot (box-and-whisker) diagram (right column) showing raw (A, B, C and D) and transformed (E, F, G and H) data [generated using XLSTAT add-in for Microsoft Excel]

Table 1. Comparative descriptive statistics of microbial count results before and after transformation

Column Statistical Test[¥]	**SP**	**PW**	**LSP**	**LPW**
ROUT (Q = 1.000%)				
Outliers	18	3	0	0
Data Spreading Description				
25% Percentile[§]	11.25	20.50	1.082	1.332
Median[§]	80.50	110.5	1.911	2.047
75% Percentile[§]	1075	1250	3.032	3.096
Maximum[£]	19700	16200	4.294	4.210
Std. Deviation	2664	2337	1.151	1.127
Std. Error of Mean	323.1	283.4	0.1396	0.1366
Lower 99% CI of mean	24.02	183.1	1.535	1.687
Upper 99% CI of mean	1739	1688	2.276	2.412
Coefficient of variation	302.13%	249.80%	60.42%	54.96%
Skewness	5.983	5.170	-0.2312	-0.4060
Kurtosis	39.56	30.20	-0.8407	-0.6884
Kolmogorov–Smirnov (KS) normality test				
KS distance	0.3703	0.3445	0.1165	0.09928
P value	< 0.0001	< 0.0001	0.0228	0.0935
Passed normality test (α = 0.01)?	No	No	Yes	Yes
P value summary	****	****	*	ns
Sum	59959	63608	129.6	139.4

Note: ****: P ≤ 0.0001. *: P ≤ 0.05. ns: P > 0.05. ROUT: **Ro**bust regression and **Out**lier removal. SP: Sanitization Point. PW: Purified Water. L: Stands for Logarithmic transformation to the base ten for SP and PW. CI: Confidence Interval.

¥: Generated using GraphPad Prism version 6.01. Median: 50th percentile.

§: V = C × (d + 1)/100, where: *V* is percentile value, *C* is the target centile and *d* is the number of values in the dataset.

£: Minimum values of zeros are not shown in the table for no microbial count recovered.

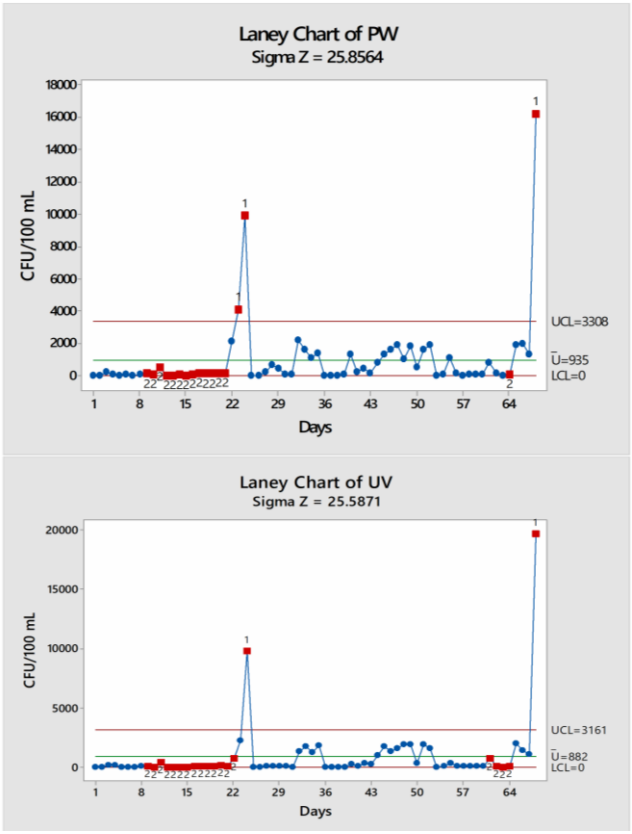

Figure 5. Laney attribute process-behavior charts of two PW point-of-uses in water treatment plant using raw dataset (generated using Minitab version 17.1.0)

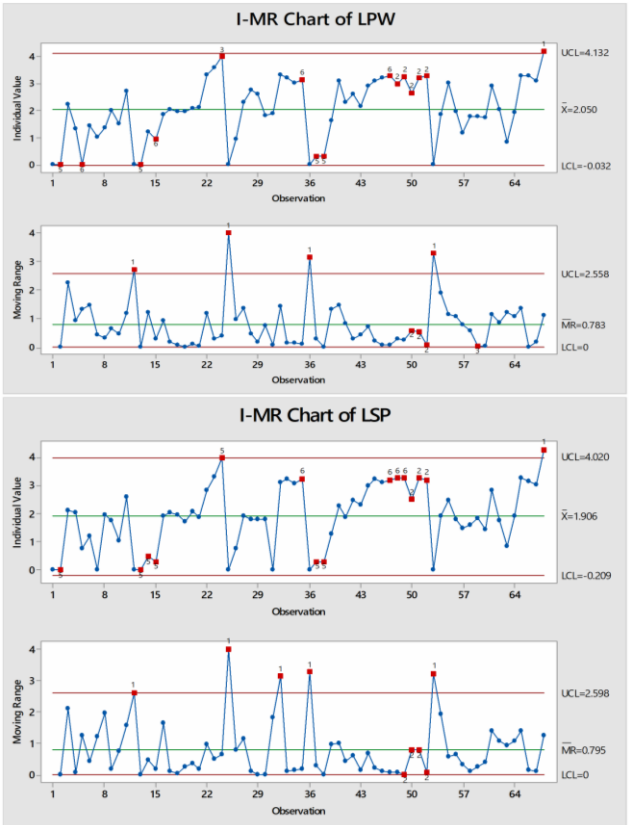

Figure 6. I-MR process-behavior charts with logarithmic transformation of two PW point-of-uses in water treatment plant generated using Minitab version 17.1.0)

DISCUSSION

Basically, control charts were designed to be used in the observation, control and monitoring of industrial objects such as product weight and dimensions (Hamed, 2017). However, process behavior charts gained popularity in other non-industrial applications (Wheeler, 2014). While setting exact parameter values in the trending charts might not be as critical for non-industrial properties as for the manufacturing ones by virtue of the processes or the inspection properties. Yet, they are still important to obtain quantitative outputs from the analyses that are reasonably informative and useful in the investigation of the inspection properties such as bioburden in water and to demonstrate acceptable and predictable levels of quality for ensuring acceptable consistency (Bhagwat, 2019). Controlling water quality for pharmaceutical purposes is at all costs a critical task that should not be underestimated and ignoring it would lead to devastating consequences (Bhagwat, 2019).

Recommendations have been postulated on the advantages of the variable over attribute trending charts as it could detect as twice as the known types of alarms in the charts that are used for discrete data type, in addition to the inclusion of another chart that accesses the process variability i.e., MR (Eissa, 2018b). This could be evident when comparing both types on the same set of data under the same conditions. In previous studies, comparison between variable and Laney-modified or corrected attribute charts has demonstrated agreement in the outcome interpretation between both types when conducted on the same dataset (Eissa, 2018b; Essam Eissa, 2018). However, transformation in the present case has changed the output derived from the I-MR chart when compared with the Laney chart projected on the original raw data.

Normality assumption for the use of variable control charts has been stated by various statisticians and experts (Khakifirooz et al., 2021). Despite the robustness of the I-MR chart has been demonstrated previously by some researchers, the Normalization procedure has been adopted in the present study to evaluate the pros and cons against the counterpart attribute chart through one of the well-known transformation paths (Keller, 2011; Elisson, 2017). This transformation tactic – for the original data - has been proved formerly to minimize data scattering and reduce outliers. Thus, Log_{10} [(CFU/100 mL) + 1] formula was used in the construction of data points in I-MR charts (Eissa, 2015; Smarter Solutions Inc., 2022). Number "1" in the equation is necessary to correct for zeros in the data record without distorting the data significantly.

On the same line, Laney's correction of the attribute chart was used for datasets that failed to show Poisson distribution assumptions of u or c charts. In this case, Laney modification was used to mitigate the risk of possible elevated alarm rates (Laney, 2002). This correction would be important to keep an eye on a process' defect rate and make any necessary adjustments for over- or under-dispersion in the present data. A conventional chart (for multiple defects per unit (D/U)) may display more points outside the control limits due to over- dispersion (Jones & Govindaraju, 2001). On the other side, a typical chart for this type of defect can display too few points outside of the control bounds due to under-dispersion. The Laney U' chart makes these adjustments of the control limits to avoid false warning signals.

A Laney-corrected chart's Sigma (σ) Z value shows how much the control limits are modified to account for over-dispersion or under-dispersion. In order to account for over-dispersion (as in the present case), the control limits on the Laney modified chart are wider than the control limits on a conventional D/U chart when the σ Z value is greater than the unity. An advantage of using this attribute chart over the I-MR chart is bypassing the transformation time and effort back and forth with less possibility of errors (Moon, 2020). Also, the merit of using the Laney chart in the present situation cannot be denied by being able to retain the same shape and profile of the original data without distortion so that abnormal points could be traced back to the original dataset chronologically.

The presence of additional types of alarm sensitivity did not seem to add much to the investigation of microbiological water quality in this work. However, the mean of the variable charts appeared much lower than that of the attribute ones for PW and SP points with values of about 113 and 81 CFU/100 mL (derived from the statistical software results) versus 935 and 882 CFU/ 100 mL, respectively (Skinner, 2018). Nevertheless, the Upper Control Limits (UCLs) for I-MR charts appeared much higher than that of Laney charts with values of 133552 and 10472 CFU/100 mL for PW and UV points which exceeds the Upper Specification Limit (USL) of 10000 CFU/100 mL. Accordingly, the Laney attribute chart was found more suitable for the initial preliminary examination of PW microbial quality with UCL values of 3308 and 3161 CFU/100 mL, respectively. Thus, the rate of detection of out-of-control points in the Laney charts was shown to be higher than in the corresponding I-MR graphs.

It should be noted that SL is a threshold value set by the formal authority or regulatory agent for an acceptable criterion of a certain subject while CL is the threshold computed from collected dataset arranged chronologically and would change based on the progressing variability of the monitored process or characteristic. When both limits are exceeded, it would be considered a critical chaotic issue that requires immediate extensive actions and investigation to prevent the occurrence of this incidence in the future. Water of poor quality will be produced if the process is under control, but the control limits are outside of the specification limits. The control limits will, in the ideal case, if centered inside of the specification limits with space to spare. The microbiological quality of water is stable and within the required process capacity if the CLs are within the SLs. It produces the best quality and has few inspection problems. The process also complies with consumer requirements. However, if one or more of the water samples showed results above CLs even if within SLs, then the process is unstable with out-of-control points detected then fast corrective and

preventive actions (CAPA) are required to bring the process again within the stability window.

Laney charts could be easily correlated and interpreted along with time series plots and Pareto diagrams as could be seen from Figures 1 and 2 which facilitate the investigation of defect spot areas to correct the problem as a part of Corrective Action and Preventive Action (CAPA) plans (Motschman & Moore, 1999). This might not be quite evident in the transformed I-MR charts except when reviewed with the original series point-by-point to understand the pattern. Transformation minimized the scattering of the data points with the subsequent bringing on the extreme results closes together. Thus, low values appear higher and the high points become lower. Accordingly, the low critical points might appear exaggerated and the more crucial results would be underestimated. Experts in the quality field should understand when to use trending chart types by evaluating the confronted situation case by case depending on the nature of the inspection characteristics and the aim of the process. The authors suggest using the Laney attribute chart in the present case for simplicity and convenience, especially since the important aberrant patterns could be easily spotted in the time series charting.

Compliance with Ethical Standards

Authors' Contributions

MEE & DEE: Study conception and design

ER: Data collection

MEE, ER &DEE: Analysis and interpretation of results

MEE & DEE: Draft manuscript

All authors reviewed the results and approved the final version of the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or its supplementary materials].

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Traditional Weather Forecasting: Practices of Fishers, Sailors and Old Folks in Tawi-Tawi, Philippines

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ABSTRACT

Weather forecasting using traditional methods in the province of Tawi-Tawi, Philippines, is an important indigenous knowledge and is heavily applied to predict the weather in preparation for fishing and sailing and also for disaster prevention. Here, we documented the practices of the local people of Tawi-Tawi, situated in the southern Philippines, in forecasting weather using traditional methods. The informants of the study were fishers/Badjaos (n=15), sailors/boat captains (n=15) and old folks (n=15), which were interviewed at the Chinese Pier, Bongao, located in Tawi-Tawi, Philippines. Results revealed that traditional knowledge/methods using atmospheric (clouds, wind, temperature, and visibility), astronomical (sun and moon) and biological (seagrasses/algae, ants, birds, bees, and earthworms) indicators were used to forecast weather, which generally predicts an incoming rainy season as well as adverse weather (typhoons and floods). This study indicates that traditional weather forecasting is still used by the local people of Tawi-Tawi crucial for fishing and sailing activities.

INTRODUCTION

Fishing is an important and major livelihood for coastal settlers in Tawi-Tawi, southern Philippines, owing to its favorable location in the heart of the coral triangle, which is deemed as the world center of coral reef biodiversity (Muallil et al., 2020; Mohammad et al., 2022; Tahiluddin & Sarri, 2022). Hence, fish and other fishery aquatic products are considered as a significant source of protein for Filipinos (Tahiluddin & Terzi, 2021; Tahiluddin & Kadak, 2022). In particular, small-scale fishing is a practice by fishers in Tawi-Tawi using various fishing gear with the help of motorized boats (Muallil & Hapid, 2020; Ajik & Tahiluddin, 2021; Mohammad et al., 2022). In fishing, the fishers have to be equipped with traditional knowledge of weather forecasting in order to plan incoming fishing activities safely. Moreover, sailors, mainly the boat captains of the "lansa"- a big wooden-hulled passenger and cargo vessel, are also adept at reading the different indicators as a clue or basis for the onset of rain or extreme

weather conditions. Fishers and sailors in Tawi-Tawi are mostly depended highly on traditional weather forecasting passed on from generation to generation (Personal Communication).

Typhoons are considered as serious threats to human lives and urban development due to their huge destructive power and high frequency of occurrence (Liu et al., 2020). Of 20 tropical cyclones on average coming from the Western Pacific Ocean, eight or nine of them cross the Philippines, which typically develop from July to November (Santos, 2021). Among the most devastating typhoons that struck the Philippines was Typhoon Haiyan (Yolanda), which occurred on November 8, 2013 (Lum & Margesson, 2014). In Tawi-Tawi, Philippines, during typhoon seasons, rough seas usually occur. Occasionally, fishers who went fishing were reported to be missing, and sailing "lansa" went sinking. Hence, weather forecasting plays an important role in safe water transportation and activities.

Traditional weather forecasting is considered a belief designed to explain mysteries of nature that old folks could hardly explain in any other way (Galacgac & Balisacan, 2009). Traditional weather forecasting is a global practice by various indigenous communities and serves as a guide in order to cope with harsh incoming weather (Risiro et al., 2012; Enock, 2013; Zachary et al., 2021). Hence, preparing in advance for their livelihood-related activities to avoid extreme damages (Galacgac & Balisacan, 2009). In the Northern Philippines, like in Ilocos Sur province, traditional weather forecasting is one important piece of knowledge used by the local people to predict weather conditions for fishing and farming (Galacgac & Balisacan, 2001). Despite the existence of modern technology, such as the Philippine Atmospheric, Geophysical and Astronomical Services Administration (PAGASA), locals claimed that PAGASA forecasts are not specific to local forecasts and are applicable in wider coverage (Galacgac & Balisacan, 2009). In Tawi-Tawi, PAGASA stations are lacking, and weather forecasting is only available via television and smartphones. However, since not all fishers and sailors own these technologies, relying on old folks' knowledge for forecasting weather remains useful. Hence, in this study, forecasting weather using traditional methods used by the fishers (Badjaos), sailors/boat captains, and old folks was determined through one-on-one interviews.

MATERIAL AND METHODS

Study Location

The study was conducted at a Chinese pier, Bongao, Tawi-Tawi, Philippines (Figure 1). The study location is the central pier of Tawi-Tawi, where all passenger boats (lansa), fishing boats, and other water transportation can be seen.

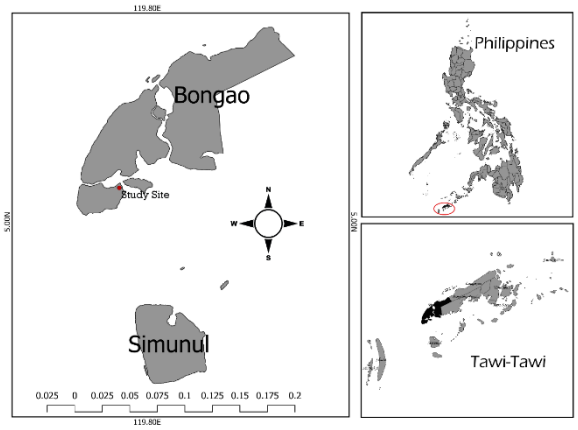

Figure 1. Study site

Data/Information Gathering

In this study, the respondents or traditional weather forecasters (n=45) were composed of fishers (Badjaos, n=15), sailors (boat captains, n=15) and old folks (n=15). Using the local dialect (Sama), the respondents were personally interviewed with the help of an unstructured questionnaire. Traditional weather forecasters were asked how they predict the weather, such as the upcoming rain or wet season, adverse weather factors (typhoons and floods) and others, using atmospheric, astronomical, and biological indicators.

Data Analysis

Descriptive statistics, i.e., frequency statistics, were used in analyzing the data with the help of Microsoft Excel 2021.

RESULTS AND DISCUSSION

This study revealed that fishers (Badjaos), sailors (boat captains), and old folks used traditional knowledge to forecast the weather in the local area. These methods of forecasting include various indicators, such as atmospheric (clouds, wind, temperature and visibility), astronomic (moon and sun), and biological (seagrasses/algae, ants, birds, bees and earthworms) indicators. Globally, this form of indigenous knowledge has been practiced since immemorial (Muguti & Maposa, 2012; Enock, 2013).

In the present study, the widely used traditional knowledge for weather forecasting was the atmospheric indicators namely clouds, wind, temperature, and visibility. All of the respondents (100%) relied on clouds to predict the weather. The presence of cumulonimbus clouds would predict rain, while the appearance of hair-like clouds (cirrus clouds) would serve as an indicator of an incoming storm (Table 1). In Africa, old folks interpret the presence of clouds (cumulonimbus) as an approaching heavy storm with thunder and lightning (Enock, 2013), similar to modern meteorology forecasts (Barry & Chorley, 1998; Ahrens & Henson, 2019). The wind is another atmospheric indicator used by the Tawi-Tawian people (80%) in the present study; when the wind blows from the south, adverse weather, like typhoons and floods, can be expected (Table 1). This claim is parallel to those reported by the old folks in Ilocos Sur, Philippines (Galacgac & Balisacan, 2001). Southwest monsoon is typically carried heavy rainfall, which could intensify when associated with tropical cyclones (Cayanan et al., 2011). Another meteorological indicator in the present study used by traditional weather forecasters (44.44%) is when a warm temperature is felt during nighttime, indicating that rain will occur within one or two days (Table 1). Galacgac & Balisacan (2001) reported that the old folks in Ilocos Norte, Philippines, also predict an incoming short-range rain once the temperature is warm at night. Moreover, Malaysian farmers used this human behavior, that is, feeling warmth associated with lethargy, irritability, and insomnia, as a rain indicator (Garay-Barayazarra & Puri, 2011).

Table 1. Atmospheric indicators utilized as traditional weather forecasting by the Tawi-Tawian people

Atmospheric Indicators	**No. of Respondents**				**Forecast**
	Boat captain	***Fishers (Badjaos)***	***Old folks***	***Total***	
A) Clouds	15	15	15	45	
The presence of cumulonimbus clouds					There is an incoming rain.
The appearance of hair-like clouds (cirrus clouds)					There will be a storm.
B) Wind	12	13	11	36	Adverse weather, like a typhoon, is coming.
Wind blows from the south					
C) Temperature	8	5	7	20	Rain will occur within one or two days.
Warm temperature is felt during nighttime.					
D) Visibility	7	7	6	20	
When the mountain or island is seen, or visibility is very high.					Adverse weather conditions will occur.

When the visibility is low or dull/foggy.	The weather is good.

Table 2. Astronomic indicators utilized as traditional weather forecasting by the Tawi-Tawian people

Atmospheric Indicators	No. of Respondents				Forecast
	Boat captain	*Fishers (Badjaos)*	*Old folks*	*Total*	
Moon and sun A luminous ring around the moon and sun	10	11	10	31	Rain will occur after one or two days.

Table 3. Biological indicators utilized as traditional weather forecasting by the Tawi-Tawian people

Atmospheric Indicators	No. of Respondents				Forecast
	Boat captain	*Fishers (Badjaos)*	*Old folks*	*Total*	
A. Plant/Algae The appearance of seagrass and algae floating in the sea	4	1	6	11	A typhoon is coming.
B. Animal Migration of armies of ants (Formicidae) typically carrying stored food Migration of birds, such as frigate birds, waders or shorebirds, and bees. Earthworms walking on land.	15	2	15	32	Wet/rainy season is about to start.

The moon and sun are typical astronomical indicators to predict weather conditions. In this study, a rain will occur within one or two days when a luminous ring around the moon and sun is observed (Table 2). According to Galacgac & Balisacan (2001), the appearance of a luminous ring around the moon (lunar corona) indicates extreme weather conditions, i.e., floods or typhoons. Enock (2013) also found out that astronomical indicators, such as the moon, are used in Africa to predict the approaching rainfall but in different ways, while the sun is typically used to signal temperature changes. Ahrens & Henson (2019) mentioned that a halo (a light ring that encircles the moon or the sun) is produced when the light passes through the crystals of ice in cirrostratus clouds. In addition, the formation of thick cirrostratus clouds often occurs ahead of a proceeding cyclonic storm; therefore, they are usually used to predict rain within 12-24 hours. Other astronomical indicators used by traditional weather forecasters are the stars, as reported by some study (Muallil et al., 2020). For instance, the agricultural farmers in Sibutu, Tawi-Tawi, used the presence of seven stars, being positioned directly overhead at sunset, to signal the farming season. In addition, rain would be expected when the three stars are noticed roughly 100 degrees

westward from the east (Muallil et al., 2020). Fishers also used the aggregation of stars observed 45 degrees from the east as a sign of a fishing season, especially, capturing *Megalaspis cordyla, Sphyraena barracuda*, and *Rastrelliger kanagurta* (Muallil et al., 2020).

Biological indicators for traditional weather forecasting were not commonly used by the Tawi-Tawian people since they typically live near coastal belt. Only one indicator was used: the appearance of seagrasses and algae floating in the sea, indicating that a typhoon is coming (Table 3 A). According to Navarrete-Fernández et al. (2022), the litter of seagrasses on the beach is three-fold higher after heavy rainfall. Other studies indicated that the most commonly used biological indicators in forecasting weather conditions, especially by agricultural farmers, are the terrestrial plants. Terrestrial plants are good rainfall predictors due to their high affinity to water, which respond easily to significant changes in atmospheric moisture content (Zachary et al., 2021). Plant indicators such as early rotting and ripening of some fruits, development of flower/buds/shoots, and flowering of plants predict the onset of the rainy season (Galacgac & Balisacan, 2009). Animals are also key indicators of premature and late rainfall onset, especially migratory birds, amphibians, and insects (black ants and spiders) (Zachary et al., 2021). The presence of plenty of spider webs is also used to indicate an upcoming extreme wet season (Enock, 2013). The appearance of a local African type of kingfisher has been linked to heavy falls, which may occur within days (Enock, 2013). In the present study, animal indicators, like the migration of armies of ants (Formicidae) typically carrying stored food, migration of birds (frigate bird and waders or shorebirds) and bees, as well as earthworms walking on land, are all indicators of incoming wet/rainy season (Table 3B). The ant exodus from their caves and the low-flying behavior of dragonflies are used by traditional weather forecasters as a clue for the upcoming rainfall (Galacgac & Balisacan, 2009). Galacgac & Balisacan (2001) also reported that the migration of honeybees predicts the onset of the rainy season. Insects are proficient at monitoring the conditions of the atmosphere since their survival is dependent on it (Galacgac & Balisacan, 2009).

CONCLUSION

Weather forecasting using traditional methods is an important indigenous knowledge that has been used by the local Tawi-Tawian people in the southern Philippines since ancient ages, handed to them by their ancestors. Atmospheric, astronomical, and biological (plants and animals) indicators were all used for traditional weather forecasting, which generally predicts the onset of the rainy season and incoming adverse weather conditions, such as typhoons and floods. The locals apply this indigenous knowledge for safe trips during fishing and sailing.

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Compliance with Ethical Standards

Authors' Contributions

ABT: Manuscript design, Drafting, Revising

AHU & AMJ: Conducted the survey, Data analysis, Drafting

JOA, JHE & WMJ: Reviewing, Editing

All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author, [ABT], upon reasonable request.

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Effects of the Combination of Treflan and Furazolidone as Therapeutants on Molting, Survival, and Growth Performance of Blue Swimming Crab Instar *Portunus pelagicus*

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ABSTRACT

A variety of antibiotics and other chemicals are used in aquaculture for various purposes, including to promote growth and reduce mortality, as well as to treat and prevent disease. As part of the larval rearing of crustaceans such as prawns and shrimp, antibiotics such as Treflan and Furazolidone are used to increase the survival rate of the post-larvae. In this study, the combined effect of Treflan and Furazolidone on the molting frequency, growth performances, and survival of blue swimming crab (BSC, *Portunus pelagicus*) instar reared in fiberglass tanks was determined. Two treatments with three replicates each were tested. Twenty (n=20) individual crab instars of uniform size (average length 1.5 ± 0.00 cm and average weight 10.33 ± 0.00 g) were stocked per circular fiberglass tanks, each with a water capacity of 200 L for 37 days culture period starting from the crab instar up to the juvenile stage. Treatment 1 corresponds to the control without the application of Treflan and Furazolidone (NTF). Treatment 2 represents the use of Treflan and Furazolidone (TF) at the dosage of 0.2 ppm and 0.3 ppm, respectively. The results showed that the growth and molting frequency of BSC instar were not affected by TF application at the end of the culture period. In both treatments, survival rates declined due to the frequent and excessive use of TF, which might be the reason for descending rates. Hence, the combined effects of TF did not enhance the molting, growth, and survival performance of BSC instars when administered as therapeutics.

INTRODUCTION

There are a number of economically important aquatic species in the Philippines, including the blue swimming crab (BSC, *Portunus pelagicus*), known locally as "alimasag" and providing family living opportunities (Camacho & Aypa, 2001; Efrizal, 2017; BFAR, 2020; Toring Farquerabao & Tahiluddin, 2022). In addition to being sold as a processed product or in a live state, BSC is mostly exported to the USA, Japan, Hongkong, and Taiwan (Camacho & Aypa, 2001; Soegianto et al., 2022). It can also be used for preparing fishery meatballs as an alternative raw material (Ajik-Cerbas et al., 2022). Philippine fisheries are dominated

by BSCs, and they are a major component of the domestic seafood industry, representing more than 90% of the catch since crab fishing began in the 1950s (Ingles, 2004; BFAR, 2013; Mesa et al., 2018). A total of 5,924 metric tons of fisheries products were produced by the BSC, constituting 0.01% of total production in 2018 (PSA, 2018). As of 2020, the retail price of BSC was 294 pesos/kg (BFAR, 2020).

A variety of gear is used in the BSC fishery, including gill nets and crab pots (Germano & Melgo, 2003; Del Norte-Campos et al., 2004; Ingles, 2004), gleaning, crab lift nets, and bamboo traps (Romero, 2009; Gadhavi et al., 2013), all of which are used by crabbers. The challenges associated with increasing population pressure and the inaccessibility of wild catching have all contributed to aquaculture has become an important part of rural livelihoods (Carleton et al., 2013). It is the fastest-growing subsector of the food industry, outpacing natural terrestrial meat production and capture fisheries (Tacon, 2001). A growing number of countries in Asia and Africa have introduced aquaculture as a culture to help rural communities escape poverty and improve living standards (Edwards, 2000). As reported by the Food and Agriculture Organization (FAO), there has been a great deal of farming of crustaceans in aquatic environments (FAO, 2022).

In response to increasing demands, the aquaculture industry has become more interested in BSC farming (Andres et al., 2010). The aquaculture potential of BSC is high because it grows rapidly (Josileen & Menon, 2005), has a relatively short larval life cycle, and has high fecundity (Romano & Zeng, 2008). It is essential to understand basic cultural conditions to optimize the production of this species as aquaculture interest increases (Romano & Zeng, 2006). In a lying-in hatchery, the eggs of BSC are held in an enclosed container and monitored until hatching occurs (Andes et al., 2010). Furthermore, antibiotics and other chemical compounds are frequently used in aquaculture as the industry becomes more popular. The use of antibiotics, fungicides, and therapeutants for the rearing of crustaceans such as shrimps, prawns, and crabs has been documented among progressive fish farmers in developed and developing countries (Uddin & Kader, 2006; Aftabuddin et al., 2009).

A wide variety of antibiotics have been successfully used in crab and shrimp hatcheries in order to prevent or treat disease, improve growth, and increase survival (Azam & Narayan, 2013). The most commonly used antibiotic in crustaceans' hatcheries such as shrimp and crabs are Treflan, Furazolidone, Formalin, Methylene Blue, and Malachite Green (Taufik, 1996; Uddin & Kader, 2006). The control of bacterial and fungal growth in crustacean hatcheries was achieved by using antibiotics such as Treflan and Furazolidone (Tareen, 1982; Baticados & Cecilia, 1988; Wijegoonawardena & Siriwardena, 2000). The antibiotic Treflan was found to kill fungi when applied to shrimp (Tareen, 1982). In addition, preventing the transmission of cholera by shrimp requires treating them with Furazolidone (Baticados & Cecilia, 1988). A number of researchers have considered the use of Treflan and Furazolidone as means for increasing the growth and survival as well as for treating bacteria, fungi, and other pathogenic bacteria which may endanger crustaceans, including shrimp and prawns (Tareen, 1982; Lio-Po & Sanvictores, 1986; Baticados & Cecilia, 1988; Chen, 1992; Nakamura et al., 1994). However, no information regarding the use of antibiotics, Treflan, and Furazolidone in crustacean crabs, especially in young BSCs. Thus, this study evaluates the combined effects of Treflan and Furazolidone on the molting, growth, and survival of BSC instar *P. pelagicus*.

MATERIALS AND METHODS

Study Site and Duration

The study was conducted at Multi-species Hatchery, College of Fisheries, Mindanao State University Tawi-Tawi College of Technology and Oceanography (MSU-TCTO), Sanga-Sanga, Bongao, Tawi-Tawi, Philippines. The duration of the study was the 37-day culture period from crab instar up to the juvenile stage.

Source and Transport of Berried Crab

Berried BSCs *P. pelagicus* were purchased from the local fishers in the coastal area of Bongao, Tawi-Tawi.

Two berried crabs having yellow eggs on its belly were selected for this study. The berried crabs were transported for about 30 minutes from the source to the hatchery using a plastic pale with sea water inside and each crab was placed in it.

Berried Crab Conditioning

As the crabs reached the hatchery, each was placed in a plastic basin for initial conditioning. After one hour in the basin provided with seawater (with salinity ranging from 26–30‰ and temperature ranging from 27–31°C) and aeration, each berried crab was treated with a formalin bath to disinfect the presence of fungi and bacteria on the belly. A 10% formalin solution was used for bathing at the rate of 150 ppm in 10 L seawater in the basin for 30 minutes (Quinitio & Parado-Estepa, 2008).

Hatching

A 200 L capacity circular fiberglass tank equipped with an aeration system was used for hatching the crabs. Sand-filtered seawater was used after treating it with 15 ppm chlorine and neutralized with sodium thiosulfate at the rate of 80% of the amount of chlorine applied and allowed aeration to take place for a 24-hour period prior to stocking the berried crab (Quinitio & Parado-Estepa, 2008). The berried crabs were stocked into the hatching fiberglass tank and took observation on the behavior of the crab and monitored the water parameter during the period of hatching. The berried crabs having yellow eggs on its belly are usually hatched three to five days in the hatchery (Allan & Fielder, 2003). As hatching happened, the mother crab was removed from the hatching tank. The newly-hatched larvae were transferred into a previously prepared larval rearing tank with a water capacity of 5 tons

Larval Rearing

The method of Quinitio & Parado-Estepa (2008) was followed for the larval rearing of BSC. A 5-ton capacity rectangular tank was used for the rearing of hatched zoea with a stocking density of 80,000 per ton. The cleaned tank was filled with 5 tons of treated seawater with subsequent aeration was fitted into the larval rearing tank at 1-meter intervals to uniformly aerate the tank. Feeding with the use of a rotifer at the rate of 30 individuals per liter of water was done twice a day. The supplementary artificial feed of # 0 feed for *Penaeus japonicus* (Grobest Feeds Philippines Inc.) was also added at a rate of 2 g/ton given twice a day. After a week of larval culture, *Artemia* sp. nauplii were introduced as live feed at the rate of 1 L per 5 tons. Harvest of crab instars was done at the end of 23 days period. Active and uniform-size (average length 1.5±0.00 cm and average weight 10.33±0.00 g) instars were selected for this study. Length-weight measurement as well as the width of the instars, was taken to determine the initial biomass for stocking.

Tank and Aeration Preparation

Calcium hypochlorite at a rate of 30 mg/L was used to clean and disinfect the inside surfaces of the tanks (Quinitio & Parado-Estepa, 2008). The hypochlorite mixture was allowed to stay for at least overnight. After rinsing, the tanks were sun-dried for at least 2 days. The aeration system was set up by connecting the tank's aeration through plastic hoses to the air pipes above the tanks. Each tank was provided with one aeration hose with an air stone attached to the submerged end of the air hose.

Seawater Treatment

Following the method of Quinitio & Parado-Estepa (2008), sand-filtered seawater was used as a culture medium and placed in the six tanks (200 L capacity each). Calcium hypochlorite was applied at a rate of 15 mg/L and allowed the mixture to stay overnight. Sodium thiosulfate was also added at the rate of 80% of the amount of hypochlorite applied to neutralize the residual content of chlorine in the water. Thereafter, continuous aeration of the tanks for 24 hours was employed. Each tank of water was ready to receive the seed stocks.

Installation of Shelter and Stocking

Artificial shelters made of black net material with a 1cm mesh size were installed in each tank. Ten net shelters with a dimension of 30×30 cm each with attached sinker were submerged into each tank to minimize cannibalism and to provide additional surface area for the crabs to attach and hide, especially

during molting. Stocking was done in the early morning with twenty active and uniform-sized BSC instars for each tank.

Experimental Design

The set-up was arranged using a completely randomized design (CRD). A total of six circular fiberglass tanks (200 L water capacity) were used. Two treatments with three replicates each were tested and evaluated. Treatment I corresponds to the control with no application of Treflan and Furazolidone. Treatment II represents the use of Treflan and Furazolidone at the dosage of 0.2 ppm and 0.3 ppm (Tareen, 1982; Baticados & Cecilia, 1988), respectively. Application of these antibiotics was scheduled after every partial water exchange, which was also done every three-day interval (Quinitio & Parado-Estepa, 2008). Fifty percent (50%) of tank water was uniformly drained from each tank and replaced with new filtered seawater to the desired original level of 200 L.

Feeding

Instars of BSC were fed twice daily at a five percent (5%) feeding rate of their total body weight. The rations were divided into two equal portions and given in the morning and afternoon, respectively. Spider conch shell meat (*Lambis lambis*) was chopped and blended into very small pieces before being fed to young crabs.

Sampling

The molting of the crab instars was monitored weekly, starting when they were stocked. This was observed by physically examining each net shelter and counting how many times they molted by looking at their appearance and by observing molted exoskeletons on the shelters. The bottom of the tank was also observed for the presence of molted crabs and their exoskeletons. A weekly sample was taken to determine the growth rate and survival rate of the young crabs. For each tank, 25% of the BSCs were measured for length and weight. Afterward, the samples were returned to their respective tanks. In addition, crab populations were counted in each tank to determine how many crabs survived. The following formulas were used to determine a specific growth rate (SGR) and survival rate (Kader et al., 2017):

$$SGR = \frac{ln\,(Wf) - ln\,(Wi)}{Days\ of\ culture} \times 100 \quad (1)$$

Where:

Wf = final weight

Wi = initial weight

$$Survival\ rate = \frac{Final\ number\ of\ stocks}{Initial\ number\ of\ stocks} \times 100 \quad (2)$$

Statistical Analysis

IBM SPSS software version 20 was used to analyze the significance of differences between the molting, growth, and survival of the two treatments using an independent sample *t*-test. This study used a 0.05 significance level. Data were presented as mean±SE (standard error).

RESULTS

Figure 1 shows the molting rate of the BSC instar. In both experimental groups, the rate of molting increased on Days 7, 14, and 21, with T1 slightly higher than T2, although there was no significant difference ($p>0.05$) in the rates between the two groups. Moreover, T1 (3.00±0.00%) was significantly higher ($p<0.05$) than T2 (1.67±0.67%) on Day 28. In addition, on Day 35, T1 (1.67±0.33%) was significantly different ($p<0.05$) than T2 (1.00±0.00%) in terms of molting rate. The specific growth rate (SGR) of the BSC instar in terms of weight is shown in Figure 2. SGR of T1 and T2 were 11.12±0.00% day^{-1} and 10.24±0.00 day^{-1} respectively. T-test showed that T1 was significantly higher ($p<0.05$) than T2. In addition, the SGR of the BSC instar in terms of length revealed that T1 (3.05±0.00% day^{-1}) significantly improved than T2 (2.35±0.00% day^{-1}) (Figure 3). Figure 4 shows the survival rate of BSC instar. The survival rate of T1 and T2 was 61.67±6.01% and 48.33±6.01%, respectively, indicating no significant difference ($p>0.05$).

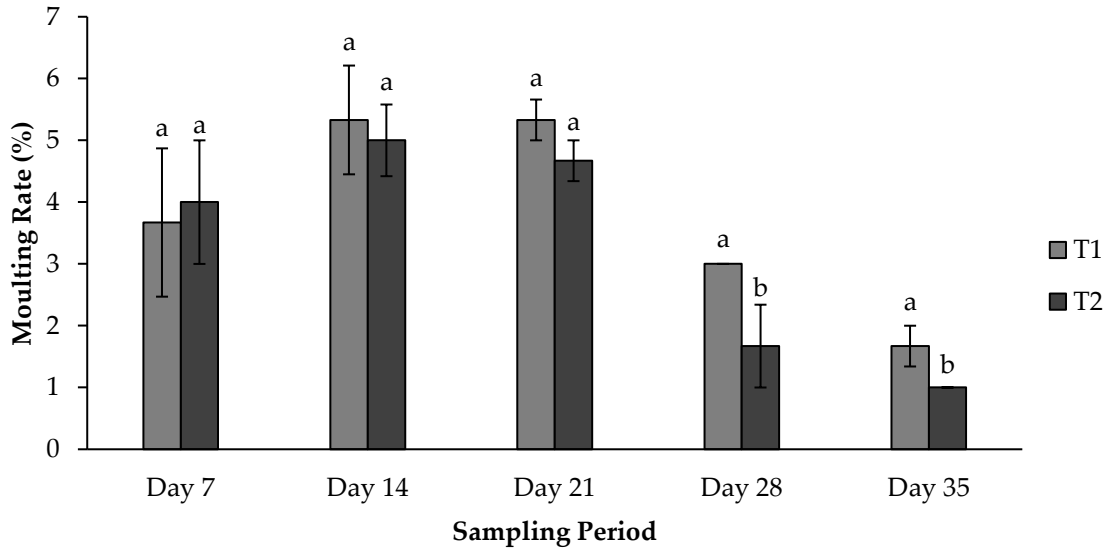

Figure 1. Molting rate of BSC instar every sampling period. T1 (No application of Treflan and Furazolidone) and T2 (combination of Treflan and Furazolidone). The bar with the different letters is significantly different ($p<0.05$). Error bars in SEM (standard error mean), n=20.

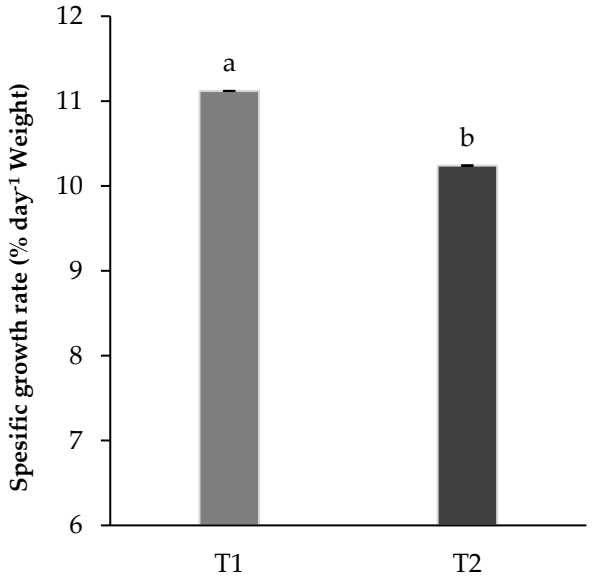

Figure 2. Specific growth rate of BSC instars in terms of weight. T1 (No application of Treflan and Furazolidone) and T2 (combination of Treflan and Furazolidone). The bar with the different letters is significantly different ($p<0.05$). Error bars in SEM (standard error mean), n=20.

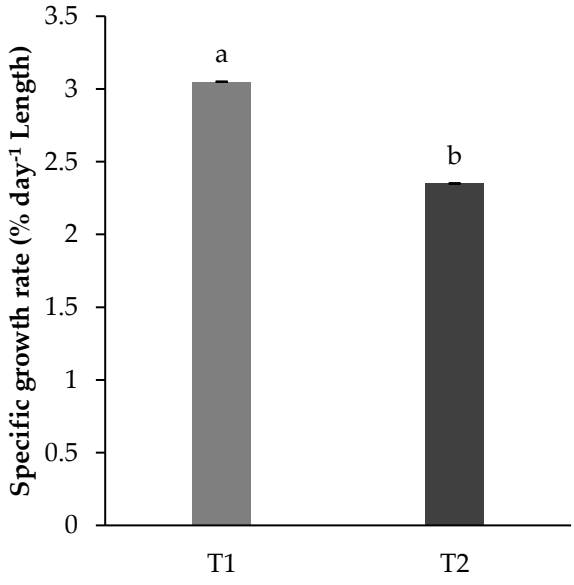

Figure 3. Specific growth rate of BSC instars in terms of length. T1 (No application of Treflan and Furazolidone) and T2 (combination of Treflan and Furazolidone). The bar with the different letters is significantly different ($p<0.05$). Error bars in SEM (standard error mean), n=20.

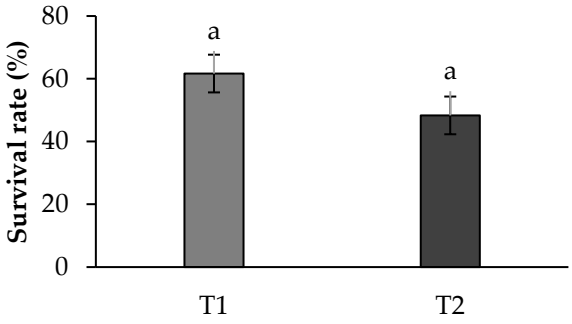

Figure 4. Survival rate of BSC instars. T1 (No application of Treflan and Furazolidone) and T2 (combination of Treflan and Furazolidone). The bar with the same different letter is not significantly different ($p>0.05$). Error bars in SEM (standard error mean), n=20.

DISCUSSION

As aquaculture has become increasingly popular, the use of antibiotics and other chemicals has become widespread. It has been found that these antibiotics, fungicides, and therapeutants are used to some extent by progressive fish farmers in both developed and developing countries for larval rearing of fin fish and crustaceans such as prawns/shrimps and crabs (Uddin & Kader, 2006; Aftabuddin et al., 2009; Tahiluddin & Terzi, 2021). In the present study, we examined the effects of combined antibiotics such as Treflan (0.2 ppm) and Furazolidone (0.3 ppm) on the growth, molting frequency, and survival rate of BSC instar. According to the results of our study, these antibiotics had no significant impact when administered as therapeutants. Other researchers have stated that the application of 0.1 ppm Treflan has a mild impact on farmed shrimp larvae growth (Chen, 1992; Nakamura et al., 1994). It has been found that Furazolidone (2 ppm) was less effective in terms of survival of the farmed shrimp; however, it was more effective against bacteria such as *Vibrio* sp. (Tareen, 1982). Comparatively, eggs, larvae, and post-larvae of crustaceans such as shrimp could be disinfected with Treflan at therapeutic levels up to 0.2 ppm, followed by complete water changes just before hatching if the water is changed completely before hatching (Lio-Po & Sanvictores, 1986; Baticados & Cecilia, 1988). The use of Treflan as an antifungal agent has been previously studied (Tareen, 1982; Wijegoonawardena & Siriwardena, 2000). Tareen (1982) found that the application of the antibiotic Treflan to shrimp killed fungi's biflagellated zoospores at 0.1 ppm. Disinfecting shrimp spawners with high concentrations of Treflan (5 ppm) followed by thorough rinsing may be effective (Gacutan, 1979; Baticados & Cecilia, 1988). Furthermore, the bacterium *Vibrio* sp., which is considered to be an essential part of shrimp microbiology, caused high mortality within postlarval and juvenile shrimp, resulting in redly pereopods and pleopods and opaque abdominal muscles (Vanderzant et al., 1971). Using Furazolidone (2 ppm) with Terramycin increased the effectiveness of the treatment against bacteria such as *Vibrio* sp. (Tareen, 1982). Imported shrimp spawners should be treated with Furazolidone to prevent cholera transmission (Baticados & Cecilia, 1988). Wijegoonawardena & Siriwardena (2000) stated that correct diagnosis and treatment are essential to an effective therapeutic treatment. Despite the fact that antibiotics enhance growth, survival, and molting, their excessive and frequent use creates and spreads antibiotic-resistant bacteria, which in turn reduces the growth rate of crab larvae and results in mass mortality of prawns (Karunasagar et al., 1994; Defoirdt et al., 2011; Azam & Narayan, 2013). In the present study, antibiotics (Treflan and Furazolidone) were combined with a high dosage, which may have adverse effects on BSCs instar. Furthermore, antibiotic treatment can lead to morphological changes in crab *Scylla serrata* larvae and juveniles, including deformities in their dorsal, lateral spines, abdominal, and rostral (Pates et al., 2017). Giant crab *Pseudocarcinus gigas* morphology has also been observed to change as a result of excessive antibiotic treatment (Gardner & Northam, 1997). In aquaculture, antibiotic residues may also be found in products. These residues might affect microbe communities, deteriorate the quality of water, and affect human health (Gräslund & Bengtsson, 2001; FAO, 2002). Although crab larvae may survive and grow better with chemicals when compared with controls, however, can suffer adverse effects with prolonged usage at high concentrations (De Pedro et al., 2007). Therefore, crab hatcheries should be careful when using chemicals. In addition to their adverse effects on

the environment and the host, antibiotics and chemicals may pose a serious threat to health risks (Gräslund & Bengtsson, 2001). Hence, the use of alternative biocontrol techniques is recommended as an alternative to eliminating these substances (De Pedro et al., 2007; Pates et al., 2017). In particular, due to their beneficial effect on the crab larvae, probiotics are recommended as a preferred prophylactic approach in instar BSC rearing.

CONCLUSION

Due to the prevalence of diseases in aquaculture food commodities, antibiotics are now used in aquaculture for a variety of reasons, including promoting growth and reducing mortality, as well as treating and preventing diseases. Among the antibiotics used in the larval rearing of tiger prawns and other shrimp are Treflan and Furazolidone. As a result of the combination of antibiotics such as Treflan and Furazolidone, Blue swimming crab *Portunus pelagicus* instars are adversely affected in terms of their growth, survival, and molting frequency. On the other hand, it may also be due to the immoderate and persistent use of antibiotics that crab weights have declined over time, resulting in descending rates. The use of specific treatments, such as Treflan and Furazolidone, and their positive reactions on other crustaceans, specifically prawns and crabs, needs to be confirmed in more similar studies.

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Compliance with Ethical Standards

Authors' Contributions

IPM: Manuscript design, laboratory experiment, draft checking, statistical analyses, writing, reading and editing.

AMA & AAE: Manuscript design, laboratory experiment, draft checking.

JHS: Statistical analyses, writing, draft checking, reading, and editing.

All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Port Competitiveness Criteria for Transshipment Container Market: A Turkish Port Industry Application

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ABSTRACT

Since the 1970s when the hub and spoke system entered commercial life, it becomes a major distribution pattern in the transshipment container market. Many feeder ports feed the mega-ports with containers, they are not large by size, but they have great capacity of being flexible, agile, and close to the shippers in the local hinterland. Although it is not enough to continuously feed the maritime transportation system with different ships, this must be continuously fed by the maritime and hinterland connection. However, the connectivity of the ports is not the only criterion to have sustainable port competitiveness for terminals. There are other criteria to be identified and measured which one is important for terminal operators and users. Therefore, this study aims to determine the criteria to be followed by container terminals and to sort them in order of importance to have a sustainable competitive advantage in the transshipment container market. For this purpose, a comprehensive literature review and a quantitative research process were carried out with container line and container terminal operators, the importance levels of these criteria were defined by the Fuzzy Analytic Hierarchy Process (AHP) method, which is a multi-criteria decision-making method. The study has not only defined competitive criteria for the transshipment market but also the opinions of both parties were compared. According to the results Port Infrastructure and Superstructure criterion is defined as the most important criterion for both parties.

INTRODUCTION

Transferring the container between the ports in the maritime supply chain is a requirement of economies of scale and commercial structure (Haralambides, 2019; Notteboom et al., 2019). A total of 197 million TEU containers transported worldwide in 2019 reached a volume of approximately 800 million TEU in ports (UNCTAD, 2019). This reveals that the transported container is transferred approximately 4 times. Container terminals are located in regions such as the China Sea, Singapore, and the Mediterranean where the transshipment traffic is heavily competed to get a share of this traffic. Competition of more than

one terminal in the transshipment regions has different advantages and disadvantages of these terminals leading the container lines to make a trade-off in port selection. As the study (Campbell & Kelly, 1994) stated in their study entitled Trade-Off Theory, "Trade-offs are at the heart of economics because neither the decision-maker nor society can have everything it wants". The trade-off theory put forward by the study (Kraus & Litzenberger, 1973) is based on choosing between funding through debt or equity by balancing the costs and benefits of each source (Notteboom & Winkelmans, 2002). In other words, businesses have an optimal financial structure, or they always want to stay close to optimal. If there is a deviation from the optimal targets, measures are taken to eliminate this deviation. The optimal level achieved should strike a balance between the ratio of earnings to losses (Singh & Kumar, 2012). The trade-off is a balancing and this balancing can be seen in different sectors under different headings and different structures. A container ship operator must consider many trade-offs when choosing the transshipment port. For this reason, the criteria that container ship lines will consider in the port selection are vital. Container terminals, which create service supply and carry out marketing activities for container ship operators, are the interface connection of sea and land transportation and an integrated transportation platform. With these features, they serve logistics, manufacturing, international trade, and information transfer as an interface for the economic development of the hinterland. Ports must serve ships and other modes of transport efficiently and effectively. Because of their importance in trade, ports play a critical role in the transfer of economic prosperity to national and international economies. In this context, the ports that want to attract transit container traffic must be directed in a strategic and correct direction to increase their competitiveness in the region.

This research aims to determine the criteria that container terminals will follow to achieve sustainable competitive advantage and to sort these criteria according to their importance. These criteria should be considered both by ports that offer service supply and by container ship operators requesting this service. In the research, a theoretical framework was first established, and a mixed research method was preferred to reach the findings.

The study first explores the port choice literature. Later the paper conducts a content analysis to categorize and sub-categorize the findings by using computer-aided content analysis software. Afterward, the Fuzzy AHP process (F-AHP) is performed over container line operators and Turkish container port terminals to determine the importance of the corresponding criterion. Finally, our research's results are compared with similar research for a superior outlook.

A Theoretical Framework: The Elements of Sustainable Port Competitiveness

Most the studies (van Dyck & Ismael, 2015; Hales et al., 2016; Parola et al., 2017) divide the elements of sustainable port competitiveness into four thematic sections, including competitiveness, business sustainability, and sustainable port competitiveness.

Competitiveness

The term "competitiveness" is defined by Porter (Porter, 1980) as the skill of companies to create goods and services using efficient methods. When the companies produce goods and services, they apply a sustainable competitive strategy to achieve long-term business. UNCTAD (1995) identifies the competitiveness of a firm as the ability to build market positions by distributing quality products or services on time and at competitive prices. This ability is the reflection of the firm flexibility to respond quickly to changes in demand and manages service differentiation by establishing the appropriate capacity and greater marketing management.

Business Sustainability and Its Relatedness with Competitiveness

Business sustainability has been continuously examined by the scholars in different aspects, including internalization, organization-specific advantages, organizational strategy and competitiveness (Kolk & Pinkse, 2008). At the same time, many scholars (Yeo et al., 2008) examined the

linkages between the competitiveness and business sustainability in different business settings, such as port industry. With regard to term "business sustainable competitiveness", the term is identified as the company's capacity to enable competitiveness all its assets in order to ensure great sustainability, profitability, productivity, and effectiveness on the long term (Herciu & Ogrean, 2018). Besides, the term "competitiveness" and "business sustainability" have found its place in many areas of strategic business management and has also been a widely studied topic in the maritime discipline. One of the main fields in this discipline is the port competitiveness and several authors (Parola et al., 2017; Notteboom & Winkelmans, 2002, Bichou, 2014) argues ports as networks where each business' success is highly connected to the sustainable competitiveness.

Sustainable Port Competitiveness

Sustainable port competitiveness is determined by many different factors, with the multidimensional use of ports and various demands related to logistics and it is mainly focused on port selection criteria from a different perspective of both port users and terminal operators (Sayareh & Alizmini, 2014). From the perspective of shipping lines, the study (Tongzon & Sawant, 2007) argued port cost and range of port services to be the only significant criteria in the port competition. A study also (Tongzon & Sawant, 2007) focused on sustainable port competitiveness for a particular region and used the following criteria to determine competitive ports: port services (quick response, zero waiting time for ships, 24 hours a day, 7 days a week service), hinterland connections, accessibility (suitability of the port at the port of destination, port congestion), availability (depth of approach channel and water in the dock, port information systems and the scope of application versatility, consistency of port workers), logistics costs (inland transportation costs, the entry of the ship and cargo into the port costs, time given for free storage at the terminal), becoming a regional hub (accessibility to the port, deviation distance to major routes) and connectivity with other ports (land distance and connectivity to key load holders, effective internal transfer network). Besides, the hinterland proximity and connectivity improve the hub port competitiveness by feeding ports with inland transport networks (Parola et al., 2017). In transshipment hub ports, port service quality (i.e., berth availability) was found important criterion in hub and spoke networks (Kavirathna et al., 2018). Another research study (Yuen et al., 2017) the hub port competitiveness in West European ports, considering ship frequency, port costs, transit time, and service quality. A different study (Wang, 2011) examined the various port services under the topic of the service factors. The study (Yap et al., 2006) affirms that port location, feeder services, and intermodal connections, the size of the hinterland, and port efficiency are critical transit port selection criteria in Western Europe. Similar to this study, the factors affecting port competitiveness were defined as cargo volume in the port, the facilities owned by the port, the geographical location of the port, and the level of service provided, while a study (Vaidya & Kumar, 2006) focused on the port's connectivity with other ports. A study (Robinson, 2002) analyzed variables such as ship types, total handling, ship frequency, and frequencies of ship lines with a linear model developed. A scholar (Tiwari et al., 2003) used suitable geographical location, port costs, suitable infrastructure, high port efficiency, a wide range of port services, and port connectivity with other ports as port selection criteria. While the study (Bichou, 2014) developed a theoretical framework on port selection criteria, firstly it formed three main categories and classified them under these categories. These categories are route factors (location, accessibility, port connection, backyard network, frequency, and transit time), cost factors (freight rates, tariffs, and capacity), and service factors (congestion, reliability, flexibility, safety, and security). In their study, physical criteria (sufficient water depth, the capacity of port facilities, number of docks, port location, ship control, and port technology) and service criteria (port working time, port tariffs, port security, port entry, operating cost, international policies, night navigation, port management, port workers, customs formalities) are the most important port selection variables (Saeed, 2009). Regarding the study (van Dyck & Ismael, 2015), operational efficiency in port is connected to the port size, that is,

bigger ports are more efficient than smaller ones due to the quality of port infrastructures, storage, and cargo handling. On the other hand, a study (Akbayirli et al., 2016) list the port selection criteria as "port location and hinterland connections, port physical and technical infrastructure and superstructure, port management and administration, port service quality, port efficiency, and productivity, port tariffs and costs, and the number and frequency of ships belonging to container carriers". In recent years, dimensions such as "Corporate Social Responsibility" and "Alliances made by container line operators" have been added to the existing criteria (Watson et al., 2012).

Although many criteria have been evaluated in these studies, they are in fact one-dimensional. All studies have separately focused on either port users or terminal operators for sustainable port competitiveness. For example, studies (Parola et al., 2017; Notteboom & Winkelmans, 2002, Bichou, 2014) examining transit ports have mainly evaluated the port competitiveness adjacent to each other in terms of port users. Briefly, there is no study has been found that simultaneously examines the factors affecting port competitiveness in terms of neither port users nor terminal operators. A new study (Munim, 2022) states that relevant studies focusing on transshipment port's competitiveness are very limited while the availability of the studies from the port users' perspectives. Finally, the major criteria concerning sustainable port competitiveness are listed as port services, hinterland connectivity, nautical accessibility, operational efficiency, maritime connectivity, hinterland proximity, route factors, cost factors, service factors, port location, port physical and technical infrastructure and superstructure, port management and administration, the port service quality, port efficiency, and productivity, others.

METHODOLOGY

This research aims to determine and prioritize the criteria that container ports will be suggested to follow the sustainable competitive advantage. For the research, a mixed-method regarding port selection criteria was preferred. In the first stage of the study, a systematic literature review was carried out and the criteria were determined. Then these criteria, which were determined by qualitative research, were prioritized by a "multi-criteria decision-making method", Fuzzy AHP (Saaty, 2008).

Qualitative Research Process

Since systematic literature reviews are conducted meticulously and systematically, they are regarded as original studies (Rother, 2007). They (Sayareh & Alizmini, 2014) state that such studies consist of four stages, these are collecting data (1), giving descriptive statistics (2), examining categories (3), and evaluating data (4). The first sample was obtained during the data collection phase. These studies have been eliminated according to certain criteria, as can be seen in Tables 1 and 2. Besides, certain constraints/criteria (internal and external) have been introduced to ensure that existing studies in the literature are excluded from or within this research, and the final sample has emerged. In the next stage, descriptive statistics about chronological studies based on countries and research types were prepared. The selection of the categories was made in line with the theoretical framework and the data were reduced to consolidate the categories.

Collection of Data

A literature review strategy was developed using electronic databases, time flow, and keywords. This literature review was carried out in February 2020 by scanning EBSCO Host, ProQuest, Scopus, Web of Science, ULAKBİM, and Microsoft Academic databases. These databases were determined in terms of ease of electronic access and reliability of data in social sciences. Also, library scanning was carried out to enrich the literature review. Studies of many authors researching sustainable port competition (Liou & Wang, 1992; Song & Yeo, 2004) were scanned by using "business sustainability", "port competitiveness", "port selection", "container terminal selection", "transit market", and "sustainable hinterland" keywords (Table 1).

After the stage of determining the keywords, a clear search sequence ("business sustainability" AND "port competitiveness" OR "port selection" OR "container terminal selection" OR "sustainable hinterland" AND "transit market") needs to be created. The results of the search string determined have been reached 35 papers.

Table 1. Literature review strategy in data collection process

#	Databases	Restrictions				Results
		Search Area	Document Type	Time Range	Language	
1	EBSCO Host	Theme	Article	From all years to today	English	210
2	Proquest	Theme, Title, Abstract, Keywords	Article and/or critics	From all years to today	English	110
3	Scopus	Theme, Title, Abstract, Keywords	Article and/or critics	From all years to today	English	75
4	Web of Science	Theme	Article	From all years to today	English	52
5	Microsoft Academic	Theme	Article	From all years to today	English	33
6	ULAKBİM	Theme	Article	From all years to today	Turkish	10
First sampling						**490**

Table 2. Constraints used in literature review

Internal Constraints	External Constraints
Full-text studies published in scientific journals	Non-academic writings and reviews
Empirical studies	Interviews
Doctoral theses	Book reviews
Scientific articles in English	Conference summaries
In-field studies on port and sustainability in social sciences	Out-of-field studies other than social sciences
Theoretical studies	Works written in languages other than English
Case Studies	
Notes are written to the editor	
Congress papers presented in full text	

The most important point in the systematic literature review is the clear definition of the constraints (internal and external) that will keep existing studies in or out of this research. These constraints in Table 2 facilitate the elimination of non-academic, non-field, and different languages.

Descriptive Statistics

In this section, the frequency distribution technique is used in the analysis of the studies that make up the sample of the study. The frequency distribution technique is very useful for researchers. With this technique, formal features that can form the basis of content analysis can be presented (Seuring & Gold, 2012). In the descriptive statistics section, publication years of the publications, and research of the authors (empirical, theoretical, or conceptual) are examined. At the same time, the sectors and fields in which the authors work are included in this analysis. The descriptive statistics section will be presented after the creation of the main categories and subcategories.

Determination of Main Categories

In terms of the theoretical framework of the study, (1) port services, (2) hinterland connectivity, (3) nautical accessibility, (4) operational efficiency, (5) maritime connectivity, (6) hinterland proximity, (7) route factors, (8) cost factors, (9) service factors, (10) port location, (11) port physical and technical infrastructure and superstructure, (12) port management and administration, (13) the port service quality, (14) port efficiency and productivity, (15) others are the main criteria divided into main

categories. The main categories belonging to the studies that are listed according to the purpose of the study and do not provide enough information from different sources are not included in the study.

Data Analysis

The data analysis phase is the last part of the systematic literature review and consists of three qualitative steps, these steps are (1) data reduction, (2) data display, and (3) conclusion (McHugh, 2012).

While there is enough analytical framework in the deductive approach to move towards content analysis, the inductive nature of the analytical framework requires that the analyzed data be summarized under predefined themes (McHugh, 2012). Thus, the data is gradually reduced and intensified (Eisenhardt, 1989). In this study, a two-stage process development approach is proposed in creating an analytical category pattern: in the first stage, after creating the basic framework of categories and sub-criteria based on the existing theory, all categories were consolidated in the coding process. In the continuation of the data reduction, the iteration cycle method was used for coding the categories of sub-criteria.

As a result of this process, eight main categories and 53 subcategories were formed. In particular, the most studied sub-criteria were determined and sub-criteria that had a little or similar contribution to the theory were excluded from the analysis. Thanks to the work done here, all sub-criteria in the main categories were compared, and similar sub-criteria were collected under the same main category, and the data reduction phase was completed. In the conclusion stage, 53 different sub-criteria whose iteration process was completed were determined as sub-categories. For example, the route factor in the study (Bichou, 2014) was not used in this study, (1) land distance to cargo owners, (2) effective internal transfer network, (3) port-road connection, (4) port-rail connection, (5) the origin of the load and its distance from the destination and (6) as sub-criteria of location are consolidated under the main category of connectivity with ports **(**Table 3).

RESULTS

Descriptive Statistics

The first step used to summarize the data and interpret the research results on the eight main categories determined in Table 4 is descriptive statistics. In other words, descriptive statistics covers the process of statistical compilation, collection, summarization, and analysis of data. In the study, several questions have been identified to decide what the descriptive statistics issues will be:

(1) What is the distribution of publications regarding port selection criteria over time?

(2) What is the distribution of the publications regarding the port selection criteria according to the journals in which they are published?

(3) What is the distribution of publications regarding port selection criteria on a country basis?

(4) What types of analysis methods have been applied in the studies?

When attention is paid to time distribution in studies, it has been observed that studies on port selection criteria have increased in recent years. This increase has become more evident especially since 2018 and concentrated on different journals (Figure 1).

When studies are analyzed by journal names, it is seen that high-impact sea transportation journals such as “Maritime Economics & Logistics” and “Maritime Policy & Management” are at the forefront. For example, the impact factor of “Maritime Economics & Logistics” journal is 1.661 for 2018, and the impact factor for “Maritime Policy & Management” is 2.5. The fact that the impact factor of the journals is greater than 1 indicates that the journal’s contribution to the field is satisfactory (Baştuğ et al., 2013). Apart from the transportation magazines, SSCI and SCI - Expanded indexed journals belonging to different fields have been determined to work on port selection criteria. The distributions of these studies are presented in Table 4.

Table 3. Main category layout resulting from data reduction

#	Main Categories	Subcategories
1	Hinterland connections	Experts and trained workforce
		The size and activities of free zones in the port hinterland
		Cargo Volume
2	Port location and accessibility	Suitability of the dock at the port of destination
		Port congestion
		Ship waiting times
		The number of ships making calls to the port
		Ship frequencies
		Transit time for cargoes
		Accessibility to the port
		Deviation distance to main routes
3	Convenience	Approach channel and water depth at the dock
		Port information systems and versatility
		Consistency of port workers
4	Connectivity with ports	Land distance and connectivity to cargo owners
		Effective internal transfer network
		Port location
		Port-road connection
		Port-rail connection
		The distance of cargo from origin and destination
5	Cost factors	Freight rates
		Tariffs and capacity
		Inland transportation costs
		Costs related to the entry of the ship and cargo into the port
		The time allowed for free storage at the terminal
		Flexibility in prices
		Ease of payments
		Total logistics costs due to port preference
6	Port Infrastructure and Superstructure	Port storage area size
		Number of port equipment
		Quality and technology of port equipment
		Cooled storage area of the port
7	Port management and administration	Management type
		Feature of port management
8	Service factors	Value-added services at the port
		Reliability offered in port services
		Social responsibility
		Green port applications
		Logistics services at the ports
		Flexibility in port services
		Special services
		Providing customers with information about the installation
		Port performance related to cargo losses and damages
		Port security
		Behavior, attitudes, and competencies of port staff
		Quick reply
		24 hours a day, 7 days a week service
		Zero waiting time service

Table 4. Distribution of studies according to journals

	Journal Name	#
1	Maritime Economics & Logistics	8
2	Maritime Policy & Management	4
3	Transport Reviews	3
4	The Asian Journal of Shipping and Logistics	3
5	Int. Journal of Physical Distribution & Logistics Management	1
6	Journal of ETA Maritime Science	1
7	Applied Economics	1
8	Journal of Shipping and Trade	1
9	Energy Policy	1
10	Marine Policy	1
11	Journal of Global Business and Social Entrepreneurship	1
12	Planning Perspective	1
13	Transportation Research Part D	1
14	Transportation	1
15	Transportation Research Part A	1
16	Energy Policy	1
17	Research in Transportation Business & Management	1
18	Sustainability	1
19	EconStar	1
20	Utilities Policy	1
21	Transport Policy	1
22	Thermal Science	1

In studies, China has seven (7) scientific types of research. After China and Turkey, developed economies have a significant proportion of work. In research, especially in Western Europe and Asia effect can be seen (Figure 2).

Since it does not require sampling, facilitates the comparative analysis, and is easy to repeat, case analysis (Kavirathna et al., 2018) is mostly preferred. Mathematical modeling and analytical hierarchy process analysis, which are frequently encountered in the studies on decision-making processes in social sciences, have also been identified as the most used analysis methods. However, the SWOT analysis method was preferred in studies that are very easy to perform and require basic analysis (Figure 3).

Content Analysis

In this section, content analysis was carried out on the articles that make up the sample, and the categories that were studied the most were determined. "NVIVO 12.0 Qualitative Content Analysis Software" was used to analyze the data in the sample. In content analysis, themes and clusters are the main categories and sub-categories found as a result of a systematic literature review. The articles were converted into digital texts with NVIVO software for analysis.

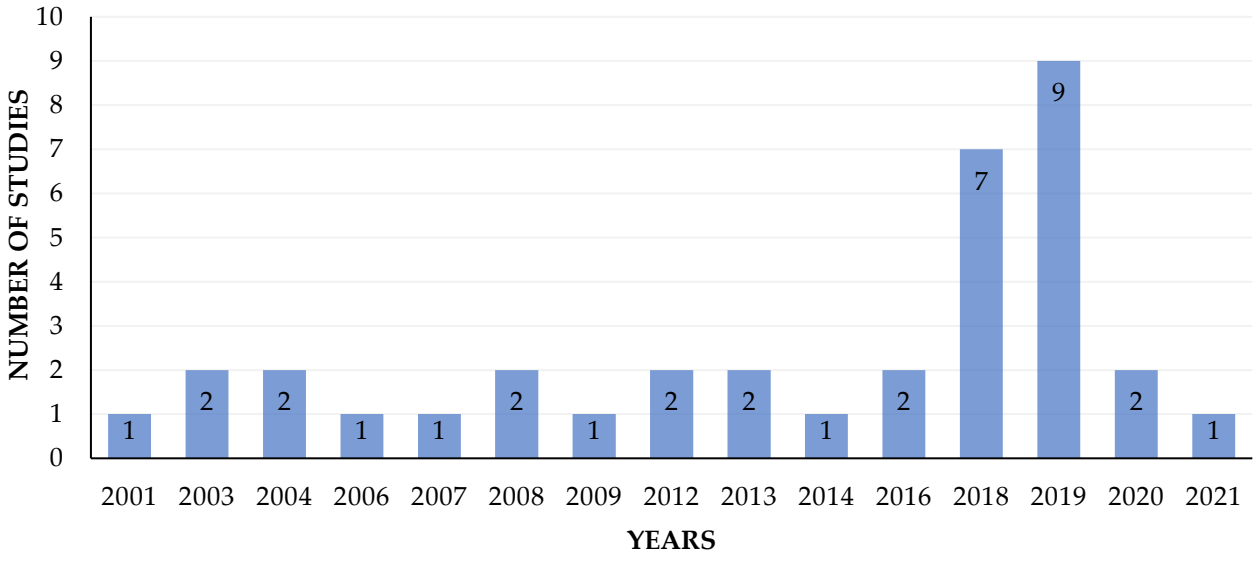

Figure 1. Annual distribution of studies on port selection criteria

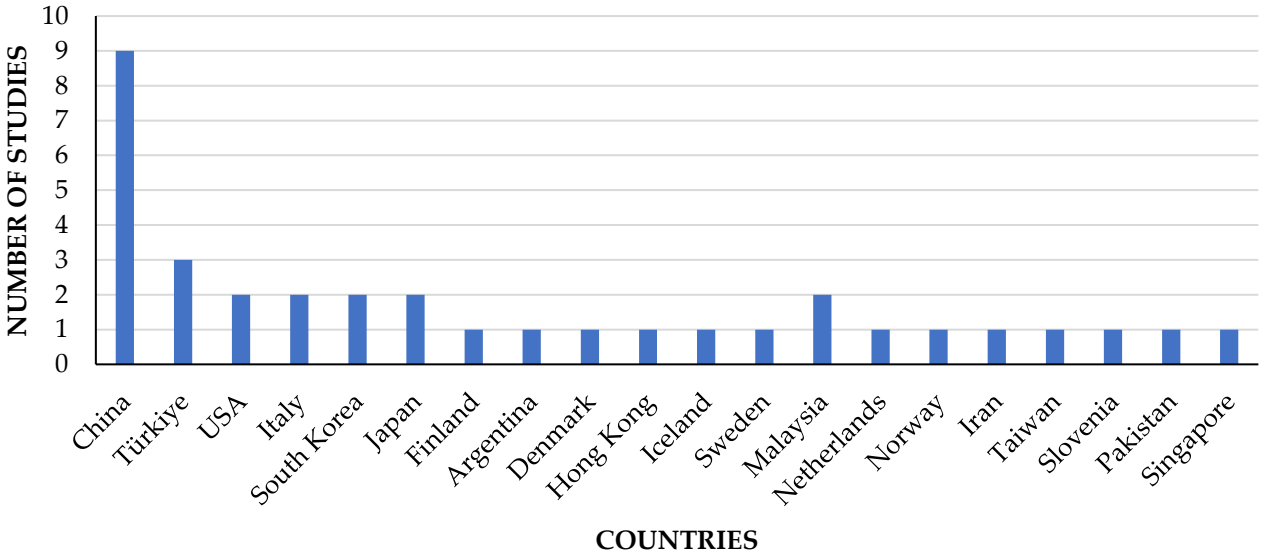

Figure 2. Territorial distribution of studies on port selection criteria

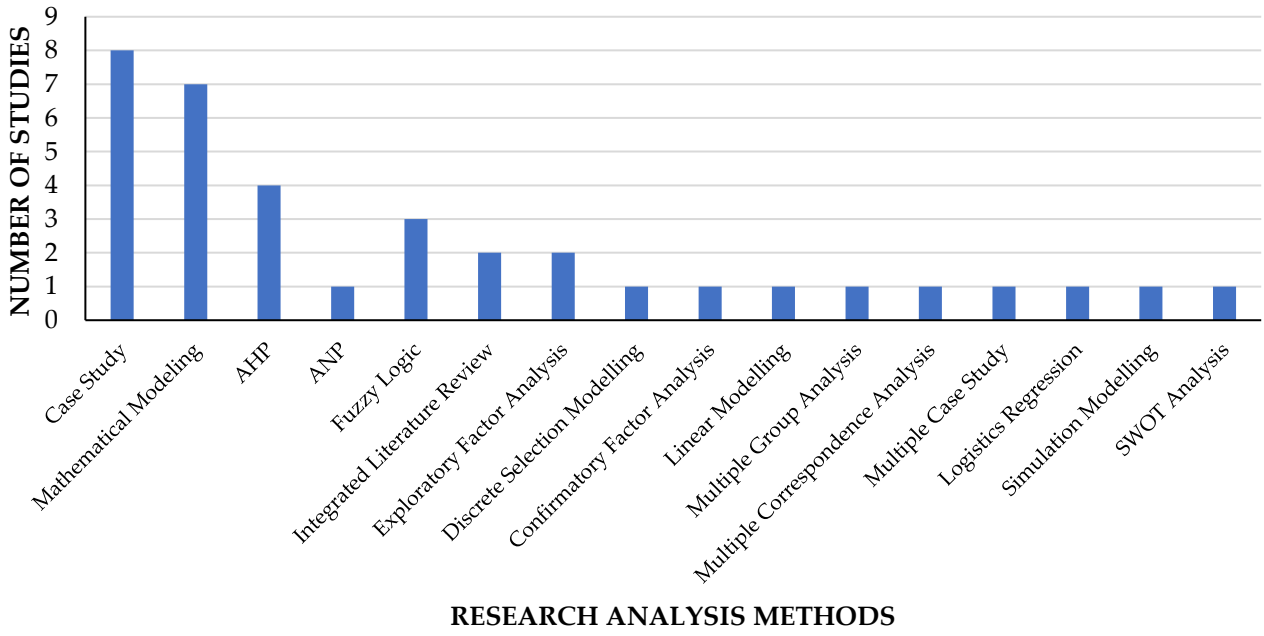

Figure 3. Distribution of Studies on port selection criteria according to research analysis methods

In traditional content analysis, the research team creates a coding scheme and trains the coders before analyzing the content. Scholars have evolved numerous algorithms and software to reduce subjective interpretations among coders (Krippendorff, 2018). Thanks to the software, a frequency table can be created, and content analysis can be performed on similar documents many times.

To increase reliability among encoders, two coders were chosen from professionals who previously attended related studies and were trained to code. As a result of comparing the coding of the two encoders, it was observed that the Kohen-Kappa coefficient scores were not less than 0.65. This shows that there is an agreement between the coded texts (Mayring, 2000). If both encoders have reached full agreement on what content to encode, the Kappa coefficient is 1. If there is no agreement between the two coders (except for random events), the Kappa coefficient is ≤ 0. The value between 0 and 1 suggests a partial agreement. In this case, it is seen that part of the study has been agreed upon.

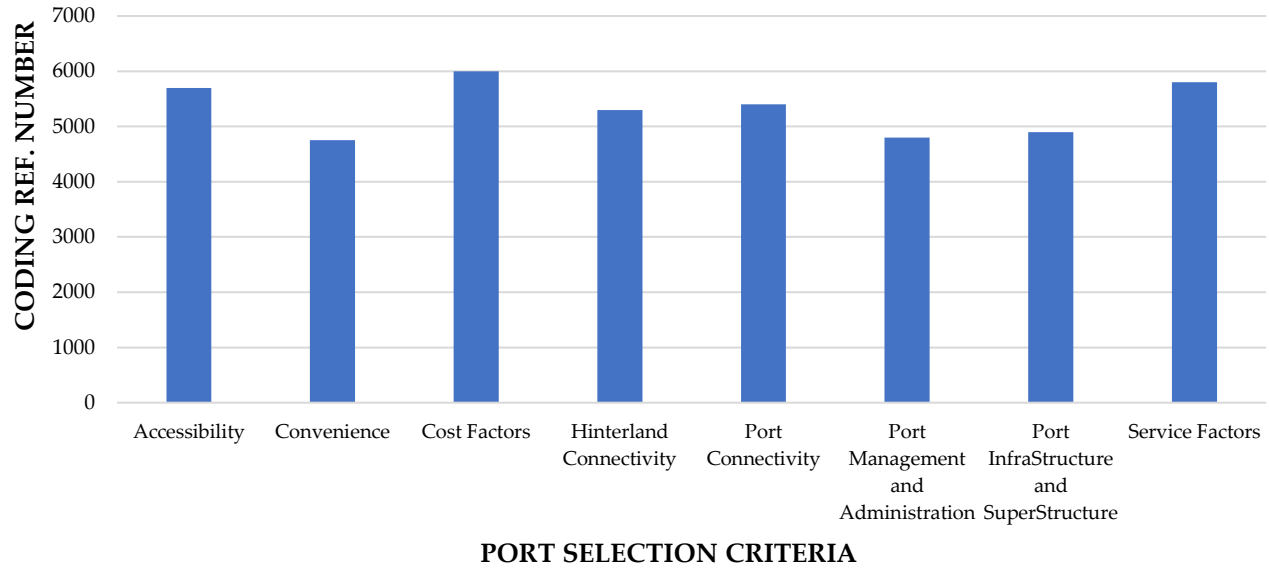

Figure 4. Coding frequencies of main criteria for port selection criteria (According to coding ref. number)

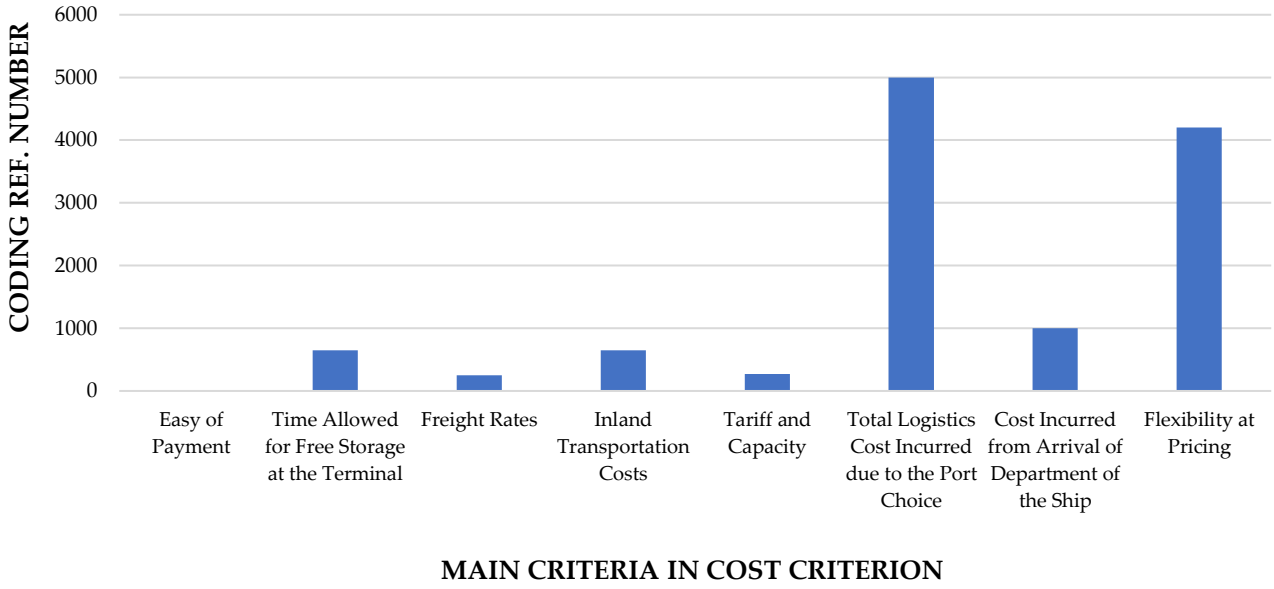

Figure 5. Coding frequencies of main criteria for cost criterion (According to coding ref. number)

For the research, matrix coding was performed for checking the relationships between the categories. Matrix coding queries are accustomed to define the scope of the main categories and concepts to make numerous comparisons between concepts and cases. Particularly, matrix encoding can match various collections of keywords in categories. They display information in Tables about the item pair corresponding to each row (for example, clusters, category-coded references, or percentage of corresponding coded data). Matrix encoding can query data like frequency, duration, encoding references, row, and column percentages, and encoding rate. In this study, coding references were accustomed to match coding differences between categories. Coding references represent a context of keywords about information collected from data sources such as focus groups, internet pages, interviews, scientific articles, social media messages, or surveys. Special words and characters can be used as operators in matrix coding.

Qualitative Research Findings

Content analysis of port selection criteria (Figure 4) shows that cost factors are the most studied topic in the 30 years between 1990 and 2020. The port operator,

which wants to create better customer value, must reduce its costs to have an advantage in the market against its competitors. The decrease in costs is very effective in determining the price. Many studies examined in the research (Tongzon & Sawant, 2007; Esmer, 2011) emphasize that pricing strategies should be strategic due to the high cost of port investments and the length of return on investment.

The most studied subject among cost factors is the total logistics costs arising from the port preference and the flexibility in prices (Figure 5). Logistics costs due to port preference are stated in the literature as THC (terminal handling cost), LCL (less than container load), documentation, handling, storage costs, and port taxes. Also, static or dynamic methods are used to calculate port logistics costs in these studies.

On the other hand, port pricing strategies can be analyzed in three categories (Frankel, 1987; Esmer, 2011): cost-based pricing strategies, value-based pricing strategies, and competition-based pricing strategies. Findings show that cost-based pricing strategies are the most studied and produced information criterion in the 30 years.

In studies conducted on port selection criteria, it was determined that the suitability category was the least studied criterion. The scarcity of studies on port workers and the innovation-oriented development of new technologies (especially Industry 4.0) in port management down the coding reference of this criterion.

Quantitative Research Process: Fuzzy AHP

The systematic literature review is ideal to ensure the first outlook into a phenomenon; however, it does not provide which criteria are to be rank-ordered from most important to least important. Hence, Fuzzy AHP was applied to find the importance of weights for the selection criteria.

The AHP method was first announced by a study (Robinson, 2002). The method is referred to as "a theory of measurement owing to pair-wise collations and depends on the judgments of professionals to evolve priority scales" (Robinson, 2002). Thanks to its ability to handle multiple qualitative and quantitative criteria, it is widely used in different fields including personnel selection, energy alternative selection, performance evaluation, job selection, factors influencing maritime transportation, and port selection and competition studies (Eisenhardt, 1989; Tongzon & Sawant, 2007; Seuring & Gold, 2012; Herciu & Ogrean, 2018; Krippendorff, 2018; Munim et al., 2022).

However, the AHP method, as Saaty (2008) first developed, has been criticized by many authors for not fully reflecting human thoughts (Durán & Aguilo, 2008). Since the pairwise formulation of the AHP contains explicit numbers from 1 to 9, scholars are not able to convey their thoughts to this certainty (Kim, 2016). At this point, fuzzy logic supports the AHP to achieve a comprehensive judgment for persons in charge of decision-making (Sarfaraz et al., 2007). Like many other studies, Fuzzy AHP is implemented in port selection studies as well (van Dyck & Ismael, 2015; Balci et al., 2018).

In the study, an analysis (Chang, 1996) has used and crisp numbers were converted into triangular fuzzy numbers. Afterward, the fuzzy numbers had created the comparison matrix and had calculated the synthetic values, extent analysis method was used. Afterward, defuzzification was performed regarding the study of (Liou & Wang, 1992). Finally, the weight of each criterion was calculated.

The AHP survey consists of a total of 8 criteria (ease of payment, the time allowed for free storage at the terminal, freight rates, inland transportation cost, tariff and capacity, total logistics cost incurred due to the port choice, costs incurred from arrival and departure of the ship, flexibility at pricing) collected from the interviews. The criteria were discovered to be comprehensive by the systematic literature review and three industry experts.

After identifying the criteria, a questionnaire was developed for the pairwise comparisons of the eight substitutes. As the study (Robinson, 2002) suggests, the 9-point comparative scale was used in the form. The studies of (Balci et al., 2018) and (Lirn et al., 2004) were preferred in the design of the comparison questions.

A cover letter has been attached to the first page of the questionnaire including instructions regarding the completion of the questionnaire. The cover letter has stated that general/deputy managers and department managers should complete the questionnaire to provide robust findings regarding their decision-making choices. Afterward, the questionnaire which consists of two sections has been prepared. In the first section of the questionnaire, the profile questions have been asked to the respondents. The second part consists of a pairwise comparison of the variables collected from the literature review.

In Table 5, it is seen that the total number of respondents was 20 and the survey was collected between May and June 2020. 10 of the survey participants are container terminal operators and the rest are container line operators (see Table 5). While container terminal operators are selected among competing container terminals that handle transshipment containers in the Eastern Mediterranean region, container ship operators have been selected from the ship lines that make calls to these ports.

The respondents were selected using judgmental sampling whereby interviewees are chosen based on the researcher's knowledge and judgment. Container terminal operators were chosen from container terminals in the Eastern Mediterranean region, whereas container line operators were chosen from the shipping lines calling at those ports. 40 questionnaires were back in April 2021, corresponding to a 65% response rate. In Table 5, both sets of respondents were key decision-makers in top management roles, having had long experience in the industry, with 12 managers having between 11-18 years of experience, and 8 having between 7-10 years' experience.

Quantitative Research Findings

The fuzzy comparison matrix is developed as shown in Table 6 and the fuzzy synthetic values are reached by using the extent analysis method (Chang, 1996).

The fuzzy synthetic value calculation for the container terminal selection criteria are as follows:

S_{HC} = (0.0416, 0.0592, 0.0860)

S_{PLA}= (0.1044, 0.1520, 0.2212)

S_{C} = (0.0395, 0.0564, 0.0840)

S_{CWP} = (0.0749, 0.1058, 0.1515)

S_{CF} = (0.1275, 0.1846, 0.2644)

S_{PIAS} = (0.2000, 0.2897, 0.4134)

S_{PMAA} = (0.0424, 0.0606, 0.0886)

S_{SF} = (0.0631, 0.0917, 0.1335)

Table 5. Profile of respondents

Container Terminal Operator			**Container Line Operator**		
#	**Position**	**Years of Experience**	**#**	**Position**	**Years of Experience**
1	CSR	10	11	Sales Vice-Manager	11
2	Agency Manager	17	12	Port Manager	16
3	CSR	10	13	General Manager	8
4	Agency Manager	9	14	Operations Director	18
5	Marketing Manager	13	15	CSR (Senior)	9
6	Agency Manager	12	16	Vice-Sales Manager	11
7	CSR	16	17	Marketing Manager	14
8	Agency Manager	8	18	General Manager	15
9	CSR	14	19	Marketing Manager	10
10	Marketing Manager	11	20	General Manager	9

Note: CSR: Customer Service Representative

Table 6. Fuzzy table comparison matrix

	Hinterland connectivity (HC)	**Port location and accessibility (PLA)**	**Convenience (C)**	**Connectivity with ports (CWP)**
HC	**(1.00, 1.00, 1.00)**	(0.32, 0.38, 0.44)	(0.87, 1.09, 1.34)	(0.72, 0.83, 0.96)
PLA	(2.26, 2.66, 3.08)	**(1.00, 1.00, 1.00)**	(3.07, 3.87, 4.65)	(1.26, 1.53, 1.91)
C	(0.75, 0.92, 1.15)	(0.21, 0.26, 0.33)	**(1.00, 1.00, 1.00)**	(0.56, 0.70, 0.87)
CWP	(1.04, 1.20, 1.39)	(0.52, 0.65, 0.80)	(1.15, 1.43, 1.78)	**(1.00, 1.00, 1.00)**
CF	(2.94, 3.65, 4.44)	(0.92, 1.18, 1.53)	(2.44, 3.07, 3.67)	(1.10, 1.39, 1.66)
PIAS	(4.25, 5.40, 6.49)	(1.91, 2.45, 3.08)	(3.33, 4.34, 5.28)	(2.42, 2.90, 3.43)
PMAA	(0.98, 1.21, 1.51)	(0.43, 0.54, 0.70)	(0.73, 0.91, 1.12)	(0.36, 0.44, 0.55)
SF	(1.12, 1.51, 1.97)	(0.45, 0.57, 0.72)	(1.60, 2.13, 2.66)	(0.54, 0.64, 0.77)
	Cost factors (CF)	**Port infrastructure and superstructure (PIAS)**	**Port management and administration (PMAA)**	**Service factors (SF)**
HC	(0.23, 0.27, 0.34)	(0.15, 0.19, 0.24)	(0.66, 0.83, 1.02)	(0.51, 0.66, 0.89)
PLA	(0.66, 0.85, 1.08)	(0.32, 0.41, 0.52)	(1.42, 1.85, 2.31)	(1.40, 1.77, 2.23)
C	(0.27, 0.33, 0.41)	(0.19, 0.23, 0.30)	(0.90, 1.10, 1.38)	(0.38, 0.47, 0.63)
CWP	(0.60, 0.72, 0.91)	(0.29, 0.34, 0.41)	(1.81, 2.25, 2.81)	(1.30, 1.57, 1.85)
CF	**(1.00, 1.00, 1.00)**	(0.50, 0.60, 0.72)	(3.23, 3.98, 4.72)	(1.55, 1.92, 2.38)
PIAS	(1.39, 1.67, 2.00)	**(1.00, 1.00, 1.00)**	(2.97, 3.70, 4.54)	(2.48, 3.03, 3.63)
PMAA	(0.21, 0.25, 0.31)	(0.22, 0.27, 0.34)	**(1.00, 1.00, 1.00)**	(0.53, 0.63, 0.77)
SF	(0.42, 0.52, 0.64)	(0.28, 0.33, 0.40)	(1.30, 1.57, 1.88)	**(1.00, 1.00, 1.00)**

Table 7. The overall importance of criteria

Criteria	**Weight**	**Rank**
Hinterland connectivity (HC)	6.2%	7
Port location and accessibility (PLA)	15.9%	3
Convenience (C)	6.0%	8
Connectivity with ports (CWP)	11.1%	4
Cost factors (CF)	19.2%	2
Port infrastructure and superstructure (PIAS)	30.1%	1
Port management and administration (PMAA)	6.4%	6
Service factors (SF)	9.6%	5

Table 8. Respondent groups' comparisons

Criteria	Line Operators		Terminal Operators	
	Weight	Rank	Weight	Rank
Hinterland connectivity (HC)	8.6%	6	4.3%	8
Port location and accessibility (PLA)	21.7%	2	11.5%	3
Convenience (C)	6.8%	7	5.1%	7
Connectivity with ports (CWP)	11.8%	4	10.0%	4
Cost factors (CF)	17.3%	3	20.5%	2
Port infrastructure and superstructure	25.7%	1	34.0%	1
Port management and administration (PMAA)	4.5%	8	8.8%	6
Service factors (SF)	9.8%	5	8.9%	5

In whole tables, the weight of criteria and rankings were listed. All criteria have been also named with capital letters. While the results obtained from all the collected questionnaires are shown in Table 7, the answers of the respondent groups are also analyzed separately in Table 8. The overall results prove that the most important criterion is Port infrastructure and superstructure, followed by Cost factors, Location, and Port location and accessibility. The least and second least important criterion are the Conveniences of port services and Hinterland connectivity (Table 7).

On the other hand, container line and container terminal operators generally made different evaluations with their perspectives (Table 8).

While the Port Infrastructure and Superstructure criterion is still defined as the most important criterion for both parties, the Convenience criterion is defined as the second most insignificant criterion. Apart from these two criteria, there were differences in other criteria. While the Port Location and Accessibility criterion are determined as the second most important criterion according to container terminal operators, this criterion is defined as the Cost Factor for container terminal operators. Among the eight criteria, the Port Management and Administration criterion was the most unimportant criterion for line operators, while terminal operators determined hinterland connectivity as the least important criterion.

DISCUSSION

This research is based on (1) qualitative method and (2) quantitative method. In the view of a qualitative study, a study has performed a deductive-based content analysis to find out the most important determinant of the sustainable port competitiveness in the literature, and secondly, the study has checked the accuracy of content analysis findings by performing Fuzzy AHP methodology. At the first stage, the study found that "total logistics costs incurred by port choice" are the most important determinant of the cost in the relevant literature. A study (Chou, 2009) suggests the fact that shippers concentrate on decreasing all logistics costs, and not only the inland costs, which was neglected by prior studies. Shippers prefer using the nearest port as it takes lower logistics cost, transport time, and less cargo damage. Historical studies showed cost-related determinants as the most important criteria for shippers; a port's infrastructure did not carry much significance (Mittal & McClung, 2016). However, this has changed dramatically over the years. A study (Cullinane et al., 2004) found the efficiency of the port infrastructure influences a shipper's port choice decision. From this research, there is no further study observed so that the cost determinant is not the most important compared to the port infrastructure. From the port operator perspective, a study (Kavirathna et al., 2018) proves that the berth availability is the most significant criterion for the transshipment ports. In many articles, berth availability is the one of the elements for the port infrastructure. Feeder market needs high circulation of vessels to avoid any delay in the line operator's schedule. Otherwise, the ports should face big penalties for jeopardizing performance guarantees of the transshipment ports in the hub and spoke system. A study (Pham et al., 2019) also confirms that terminal

accessibility is the most important criterion for the transshipment port from the perspective of line operators. A study (Yeo, 2010) confirms that, the selection decision on transshipment hub port has become more complex because of the competitive offerings at the same time. Hence, a great competition between hub ports in neighboring regions have supported the hub-hopping nature of the container shipping sector. Although port infrastructure investments are extremely expensive and long-term projects, they play an effective role especially in sustaining the transshipment port competitiveness. To ensure sustainable competitiveness in shipping, many carriers have invested in mega-container ships, which dictates extraordinary operational challenges to the port industry. Especially, they require deeper terminal water and channel as well as longer quays and larger terminal areas and they also mean bigger container cranes, wider storage space, and a more developed logistics infrastructure. These provisions become mandatory for those ports to keep their market shares and defend their competitiveness. The scholars (Cullinane et al., 2006; Guy & Urli, 2006; Hales et al., 2016) mentioned that capacities and efficiencies of the port infrastructure and superstructure are of particular importance to carriers and shippers because ports operate during peak and off-peak periods. Another challenge for sustainable port competitiveness is the direct calls of container operators. There are constraints to the depth of the port access channel and the draft of the container berths, which means any type of ship can call when fully loaded. This study proves that the criterion of the port infrastructure and superstructures is really important for both sides. Huge container carriers require bigger terminal areas, wharfs, cranes and therefore, the infrastructure limits the growth of the seaport in the competition (Haralambides, 2019). If the terminal does not provide such service, the economies of scales turn into the diseconomies of scale. European ports continuously modernize and develop their terminals due to the peripheral port structure. Slack & Wang (2002) mentioned that peripheral port structure needs port centralization, economies of scale between peripheral ports, convenient water depths, and low distance to shipping lanes. This provides attractiveness for terminal users from port terminals.

Besides, the cost did not lose its importance in the port industry regarding the study findings. It still plays an important role to attract shippers in the context. A study (Parola et al., 2017) suggests that cost determinant emerges as a relevant economic-related driver of port competitiveness. In many sectors, the price of goods and services is a key element that customers pay special attention to while deciding on industrial buying. This is also the same in the port industry where tariffs (to be paid to the Port Authority) and costs (to be paid to the terminal) comprise an important part of total transportation costs for ocean carriers and shippers. The carriers can compare the tariff and port costs similar to other sectors.

On the other hand, port location and accessibility include cargo routing decisions, which are responsible for the dispatch of goods between production facilities and ports. Port competitiveness may significantly be increased by the strategic location of the port. Especially, port location deals with the concept of "diversion distance", which is the sailing deviation from main trunk routes, which is important to call a certain port. A study (Akbayirli et al., 2016) found that port users give importance to total transit time, the directness of sailing, and freight rate from the location of the loading and discharging port.

Limitation

The study has several limitations due to the difficulties in data collection and other reasons. Although the port sector has a wider place in global trade, the study was carried out only on port users and terminal operators operating in the eastern Mediterranean region, due to time and money constraints. Of course, this situation makes data collection harder and a wider sample from different regions would better enable cross-cultural validation of the constructs evolved in this study. Another limitation is generally covering the transshipment port terminals. Therefore, future research should be conducted on the other types of port terminals (hub ports, green ports, and conventional ports).

Recommendations for Future Studies

AHP is a cogent method for decision-making, however generally recognized as subjective. To secure the reliability of the study, a comparison from various methods may be used to demonstrate the superiority of the AHP method. Another study orientation may be performed to do correlation analyses between influencing determinants. Although the admonitions to these results through the underlying model selection and formulation, the study will be beneficial to terminal managers and port planners in designing and compelling policies for seizing shippers' attention. During the fierce port competition, port managers and terminal operators will clearly understand the determinants that influence sustainable port competitiveness, this study aids them to identify and explain the determinants that have freshly become more important and precisely affect their port operations.

CONCLUSION

This study proposed an adaptation of AHP implementation for the port competitiveness from dual perspectives (port users and terminal operators). It also offers an analytical approach to compare which criterion is more competitive for both sides. As a result, this study contributes that port infrastructure and superstructure directly affect sustainable port competitiveness. Port infrastructure and superstructure increasingly help and become integrated with port operations and hinterland logistics by changing shore extensions and offshore ports and by building resilient and flexible solutions for future ship and ship types, logistics, innovative port activities and the port's climate change. The expansion of port infrastructures and superstructures needs a long time, capital-intensive investments and hence long-run planning. That is, the design of port infrastructures and superstructures should predict the needs of customers such as container shipping lines. It is a particularly painful job at a time when the shipping sector is deeply involved with extensive transformation affecting both maritime and inland aspects (autonomous shipping and cargo handling, zero emissions, new IT architecture, etc.). Therefore, innovative facilities (generation of zero-emissions energy or green supply, chain for ships) are much necessary for keeping port competitiveness from the perspective of shipping lines. Port infrastructures and superstructures are more resilient to environmental problems. To ensure the quick application of the energy transition, clarity in port terminal management is required on the most likely transition way. Against any type of IT based risks (i.e., cyber-attacks, or data losses), adaptive secure communication is required for benefit of strategic port and traffic management and infrastructures (towage, moorings, smart berths, bunkering and etc.) which assists the ship's services for terminal operators. Moreover, city-port-nature-oriented infrastructures will contribute to leisure and business integrated centers for sustainable port competitiveness. On the other hand, transshipment is an important section of port infrastructure development plans in many places. Türkiye is not at the center of the main trunk routes of the container market. Turkish ports are at the end of the line rather than at the gateway to a district of feeder ports. Therefore, they would like to increase their innovative port facility investments to attract major carriers in the container shipping market. However, the priority between the determinants has dramatically been changed to keep more resilient sustainable competitiveness for the ports. The most important priority is that the terminal operators especially focus on the coordination of port investment projects so that the port may accommodate very large container ships. Especially, port access should be the main investment area to attract the mega-ship container carriers.

Compliance with Ethical Standards

Authors' Contributions

Study conception and design: SB, SE

Data collection: SE

Analysis and interpretation of results: SB, SE, EE

Draft manuscript preparation: SB

All authors reviewed the results and approved the final version of the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

Data Availability Statement

Not Applicable.

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SUPPLEMENTARY MATERIAL

Table S1. AHP and F-AHP scales

Linguistic variables	**Scale**	**Reciprocal Scale**	**Triangular Fuzzy Scale**	**Triangular Fuzzy Reciprocal Scale**
Equal importance	1	1/2	(1,1,1)	(1/1, 1/1, 1/1)
Equal to moderately importance	2	1/3	(1,2,3)	(1/3, 1/2, 1/1)
Moderate importance	3	1/4	(2,3,4)	(1/4, 1/3, 1/2)
Moderately to strongly the importance	4	1/5	(3,4,5)	(1/5, 1/4, 1/3)
Strongly importance	5	1/6	(4,5,6)	(1/6, 1/5, 1/4)
Strongly to very strong importance	6	1/7	(5,6,7)	(1/7, 1/6, 1/5)
Very strongly importance	7	1/8	(6,7,8)	(1/8, 1/7, 1/6)
Very strongly to the absolute importance	8	1/9	(7,8,9)	(1/9, 1/8, 1/7)
Absolute importance	9	1/10	(8,9,9)	(1/9, 1/9, 1/8)

Source: Adopted from Ayag et al. (2006)

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ACTA NATURA ET SCIENTIA PRENSİP

Development of High Throughput Rapid Turbidimetric Assay for Potency Determination of Gramicidin

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ABSTRACT

Gramicidin is a polypeptide antibiotic composed of a mixture of antimicrobial compounds. Thus, its antibacterial activity is preferentially assessed using a microbiological assay. The aim of this study is targeting to establish and validate a microbiological potency for Gramicidin with a view to the employment of a simple method with more than two folds output per test run (if compared with symmetrical designs) using 3×1 experimental designs with reasonably statistically acceptable results. The validation criteria of gramicidin turbidimetric assay using the USP method were tested in terms of selectivity, linearity, accuracy, precision and robustness. Moreover, the consistency of the experimental groups was examined in terms of error and difference from the target labelled concentration of 0.25 mg g^{-1} value, in addition to the uncertainty factor. Verification of the assay suitability was evaluated statistically against reference antibiotics of known activity. Calibration of the analytical curve showed a coefficient of correlation (r) = 0.9980 with none of the relative standard deviations (RSD) values greater than three. There was no observable fixed or variable deviation in the absorbance measurement with concentration increment. The accuracy output and profile were evaluated over ranges 50%, 100% and 150% having a maximum RSD of around three with reasonable results, confidence and absence of concentration-related bias. Robustness, precision and suitability verification were evaluated with no outliers and all RSDs below five. The turbidimetric assay design of 3×1 for gramicidin showed acceptable validation parameters and could be used as a substitute design for conventional higher-level parallel line assay models.

INTRODUCTION

Quality control monitoring of the medicinal product potency and activity is a crucial task that must be performed to ensure product safety and efficacy (WHO, 2007; Sardella et al., 2021). Even though many biologically active compounds could be analysed chemically nowadays, bioassay still retains its importance in the analysis of specific drugs such as a complex mixture of related antibiotics as was illustrated by other researchers (Eissa et al., 2021a; Eissa et al., 2021b). Until now, microbiological assay

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still possesses a critical role in the evaluation of several antibiotic compounds (Balouiri et al., 2016; Dafale et al., 2016). This kind of test has its advantage over HPLC, UPLC and GC methods in terms of simplicity, low cost and safety from multiple hazardous and toxic chemicals (Dafale et al., 2016). Another important feature – according to Dafale et al. (2016) – is the ability to estimate the biological activity of active products that consist of a composite of several related constituents that are hard to evaluate activity using conventional chemical means.

Gramicidin is a polypeptide antibiotic (Figure 1) that consists of gramicidin A, B, and C, three iontophoretic antibiotics that together make up around 80%, 5%, and 15% of the mixture, also known as gramicidin D (Budavari et al., 1996). The mixture has six different types of gramicidin molecules since each has two isoforms (Kessler et al., 2004). They can be produced from soil bacteria called *Brevibacillus brevis*. Gramicidin constitutes 15 amino acid linear peptides (Kessler et al., 2004). This contrasts with the associated known cyclic peptide gramicidin S. Determination of gramicidin potency is conducted through microbiological assay using the turbidimetric method according to the official monographs from international pharmacopeia (British Pharmacopoeia, 2022; United States Pharmacopeia, 2022). The commonly applied assay designs involve 4×4 or 3×3 Parallel Line Models (PLM) (Hewitt, 2003). This would limit the material batches analysed in a single assay run.

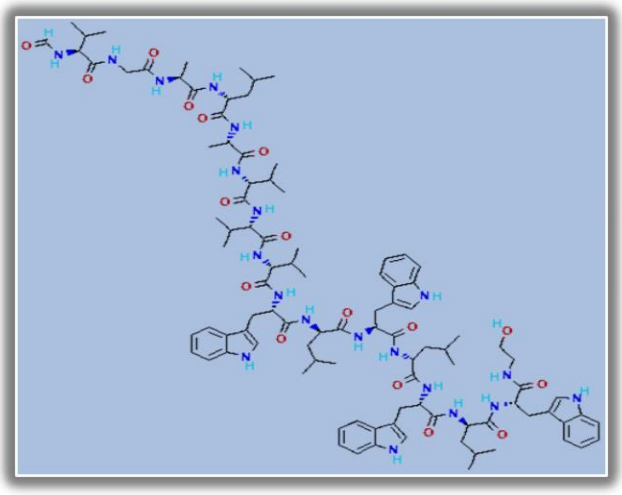

Figure 1. Two-dimensional (2D) structure of the polypeptide antibiotic gramicidin with chemical formula $C_{99}H_{140}N_{20}O_{17}$ (National Library of Medicine, 2007)

The present study herein aimed to investigate an alternative 3×1 simple design with higher throughput but maintaining the quality of the validation criteria with respect to linearity, accuracy, precision and robustness. The availability of this type of design could increase the number of batches throughput that could be tested by more than 300% from the original test design.

MATERIALS AND METHODS

Chemicals

The gramicidin standard (assigned with a potency of gramicidin 0.6019 mol kg^{-1} with batch number $1.31E^{+09}$ was obtained from a local broker, pharmaceutical dosage forms from the market containing gramicidin were obtained commercially from the market retail and it was claimed to have 5 mg 20 g^{-1} of the Active Pharmaceutical Ingredient (API) in medicinal product unit (Eissa et al., 2021a). All reagents used were of analytical reagent grade and were purchased from Oxoid, Merck and Fluka (Oppe et al., 2018).

Microorganisms and Inoculum

The cultures of *Enterococcus hirae* NCTC 13383 (Culture Collections, 2007) were cultivated from a freshly grown slant in antibiotic medium No. 3 – which would also be used in the assay in the oscillating-rack water bath - and incubated at 36.8±0.7°C for 17±1 hours (British Pharmacopoeia, 2022; United States Pharmacopeia, 2022). The stock microbial suspension was prepared at the end of the incubation time by re-suspending the solution and making the appropriate absorbance adjustments with antibiotic medium no. 3. The absorbance was adjusted for inoculum at 2.398 AU measured at wavelength $5.3E^{-7}$ m using a qualified spectrophotometer and about one cm diameter test tube of absorption cells against plain medium as blank (Francisco et al., 2014; Christ et al., 2015).

Reference and Samples Preparation

The reference and the raw material samples solutions were prepared using an amount of powder equivalent to 100 mg of gramicidin that was transferred to a 100 mL volumetric flask with dehydrated Ethanol followed by making up to the final volume with this solvent (1000 μg mL^{-1}). For a topical pharmaceutical product, an amount of about 2 g was transferred to the 100 mL volumetric flask and made up to the final volume with homogenization in absolute Ethyl Alcohol. Final aliquots dilutions range in the diluent were between 1:80 and 1:16 v/v using five equally separated increment levels so that the assay doses - expressed as ln values of (ng L^{-1}) - were 3.219, 3.912, 4.317, 4.605 and 4.828.

Calculations

To calculate the activity (potency) of gramicidin in raw material and pharmaceutical preparation a standard equation was adopted. The assay was statistically calculated by the 3×1 model and by means of regression analysis and verified using analysis of variance (ANOVA) (William, 2003; Eissa et al., 2021c; Eissa et al., 2021d).

Method Validation

The method was validated by determination of linearity, precision, accuracy, robustness and specificity. According to the ICH and the United States Pharmacopoeia (Ermer & Miller, 2006; Oppe et al., 2018), the limits of detection and quantification are not required for this category of assay.

Linearity

The calibration curve was obtained with five doses of the working standard. The linearity was evaluated by linear regression analysis, which was calculated by the least-squares regression method. Five readings were performed (Eissa et al., 2021a).

Precision

The precision of the assay was determined by repeatability (intra-assay) and intermediate precision (inter-assay). Repeatability was evaluated by assaying three samples at the same concentration and on the same day. The intermediate precision was verified by comparing the assays of two different analysts. The precision is calculated by the relative standard deviation (RSD) (Oppe et al., 2018).

Accuracy

The accuracy measurement range was assessed at 50%, 100% and 150% from the target concentration value. This was determined by adding a known amount of the Active Pharmaceutical Ingredients (API) in the samples to yield the hypothetical potency required (Eissa et al., 2021c). The accuracy determinations were evaluated with this concentration range.

Robustness

The experimental framework for the potency determination under the test conditions variation assessing the test design tolerance to deliberate changes or drifts in the proposed assay conditions to show the robustness of the experimental layout to a small deviation in pH of the antibiotic medium (0.4±0.2 deviations in pH range), incubation temperature (37±1°C of temperature drift range) and period (time variation of 210±30 minutes) of the tube assay conditions.

Specificity

The specificity was determined by measurement in presence of the active compound and blank. The blank samples were processed exactly as that containing the active antimicrobial component to examine the possible interference from other assay reagents and chemicals. This should ensure that and change in turbidity could be attributed only to gramicidin.

RESULTS AND DISCUSSION

The calibration curve for gramicidin was constructed by plotting the log (to base ten) of concentration (ng mL^{-1}) versus absorbance (AU) following a similar approach as in previous studies (Figure 2) (Zuluaga et al., 2009; Dafale et al., 2015). The corresponding mean absorbance for reference solutions was 0.163 AU (RSD% = 0.76) for the lowest dose (28.33 ng mL^{-1}), 0.154 AU (RSD% = 1.04) for the next dose (56.65 ng mL^{-1}), 0.146 AU (RSD% = 0.64) for

the middle dose (113.30 ng mL^{-1}), 0.143 AU (RSD% = 0.76) for the fourth dose (141.3 ng mL^{-1}) and 0.139 AU (RSD% = 0.70) for highest dose (169.95 ng mL^{-1}) as could be seen in Table 1. The line fit plot showed the agreement between the actual analytical curve points and the predicted values. Regression investigation showed that there is no sign of a fixed or variable trend in the residual error with concentration (Eissa et al., 2021a, Eissa et al., 2021d). Moreover, the sample points cannot be excluded from the normality assumption as they followed a normal probability plot with good regression and the correlation coefficient is 0.953 (Eissa et al., 2021b, Eissa et al., 2021c). At P = 0.05, the critical value for N = 5 is 0.8786 which is fairly below the calculated value. The variability was low with no observable pattern and RSD% below five. The regression line analysis is shown in detail in Table 2 with a good coefficient of determination and minimum error. ANOVA test showed $F_{actual} > F_{significance}$ (Hewitt, 2012; Nunes Salgado & Gomes Tozo, 2007). The calibration equation could be expressed as the following:

$$y = -0.0303x + 0.2077 \quad (1)$$

Where: y is the absorbance (AU) and x is the logarithmic transformation of the gramicidin concentration in ng mL^{-1} with R^2 = 0.9961. The lower and upper 95% confidence for the coefficients of the regression formula intercept and slope were ±0.006814231 and ±0.003495249, respectively. Statistically, there is no significant difference between the predicted and actual residuals at P < 0.05 when a two-tailed paired t-test was used (Table 3).

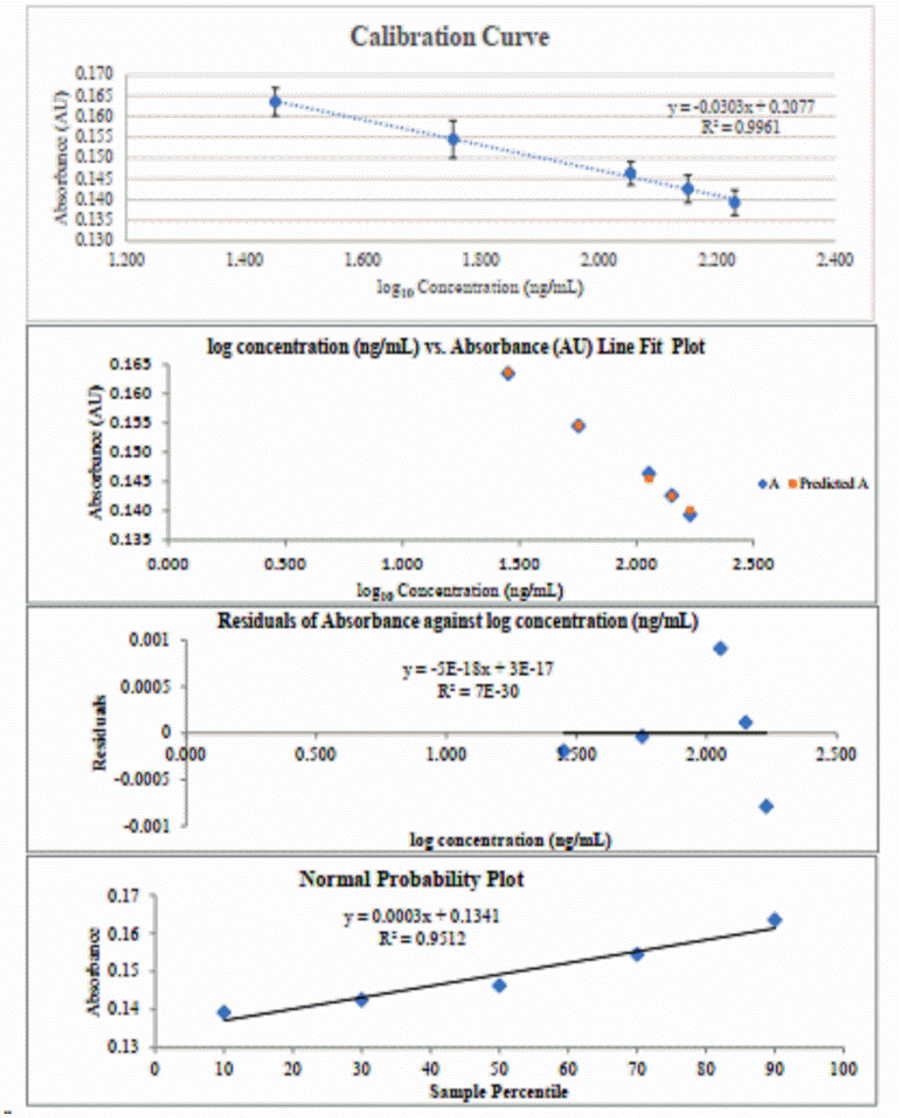

Figure 2. Analysis of linearity curve of gramicidin in turbidimetric assay with the adjusted overall absorbances of high dose (H) at 0.137 and low dose at 0.161 for range 0.024 for five observations (concentrations) levels. The correlation between absorbance and log10 (concentration) is -0.9980.

Table 1. Descriptive analysis of the absorbance readings data for six readings of gramicidin at five concentration levels

Mean Absorbance (AU)*	SD	RSD%	Variance	Combined SD	Combined RSD
0.164	0.0033	%2.04	8.93453E-06		
0.155	0.0045	%2.91	9.91826E-06		
0.146	0.0028	%1.91	6.53651E-06	0.003	%2.30
0.143	0.0033	%2.31	9.0683E-06		
0.139	0.0030	%2.17	8.19301E-06		

Note: * Average of five measurements

Table 2. Statistical analysis for the validity of the linearity curve of gramicidin in turbidimetric assay

Regression Statistics summary output					
Multiple R Correlation	0.99804				
R Square	0.996083				
Adjusted R Square	0.994778				
Standard Error	0.000707				
ANOVA*	**df¥**	**SS€**	**MS£**	**Significance F**	
Regression	1	0.000381	0.000381	0.000104	
Residual	3	1.5E-06	4.99E-07	**F**	
Total	4	0.000383		762.9703702	
Curve Parameter	**Coefficients**	**Standard Error**	**t Stat**	**Lower 95%**	**Upper 95%**
Intercept	0.207713	0.002141	97.00806	0.200899	0.214527
log conc.	-0.03034	0.001098	-27.6219	-0.03383	-0.02684

Note: *Analysis of Variance; €Sum of Square; £Mean Square; ¥Degree of Freedom.

Table 3. Residual and probability outputs for the calibration curve of gramicidin using five concentrations.

Observation	Predicted Absorbance	Residuals*	Standard Residuals	Percentile	Absorbance
1	0.163659	-0.00019	-0.31653	10	0.139263
2	0.154526	-3.8E-05	-0.06175	30	0.142567
3	0.145394	0.000908	1.48312	50	0.146302
4	0.142454	0.000113	0.184469	70	0.154489
5	0.140052	-0.00079	-1.28931	90	0.163465

Note: * The discrepancy between expected results of $\hat{Y}$ (the dependent variable) and actual values of y is the residual for each observation. Relative to projected and actual y values and according to equation 1, residual $(r_i) = y_i - \hat{Y}_i = y_i - (-0.0303x + 0.2077)$

Table 4. Evaluation of the accuracy of the turbidimetric analysis of gramicidin using 3+1 assay design

Group€	Theoretical Potency (mg/g)	Recovered Potency (mg/g)	Recovery (%)	Acceptance Range (90–130%)	%95 CI Range	Maximum G_1* (P=0.02)	SD§	RSD (%)¥
AR1	0.125	0.125	99.82	0.050	0.039	0.646	0.0054	2.95
AR2	0.250	0.251	100.54	0.100	0.087	0.619	0.0044	2.66
AR3	0.375	0.368	98.18	0.150	0.106	0.619	0.0048	3.02
Combined SD§		0.0049	**Average Absorbance (AU)**		0.170	**Combined RSD¥**		2.89

Note: * USP outlier detection: $G_1 = Z_2-Z_1 / Z_n-Z_1$, where Z is the AU reading and the subscript number is the order ascending and descending based on the magnitude The limiting Value of $G_1 = 0.846$ ¥ Relative Standard Deviation § Standard Deviation € Accuracy Result

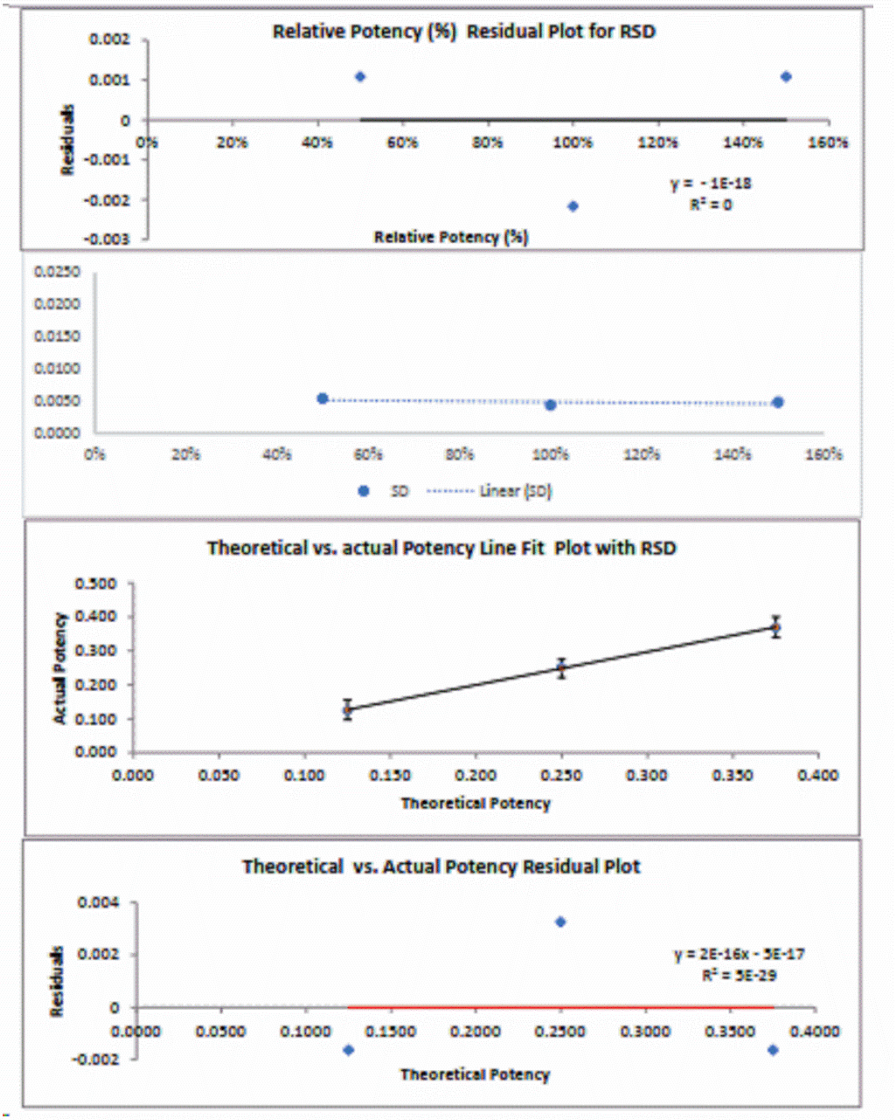

Figure 3. Accuracy profile analysis to assess the presence of signs of fixed and relative bias over the range 50% to 150% from the target potency

Table 5. Consistency and validity verification check analysis for the overall experimental groups and pairwise comparison study between the reference product potency and the 3×1 design for the turbidimetric assay of gramicidin powder for 13 separate tests at 95% confidence interval (CI)

Group	Error from Target (%)	Difference from Theoretical Value (%)	Uncertainty (%)
Control	1.13	1.12	0.011
pH	0.89	0.89	0.009
Incubation Time	0.54	0.54	0.005
Incubation Temperature	2.60	2.57	0.025
AR1	0.18	0.18	0.002
AR2	0.54	0.54	0.005
AR3	1.82	1.84	0.019
RA A	2.16	2.14	0.021
RA B	1.17	1.18	0.012
RP A	1.37	1.36	0.014
RP B	1.61	1.60	0.016
RP C	0.11	0.11	0.001
Lower Extreme G_2	0.199	0.201	0.202
Upper Extreme G_2	0.322	0.306	0.289

Gramicidin Sample Code	Comparison Groups of Gramicidin Assay Designs¥	
	3×1 Design Potency	**Manufacturer Reference Potency**
G_{549}	1066	1081
G_{869}	1029	1066
G_{200}	1093	1072
G_{382}	1053	1057
G_{568}	1086	1072
G_{549r}	1128	1081
G_{145}	1179	1093
G_{145s}	1003	1093
G_{506}	1192	1055
G_{264}	903	1038
G_{612}	1202	1051
G_{145r}	1109	1093
G_{248}	1030	1057

t-Test: Paired Two Sample for Means*		
Mean	1082.54	1069.92
Pearson Correlation	0.2499	
Hypothesized Mean Difference	0	
df	12	
t Stat§	0.5618	
P(T<=t) one-tail	0.2923	
t Critical one-tail	1.7823	
P(T<=t) two-tail	0.5846	
t Critical two-tail	2.1788	

Note: * Significance level (α) = 0.05

§ For null hypothesis of no difference t_{Stat} should be lower than $t_{critical}$

¥ Potency of not less than 900 µg of gramicidin per mg, calculated on the dried basis according to USP and BP G_2 (P = 0.02) threshold value of 0.643, for n = 8 - 13

Accuracy determinations were estimated at 50%, 100% and 150% of the target concentration of the antibiotic (Nunes Salgado & Gomes Tozo, 2007). None of the groups showed significant outliers as the maximum G_1 observed in all treatments was lower than the critical value limit (Eissa et al., 2021a; Eissa et al., 2021d; United States Pharmacopeia, 2022). In addition, the 95% confidence interval (CI) ranges were narrower than the acceptance criteria threshold (Solano et al., 2011). None of the RSD% exceeded 5%. The target and actually recovered potencies can be found in Table 4.

Statistical analysis for the regression statistics of the potency results of the three levels of the observations from the accuracy results showed that the multiple regression (R) value is 0.999732, R^2 = 0.999463, adjusted R^2 = 0.998926 with standard deviation (SD) of ±0.003989. ANOVA examination of the accuracy profile showed the value of F_{test} (1861.987) > $F_{significance}$ (0.014751) with a degree of freedom (df), sum of squares (SS) and mean squares (MS) of unity, 0.029622 and 0.029622, respectively. SS and MS of residual with df of one were estimated to be 1.59E-05 and 1.59E-05, respectively. Total SS with a degree of freedom of two was found to be 0.029638. The curve parameters of intercept and slope were analysed for fixed and variable bias with no statistical evidence that could be observed (Loureno et al., 2007). The intercept coefficient with 95% upper and lower bounds of 0.082114 and -0.07272, respectively. The standard error was calculated as 0.006093, t Stat of 0.771262 and P-value of 0.58176. The slope coefficient was calculated as 0.973607, standard error of 0.022563, t stat of 43.15074, P-value 0.014751 and the 95% upper and lower bounds were 1.260296 and 0.686918, respectively. Thus, the intercept and slope factors embrace one and zero values i.e., 0.004699±0.077417 and 0.973607±0.286689, respectively. These findings are illustrated in Figure 3 which is supported by SD, RSD and theoretical vs. actual plots to show the absence of significant bias in error with the concentration range investigated.

Investigations of the precision and robustness criteria were demonstrated in Table 5 with the outcome in the same line with as previous works (Eissa et al., 2021c). The deliberate minor fluctuations in the selected experimental conditions i.e., pH, incubation time and temperature showed acceptable average recovery (101.02% (RSD% 1.27) = 0.251 mg g^{-1} ± SD 0.00545) with all RSD% values below five. The repeatability and intermediate precision groups yielded statistically valid results with mean recovery of 100.82%, RSD% 1.33 which is equivalent to 0.252 mg g^{-1} ± SD 0.00327.

Table 5 consists of two sections for verification of the suitability of the 3×1 assay design. The first part showed the measurement of the consistency among all experimental groups by measuring percentage error, difference and certainty with reference to the theoretical target value of 100%. There was no aberrant result when using either the USP G test for outliers for n = 8 - 13 at P = 0.02 or robust regression and outlier removal (ROUT) at Q = 10.0% test which is based on the false discovery rate (FDR) (Motulsky, 2015; United States Pharmacopeia, 2022). Furthermore, these groups demonstrated homogeneity of the distribution indicated by showing signs of normality by both the Kolmogorov-Smirnov (KS) normality test and the Shapiro-Wilk normality test at α = 0.05. Thus, variation and error factors throughout the whole experiment did not demonstrate any evidence of abnormality in the consistency among the test groups.

The second aspect of verifying the assay design validity is the comparison of the gramicidin of known reference potency with that generated from the established turbidimetric design using paired t-test for a series of antibiotic materials from a well-known manufacturer source with reference potency (Table 5). The two-tailed parametric test for the customarily distributed group columns showed a significant correlation between test and reference groups without statistically substantial difference where t-actual < t-critical (Vieira et al., 2014; Martins et al., 2020). Hence,

the potency results using an alternative design are comparable with that of the reference control group.

CONCLUSION

The turbidimetric potency determination of the 3x1 design for gramicidin antibiotic showed acceptable validation parameters in terms of specificity, accuracy, precision and robustness. The proposed tube assay could be used as an alternative for the conventional 3×3 or 4×4 assay methods with comparable results that are statistically not significantly different with relatively and remarkably higher output batch analysis per assay run. The assay method is fast, simple, effective and safe without expensive instruments and no significant use of hazardous chemicals or reagents was encountered.

Compliance with Ethical Standards

Authors' Contributions

MEE: Performed laboratory experiments and managed statistical analysis.

ERD: Wrote the first draft of the manuscript.

DEE: Designed the study. Carried out the field study.

All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article.

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Geleceğin Türkiye'sinde Doğal Çayır ve Meraların Önemi

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ÖZET

İnsanların yeterli ve dengeli beslenebilmeleri için bitkisel ürünler yanında hayvansal ürünleri de tüketmeleri gerekir. Bu ise yeterli hayvansal ürün üretimine, dolayısıyla hayvanların düzenli beslenebilmelerine bağlıdır. Bu sebeple Ülkemizde hayvancılık ile ilgili karşılaşılan en önemli sorun, yeterli ve nitelikli kaba yem teminindeki zorluklardır. Çiftlik hayvanları için nitelikli kaba yemlerin ana kaynakları, tarla alanlarında üretilen yem bitkileri ile çayır ve mera otlarıdır. Türkiye'de tarla alanlarının yaklaşık %14,5'inde ot, %27,2'sinde ise tane yem (kesif yem) üretilmektedir. Diğer taraftan artan nüfus ve yükselen gelir düzeyi hayvansal ürünlere olan talebi artırmaktadır. Oysa hâlihazırda tarla alanlarının %41,7'si hayvan yemi (ot + tane yem) üretimine ayrılırken, insan için gıda üretimi tarla alanlarının %55,6'sında yapılmaktadır. Dolayısıyla bundan sonra hayvanların artan kaba ve kesif yem talebini mevcut tarım alanlarından karşılamak çok zordur. Ancak doğal çayır-meralar gıda üretim alanları ile rekabete girmeden, hayvanların kaba yem ihtiyaçlarını karşılayabilecek en önemli kaynaklardır. Bu sebeple gelecekte insanların düzenli beslenmeleri arzulanıyorsa, çayır-meralar korunmalı, amaç dışı kullanılmamalı, düşük verimli olanlar ıslah edilmeli ve sürdürülebilir bir üretim için yönetim ilkelerine uygun olarak kullanılmalıdır.

Importance of Natural Meadows and Rangelands in the Future Prospective of Türkiye

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ABSTRACT

People should consume animal as well as plant products in terms of having a sufficient and balanced diet. Therefore, it depends on the production of adequate animal products, so that animals can be fed regularly. Due to this reason, the most important problem faced in animal husbandry in our country is the difficulties in providing adequate and quality roughage. The main sources of quality roughage for livestock animals are the fodder crops produced from cultivated fields as well as meadows and rangeland hay. In Türkiye, approximately 14.5% hay and 27.2% grain feed (concentrated feed) are produced in field areas. On the other hand, increasing population and rising income levels correlatedly increase the demand for animal products. However, currently, 41.7% of field areas are allocated for the production of animal feed (hay + grain feed), while food production for human beings is carried out in 55.6% of cultivated field areas. Therefore, it is very difficult to meet the increasing demand for roughage and concentrated feed of animals from existing agricultural areas. However, natural meadows-rangelands are the most important resources that can meet the roughage needs of animals without competing with food production areas. Therefore, meadows and rangelands should be protected, and shouldn't be used for other purposes, low-yielding ones should be improved and should be used in accordance with management principles for sustainable production if it is desired to feed people regularly in the future.

GİRİŞ

İnsanoğlu varoluşundan bu yana bitkisel ve hayvansal ürünleri birlikte tüketmiştir. Başlangıçta avcı-toplayıcı olan insanlar, bitkileri toplamışlar ve hayvanları avlamışlardır. İnsan aklı avlamanın zorluklarından kurtulabilmek için ürünlerinden faydalanabildikleri ve evcilleştirilmeye uygun hayvanları yetiştirmeye başlamıştır. Bu açıdan ilk evcilleştirilen hayvanlar bugün de yetiştiriciliği yapılan at, sığır, koyun ve keçi olmuştur. Koyun MÖ 6000'lerde evcilleştirilmiş, keçinin evcilleştirilmesi ise daha eskilere dayanmaktadır. Atın en eski kemik kalıntılarına MÖ 4000'lü yıllarda Ukrayna ve Orta Asya'da Aral Gölü kuzeyindeki bozkırlarda rastlanmıştır (Baskıcı, 1999).

Bu çiftlik hayvanlarının evcilleştirilmesi, beraberinde beslenmeleri için de planlamalar yapılmasını gerektirmiştir. Bunun sonucunda doğal otlaklarda otlatma düzenlenmiş ve bu alanların beslemede yetersiz kaldığı zamanlarda, yerleşik hayata geçen insanlar tarım alanlarında ürettikleri yonca, bezelye, burçak ve arpa gibi yem bitkilerini hayvanlarını beslemek için kullanmışlardır. Bu münasebetle insanların ilk mesleği çobanlık olmuştur. İnsanlar yaklaşık 10.000 yıldır hayvanlarını meralarda otlatmaktadır (Williams, 1981). Bu hayvan besleme modeli bugün de aynı şekilde devam etmektedir. Sonuçta dünyada 3,2 milyar hektar (karaların %24,5'i) alan doğrudan otlak olarak kullanılmakta (FAO, 2019) ve bu alanlar hayvanların yem ihtiyacının yaklaşık %70'ini karşılamaktadır (Holechek & Herbel, 2011).

İnsanların hayvansal ürünlere talebi, fizyolojileri ile alakalıdır. Dünya Sağlık Örgütünün tavsiyesine göre normal bir insanın günlük protein ihtiyacı kg ağırlık başına 0,8-1 gramdır. Yetişkin bir insanın günlük kalori ihtiyacının %10-35'i de proteinlerden karşılanmalıdır. Yaş gruplarına, fizyolojilerine ve beslenme biçimlerine göre değişmekle birlikte, küresel ölçekte bir insanın tükettiği proteinin %57'sini

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bitkisel, kalanını da hayvansal proteinler teşkil etmektedir (Lonnie vd., 2018). Türkiye'de insanların enerji ihtiyaçlarının yaklaşık %10-20'si proteinden gelmektedir (TÜBER, 2015). Dolayısıyla kaliteli ve sağlıklı bir yaşam, yeterince hayvansal ürün tüketimine bağlıdır. Kuşkusuz tüketim de üretim ile mümkün olacaktır. Bu sebeple her zaman tarımsal üretimde bitkisel üretimin yanında hayvansal üretime de yer verilmiştir. Bu gereklilik sonucunda, dünyada yaşanabilir alanların yaklaşık yarısı tarımsal üretime tahsis edilmiş, bunun da yaklaşık %77 kadarını oluşturan 4 milyar hektarında (otlaklar dahil) hayvancılık yapılmaktadır (Ritchie & Roser, 2020). Ülkemizde de tarla alanlarının %41,7'si hayvan ihtiyaçlarını gidermek amacıyla ot ve dane yem üretimine tahsis edilmiştir (Tablo 3). Dolayısıyla tarımsal ekosistemlerde hayvancılık faaliyetleri için bitkisel üretimden çok daha fazla alana ihtiyaç duyulmaktadır.

ÇİFTLİK HAYVANLARININ BESLENMESİNDE KULLANILAN YEM KAYNAKLARI

Çiftlik hayvanlarının beslenmesinde kullanılan yem kaynakları kaba ve kesif yemler olmak üzere iki başlıkta toplanmaktadır. Kesif yemler yüksek enerjili, sindirimi daha kolay olan yemlerdir. Sindirilebilme oranları %65-85 arasında değişirken ham protein oranları ise %9,5 ile %40 arasında değişim göstermektedir (Tablo 1). Bu sebeple daha çok yüksek verimli ya da besiye alınan hayvanlar için önerilir. Kaba yemler ise lifli bileşikleri daha çok içerirler (selüloz %18'in üzerinde) ve bu yüzden sindirilme oranları daha düşüktür (yaklaşık %60'ın altında) (Harmanşah, 2018; Gürsoy & Macit, 2020). Fakat geviş getiren hayvanların sindirim sistemi için en uygun yemler olup, hayvanlarda yemden yararlanmayı artırır ve rumen sağlığı için önemlidir (Tekce & Gül, 2014). Bu özellikleri ile kaba yemler çiftlik hayvanlarının tükettikleri toplam yemin yarısından çoğunu teşkil ederler.

TARLA BİTKİLERİNİN EKİM ALANLARI

Türkiye İstatistik Kurumunun (TÜİK) 2020 yılı verilerine göre tarla alanlarının en büyük kısmında (11,1 milyon ha) tahılların üretimi yapılmaktadır. Yem bitkileri ekim alanı 2,3 milyon ha ile ikinci sırada yer almaktadır. Lif bitkileri ise en az ekim alanına (359 bin ha) sahiptir (Tablo 2). Ancak bu gruplamaya tütün ile tıbbi ve aromatik bitkiler dahil edilmemiştir. Bu bitki gruplarının 2020 yılı toplam ekim alanı 143 bin hektardır. Ayrıca 3,2 milyon ha alanda da nadas yapılmaktadır.

Table 1. Average of crude protein and digestibility ratios of certain roughage and selected concentrate forage in terms of dry matter

Tablo 1. *Kuru madde esasına göre seçilmiş bazı kaba ve kesif yemlerin ortalama ham protein ve sindirilebilme oranları*

Yem Kaynakları	Ham Protein (%)	Sindirilebilme (%)
Kaba yemler		
Çayır otu	8-12	60-65
Yonca kuru otu	17-22	65-80
Yaygın fiğ kuru otu	17-19	75-85
Mısır silajı	6-8	75-80
Buğday samanı	3,5-4,5	40-45
Pancar posası (kuru)	7-9	70-78
Kesif yemler		
Arpa danesi	10-12,5	80-85
Yulaf danesi	10-12,5	75-80
Mısır danesi	9,5-11	82-85
Ayçiçeği küspesi	30-35	80-85
Pamuk çiğidi küspesi	35-40	65-70

Table 2. Sowing areas of field crops (thousand ha)

Tablo 2. *Tarla bitkilerinin ekim alanları (bin ha)*

Yıl	Tahıllar	Yem Bitkileri	Yağlı Tohumlar	Dane Baklagil	Yumru Bitkileri	Lif Bitkileri
2005	13.893	942	644	1.177	490	547
2010	12.100	1.461	769	822	470	481
2015	11.713	1.863	866	690	429	434
2020	11.143	2.269	895	871	486	359

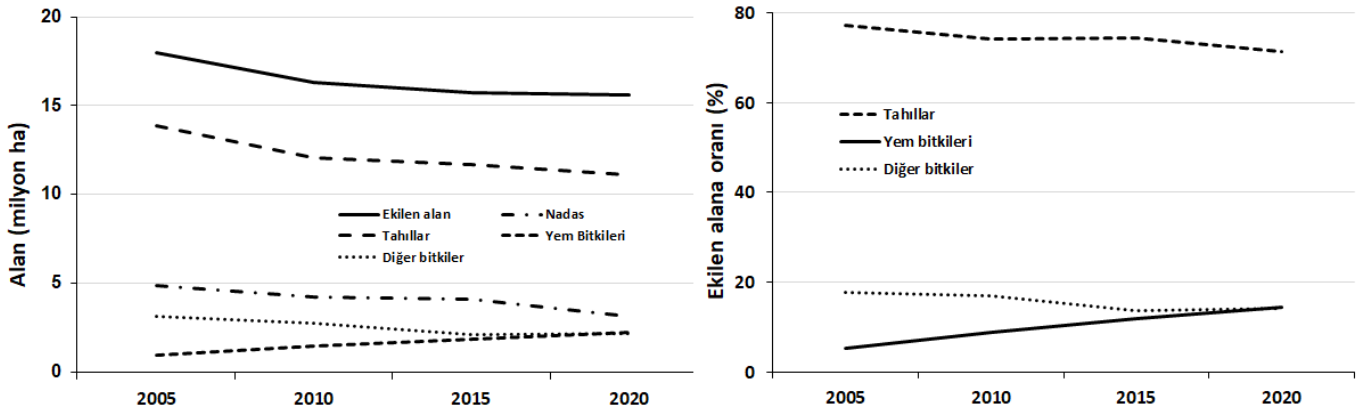

Figure 1. Sowing areas of field crops. Change in sowing areas of important field crop groups (Graph, on the left, shows the sowing area versus the fallow area; in the figure, on the right, shows only the change in sown field crops)

Şekil 1. *Önemli tarla bitkileri gruplarının ekim alanlarındaki değişim (Soldaki grafikte ekilen alan ile nadas alanı; sağdaki şekilde ise sadece ekilen tarla bitkilerinin değişimi yer almaktadır)*

Önemli tarla bitkileri gruplarının toplam ekim alanlarının 2005-2020 arasındaki 15 yıllık süreçteki değişimi yem bitkileri lehine olmuştur. Bu dönemde toplam ekilen alan ve tahıllar ile diğer bitkilerin ekim alanları sürekli olarak azalırken yem bitkileri ekilen alanlar artmıştır. Nadas alanlarında da özellikle son 5 yılda önemli bir azalma görülmüştür. Nadas dışında sadece ekilen alanlar değerlendirildiğinde ise değişim daha belirgin olarak ortaya çıkmaktadır (Şekil 1).

YEM BİTKİLERİ EKİM ALANLARI

Türkiye'de gerek kaba yem gerekse dane yemi üretim alanları 2005-2020 yıllarını kapsayan dönemde düzenli artış göstermiştir. Bu artış son beş yılda daha hızlı cereyan etmiştir. 2020 yılı itibariyle ekilen tarla alanları içerisinde kaba yem üretilen alanlar 2,3 milyon, tane yemi üretimine ayrılan alanlar ise 4,3 milyon hektar olmuştur. Buna göre toplam 6,5 milyon ha tarla alanı yem üretimine tahsis edilmiştir (Tablo 3). Bu sonuçlara göre, 2020 yılında ekilen tarla alanlarının %14,5'inde kaba yem, %27,2'sinde de tane yem üretilmek suretiyle çiftlik hayvanlarını beslemek amacıyla üretim yapılan alan ekilen alanların %41,7'sini meydana getirmiştir. Bunlara yem üretimi amacıyla tohumluk üretimi yapılan alanlar da dahil edildiğinde, oran %44,9'a yükselmektedir. Nadas alanları dahil olmak üzere tarla alanlarının içerisinde kaba yem üretim alanlarının oranı ise %12,1, dane yem üretim alanlarının oranı %25,3 ve toplam alanın oranı %37,4 olmaktadır. Bu kadar alanın yem üretimine tahsis edilmiş olmasına rağmen, halen hayvansal ürünlerin üretimi, tüketimi ve pazarlanmasında ciddi sorunlar yaşanmaktadır.

NÜFUS DEĞİŞİMİ İLE HAYVANSAL ÜRÜN İLİŞKİSİ

Son 15 yıllık süreçte Türkiye'de nüfus artışına paralel olarak hayvan sayısı ve hayvansal ürün miktarında düzenli artış olmuştur. Nüfus 67,7 milyon kişiden 83,6 milyona çıkmış, yani %23,4 artmıştır.

Buna karşılık hayvan varlığı %58,6 artarken, kırmızı et üretimi yaklaşık 2,5 katına, süt üretimi de 2 katına çıkmıştır (Tablo 4). Doğal olarak bu durum fert başına tüketilen hayvansal ürün miktarını artırmıştır. Nitekim 2005 yılında fert başına kırmızı et tüketimi 10,88 kg iken, 2020'de 21,36 kg'a yükselmiştir. Süt ve ürünlerinin tüketimi ise 164,0 kg'dan 281,1 kg'a çıkmıştır (Tablo 5).

TÜİK verilerine göre hayvan varlığı ile birlikte hayvansal ürünlerin üretimi ve tüketiminde önemli artışların yaşaması, Türk insanının beslenme kalitesinin yükseldiğini göstermektedir. Ancak Sağlık Bakanlığı ile Başkent, Hacettepe ve Hasan Kalyoncu Üniversiteleri tarafından hazırlanıp 2019 yılında yayınlanan Türkiye Beslenme ve Sağlık Araştırması'na göre, insanlarımızın %10,4'ü dana (sığır) etini, %35,7'si de koyun etini hiç tüketmemişlerdir. Yaş gruplarına göre vejetaryen olanların oranının %0,6-1,4 olduğu göz önüne alınırsa (TBSA, 2019), et üretimindeki artışa rağmen, belirli bir kesimin kırmızı ete ulaşamadıkları görülecektir. Ayrıca Türkiye'nin 2020 dana (sığır) eti ithalatı 4584 ton ve ihracatı 517 tondur (TEPGE, 2021). Dolayısıyla toplam kırmızı et üretiminin yanında, çok küçük miktarda (4067 ton) da olsa net dana eti ithalatı söz konusudur. Fakat bu ülke insanının fert başına et tüketimini etkilememektedir. Bu verilerin yanında, OECD tarafından dünyada 2020 yılı ortalama kırmızı et tüketimi 18,83 kg/fert (sığır ve koyun eti tüketimi 8,15 kg/fert), Türkiye'nin tüketim ortalaması ise 13,86 kg/fert olarak bildirilmiştir (OECD, 2020). Dolayısıyla bu veriler Tablo 5'te yer alan verilerle uyuşmamaktadır.

Table 3. Areas allocated to animals for feed production within the sowing area (excluding fallow) (in million ha) calculated from TURKSTAT data)

Tablo 3. *Ekilen alan (nadas hariç) içerisinde yem üretimi amacıyla hayvanlara tahsis edilen alanlar (milyon ha) (TÜİK verilerinden hesaplanmıştır)*

Yıl	Ekilen Alan	Kaba Yem		Tane Yemi		Toplam	
		Alan	*Oran (%)*	*Alan*	*Oran (%)*	*Alan*	*Oran (%)*
2005	18,005	0,942	5,2	3,295	18,3	4,237	23,5
2010	16,333	1,461	8,9	3,182	19,5	4,643	28,4
2015	15,723	1,863	11,8	3,582	22,8	5,445	34,6
2020	15,628	2,269	14,5	4,254	27,2	6,523	41,7

Table 4. Relationship between animal population, existence and production (TURKSTAT data)

Tablo 4. *Nüfus ile hayvan varlığı ve hayvansal üretim ilişkisi (TÜİK verileri)*

Yıl	Nüfus	Hayvan Varlığı (HB)	Kırmızı Et (ton)	Süt (ton)
2005	67.743.052	9.669.509	737.220	11.107.897
2010	73.722.988	10.186.300	879.819	13.543.674
2015	78.741.053	13.662.300	1.187.018	18.654.682
2020	83.614.362	16.498.464	1.785.952	23.503.790

Table 5. Per capita consumption of animal products (calculated from TURKSTAT data)

Tablo 5. *Fert başına hayvansal ürün tüketimi (TÜİK verilerinden hesaplanmıştır)*

Yıl	Kırmızı Et (kg/fert)	Kırmızı Et + Tavuk Eti (kg/fert)	Süt (kg/fert)
2005	10,88	24,71	164,0
2010	11,93	31,52	183,7
2015	15,07	39,32	236,9
2020	21,36	46,91	281,1

GIDA – YEM REKABETİ

Doğrudan insan ihtiyaçları için kullanılan alanlar ile hayvan yemi üretim alanları arasındaki paylaşımda değişim sürekli olarak yem üretimine ayrılan alanlar lehine olmuştur. Nitekim 2020 istatistikleri ekilen tarla alanlarının %55,6'sının insan gıdası ve %2,3'ünün de lif üretimi amacıyla kullanılmasına karşın, %41,7'sinin hayvan yemi (kaba + dane) ve yalnızca %0,4'ünün diğer ihtiyaçların üretimi için kullanıldığını göstermektedir (Tablo 6). Buna rağmen değişik araştırmacılar tarafından yapılan hesaplamalara göre, ülkemizde halen nitelikli kaba yem ihtiyacının ancak %40-65'i karşılanabilmektedir (Gökkuş, 1994; Alçiçek vd., 2010; Yavuz vd., 2020; Hanoğlu Oral & Gökkuş, 2021). Dolayısıyla kaba yem açığının kapatılmasında yem bitkileri ekim alanlarının daha çok artırılması mümkün gözükmemektedir. Zira yem üretim alanlarını artırmanın gıda üretimine ayrılacak alanlarda yaratacağı daralma, halen bu yönde yaşanmakta olan sorunların daha da büyümesine sebep olacaktır.

İnsanların temel besin kaynağı olan ve bu özelliği ile de stratejik ürün olarak kabul edilen buğdayın ülkemizdeki üretimi ile ihtiyaçların karşılanması arasındaki dengenin kurulmasında zorlanılmaktadır. Son 20 yılın yaklaşık yarısında buğday üretimi ile tüketimi arasında negatif bir dengeden söz edilmektedir (TÜİK, 2020). Esasen buğday ekim alanları 2000 yılından beri sürekli azalmaktadır (Şekil 2). Fakat birim alana verimin artması üretimdeki azalmayı telafi etmektedir. Zira 2000 yılında ortalama 223,4 kg/da olan buğday verimi, 2020 yılında 296,1 kg/da'a yükselmiştir (Şekil 2) (TMO, 2020).

Bunun yanında Türkiye'nin 2021 yılı nüfusu 84,7 milyondur (TÜİK, 2021). Kayıtsız göçmenler ile birlikte nüfusun 90 milyonu aştığı söylenebilir. TÜİK tarafından geleceğe yönelik olarak yapılan ana senaryoya göre, Türkiye nüfusu 2069 yılında kadar artarak 107,7 milyona ulaşacaktır. Bu durumda gıda talebi bugünden çok daha fazla olacaktır. Oysa bugün bile buğday üretiminde zaman zaman yetersizlikler yaşanmaktadır. Bu şartlarda yem üretiminin artırılmasına yönelik öneriler gerçekçi olmayacak ve gıda üretimindeki sorunları çoğaltacaktır. Bu yönde gelişmeler olduğunda ise gıda sorunu çözümsüz hale gelebilecek ya da sorun ithalat yoluyla çözülmeye çalışılacaktır. Hâlihazırda dünya pazarında yaşanan sıkıntılar da göz önüne alınırsa, ihtiyacın dışarıdan karşılanmasının da kolay olmayacağı veya bedelinin yüksek olacağı açıktır.

Buğdayın en çok üretildiği İç Anadolu'da etkili olması beklenen küresel ısınmanın getireceği muhtemel üretim kayıpları da (Öztürk, 2002) yem bitkileri ekim alanlarını kısıtlayabilecek başka bir sorun olarak görülebilir.

Table 6. Fields (excluding fallow) cultivated for the purpose to provide feed, hay and fiber requirements million ha) and their share in total field area (%)

Tablo 6. *Gıda, yem ve lif ihtiyaçlarının temini amacıyla ekilen tarla (nadas hariç) alanları (milyon ha) ve toplam tarla alanı içindeki payı (%)*

Yıl	Gıda		Hayvan Yemi (ot + dane)		Lif		Diğer*	
	Alan	Oran (%)	Alan	Oran (%)	Alan	Oran (%)	Alan	Oran (%)
2005	13,174	73,2	4,237	23,5	0,547	3,1	0,047	0,2
2010	11,144	68,2	4,643	28,4	0,481	3,0	0,065	0,4
2015	9,766	62,1	5,445	34,6	0,434	2,8	0,078	0,4
2020	8,685	55,6	6,523	41,7	0,359	2,3	0,061	0,4

Not: *Bu grupta tütün, haşhaş ve tıbbi-aromatik bitkiler yer almaktadır.

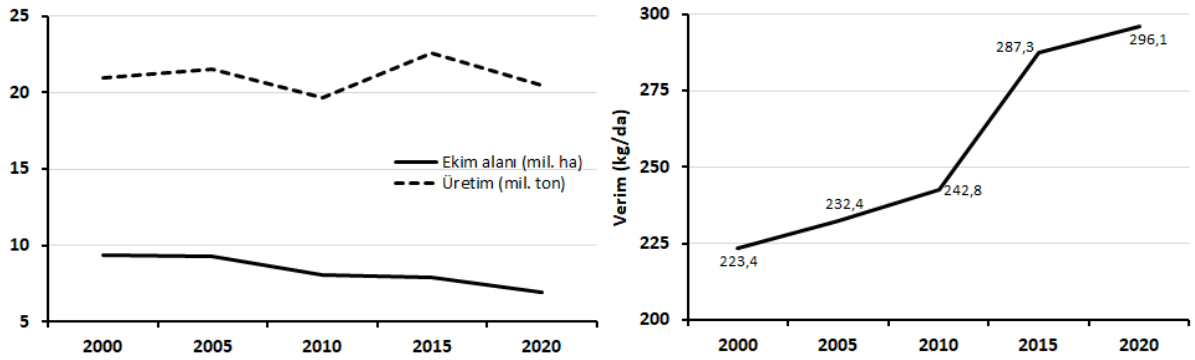

Figure 2. Changes in wheat cultivated areas (million ha) and production amounts million tons) in last two decades left), and change in wheat yield (kg/da) (right) (TMO data)

Şekil 2. *Son yirmi yılda buğday ekim alanları (milyon ha) ve üretim miktarlarındaki (milyon ton) değişim (solda) ve buğday verimindeki (kg/da) değişim (sağda) (TMO verileri)*

Bazı bilim insanları Türkiye'nin tarımsal üretim potansiyelinin 100 milyon kadar insanı ancak besleyebileceği, bunun da bitkisel ürün ağırlıklı beslenme ile mümkün olabileceğini vurgulanmaktadır (Kışlalıoğlu & Berkes, 1985). Diğer taraftan Batı tarzı beslenecek şekilde çeşitli, sağlıklı ve besleyici bir bitkisel ve hayvansal ürün diyetini sağlamak için fert başına yaklaşık 5 da ekili alan gerektiği ifade edilmektedir (Lal, 1989). Bu veri esas alınırsa, 23,4 milyon ha işlenen alana (meyve, sebze dahil) sahip Türkiye sadece 46,9 milyon insanın yeterli ve dengeli besleyeceği gıda ve diğer ihtiyaçlarını karşılayacak durumda olduğu görülecektir.

Diğer taraftan Türkiye İşçi Sendikaları Konfederasyonunun 2023 yılı Ocak ayı raporuna göre, dört kişilik bir ailenin açlık sınırı 8.874 TL'ye, yoksulluk sınırı da 28.875 TL'ye çıkmıştır (TÜRK-İŞ, 2023). Bu durum Türk insanının yaklaşık %90'ının yoksulluk sınırı altında yaşadığını göstermektedir. Bu sebeple mevcut durumda hayvan besleme sorunlarının yanında, ister istemez yüksek fiyatlı kırmızı et tüketimi de azalacaktır.

Toplumların gelişmişlik düzeyi arttıkça, gıda yelpazesi içerisinde hayvansal ürünlerin payı da artmaktadır. Bu yüzden milli geliri yüksek ülkelerde hayvansal ürün tüketimi de yüksektir. Geleceğin Türkiye'sinde de nüfus artışı yanında gelir düzeyinin de yükselmesi ile hayvansal ürünlere olan talep de artacaktır (Saygın & Demirbaş, 2018). Bu da hayvansal üretimin artırılmasını zorlayacaktır. Bunun yanında kilogram başına en yüksek arazi ihtiyacı da hayvansal ürünlerde görülmektedir. Bir kilogram koyun ve kuzu eti ile sığır eti üretimi için 300 m²'nin üzerinde araziye gerek duyulurken, bitkisel ürünlerin üretiminde gerekli arazi miktarı 4 m²'nin altındadır. Örneğin 1 kg mısır ve buğday üretimi için 2,94 ve 3,85 m² arazi gerekli iken, 1 kg biftek ve koyun/kuzu eti üretimi için 326,2 ve 369,8 m² alan gerekmektedir (Ritchie & Roser, 2020). Dolayısıyla yem bitkileri üretiminin ve ekim alanlarının artışının sınırlı kalması zorunluluğunda, sorunun çözümünde en önemli kurtarıcı doğal çayır ve meralar olacaktır.

YEM BİTKİLERİ İLE İLGİLİ ALINMASI GEREKEN ÖNLEMLER

Bu değerlendirmeler sonucunda, yem üretiminde geleceğe yönelik olarak alınması gereken önlemler aşağıda sıralanmıştır.

i. Yem bitkileri ekim alanlarını bir miktar daha artırabilmek için 1980 yılından bu yana yaklaşık 5 milyon hektar azalan (terk edilen) tarım alanı yeniden üretime kazandırılmalıdır.

ii. Mevcut üretim alanları dışında, yem bitkileri yetiştiriciliği kışlık ara ürün olarak sanayi bitkileri tarımı yapılan alanlardan daha çok yararlanacak şekilde planlanmalıdır. Bu hem yem üretimi hem de tarımda sürdürülebilirlik sağlayacaktır. Bu konuda ülkemizde birçok araştırma yürütülmüş ve olumlu sonuçlar elde edilmiştir (Soya & Ergin, 1989; Karasu vd., 2006; Kara, 2016; Gökkuş & Kaplan, 2018; Sertel, 2019).

iii. Mümkün olabildiği ölçüde yem bitkileri üretiminde nadas alanlarından yararlanma

yoluna gidilmelidir (Altın, 1984; Bakır, 1984). Örneğin yıllık ortalama 400 mm yağış alan Erzurum'da buğday verimini azaltmadan nadas alanlarının yonca ve korunga ekilerek azaltılabileceği vurgulanmıştır (Tosun & Altın, 1984).

iv. Sap-saman gibi bitki artıklarının yem maddesi olarak kullanımı sürdürülmeli, ancak bunların bir bölümü yem kalitelerini yükseltmek amacıyla üre, amonyak ve NaOH gibi maddeler ilave edilerek hayvanlara verilmelidir (Coşkun, 2021).

v. Yem bitkileri destekleri sürdürülmelidir. Ancak Orta Anadolu'nun kurak ovalarında yoğun su isteyen yonca ve mısır gibi yem bitkileri destek kapsamı dışına çıkarılmalı, en azından destek miktarı azaltılmalıdır. Diğer bölgelerdeki yem bitkileri desteklerinin sağlayacağı ekim alanı artışları da gıda alanları ile daha fazla rekabet etmeden yem üretimi için yeterli olacaktır.

ÇAYIR VE MERALARIN ÖNEMİ

Yem bitkileri ile ilgili belirtilen hususlar ve önlemlerin yanında, gıda üretim alanları ile rekabete girmeden üretilecek nitelikli kaba yemin en önemli kaynakları doğal çayır ve meralardır. Çayırlar genellikle barınak sürecinde, meralar ise otlatma mevsiminde çiftlik hayvanlarının yem ihtiyaçlarını karşılayan en önemli üretim alanlarıdır. Bu önemlerine karşılık, bugün arazinin parasal değerinin yükseldiği büyükşehirlere yakın yerlerde meralar öncelikle tahsis amacı değiştirilmeye çalışılan alanlar durumundadır. Ayrıca zamansız otlatma başta olmak üzere (Gökkuş, 2020), düzensiz mera kullanımı ülke meralarının yaklaşık %85'inin çok iyi ya da iyi durum sınıfından uzaklaşmalarına sebep olmuştur (Avağ vd., 2012; Aydoğdu vd., 2020). Aynı zamanda ekonomik, sosyal ve kültürel sebeplerle genç nüfusun köyleri terk etmesi, çayır ve mera alanlarından yeterince yararlanmayı da engellemektedir. Esasen Doğu ve İç Anadolu Bölgelerinde meralarda yoğun otlatma baskısından bahsetmek de doğru değildir. Buralarda temel sorun, mera otunun gereğince hayvansal ürüne çevrilememesidir.

Sonuç olarak, çayır ve meralarla ilgili olarak aşağıdaki hususlara dikkat edilmesi, gelecekte nitelikli kaba yem üretiminde yaşanabilecek daha büyük sorunların önünü alacaktır.

i. Çayır-mera alanları korunmalı, amaç dışı kullanılmamalı ve vasıfları değiştirilmemelidir.

ii. Verimleri azalmış ve ot kaliteleri düşmüş meralar uygun yöntemlerle ıslah edilmelidir.

iii. Sürdürülebilir bir üretim için meralar yönetim ilkelerine uyun şekilde kullanılmalıdır.

iv. Ot kalitesinde kayıplar yaşanmaması için çayırların bakım işlemleri aksatılmadan yapılmalı, uygun zamanda biçilmeli, biçilen ot kısa sürede kurutulmalı ve taşınıp depolanmalıdır.

v. Mera kanununun daha somut bir şekilde işlevsel hale getirilmesi için taşra teşkilatları, teknik elemanları ve kolluk kuvveti ile bir bütün halinde otlatma yönetimi kurumsal bir yapı altında toplanmalıdır.

Özetle, yarın pişman olmamak ve nesillerimizin kaliteli ve ucuz beslenmesini temin etmek için bugünden çayır ve mera alanları tarımsal sit alanı ilan edilerek çok iyi korunmalıdır.

Etik Standartlar İle Uyum

Yazarların Katkısı

AG: Makalenin yazımını planlamıştır.

EC: İstatistik verilerini derlemiş ve gerekli hesaplamaları yapmıştır.

Çıkar Çatışması

Yazarlar herhangi bir çıkar çatışması olmadığını deklare etmektedir.

Etik Onay

Yazarlar bu tür retrospektif çalışmalar için resmi etik kurul onayının gerekli olmadığını bildirmektedir.

Veri Kullanılabilirliği Bildirimi

Yazarlar, bu çalışmanın bulgularını destekleyen verilerin makale içinde mevcut olduğunu onaylamaktadır.

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The Effects of Diets Including Different Amount of *Tribulus terrestris* Supplementation on Spermatological Parameters and Fertilization Ability of Male Rainbow Trout (*Oncorhynchus mykiss*)

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ABSTRACT

The aim of this study was to examine how varying doses of *Tribulus terrestris* supplementation affected the fertilization ability and spermatological characteristics of male rainbow trout (*Oncorhynchus mykiss*). Male fish (mean weight of 497±5.29 g and a mean length of 33.5±3.4 cm) were fed with four experimental diets containing different amounts of *T. terrestris* (0, 100, 250, 500 mg/kg feed) for 90 days in duplicates. The motility time analysis of all groups indicates that the motility times of male rainbow trout fed with *T. terrestris* increased. Besides, the fertilization rate of the control group is lower than the groups that fed with *T. terrestris* supplementation. In comparison with the control group without any additives, especially the fertilization rate of the trout fed with 250 mg/kg was 95% ($p<0.05$). Furthermore, this study also examined how *T. terrestris* supplementation affects the blood plasma of male rainbow trout and it was found that the male rainbow trout fed with *T. terrestris* supplementation had the highest LH hormone value at 250 mg/kg.

INTRODUCTION

So far, plant supplementation has been tested for diverse uses, including the drugs, cosmetics and food supplement industries (Bhuvaneswari & Balasundaram, 2006). It has been reported that medicinal plants are powerful agents in comparison to chemical drugs and the compounds of medicinal plants enhance the therapeutic effects while reducing the toxicity and side effects of drugs. Besides, they help to accelerate gastrointestinal absorption process (Platel et al., 2002).

In recent years, there have been varieties of research conducted in the field of aquaculture which aim to decrease the use of chemicals and, as alternatives to those chemicals and antibiotics, many

different additives have been started to use as phytobiotics (Yeganeh et al., 2017; Elsabagh et al., 2018). The phytobiotics' analyses indicated that because they are harmless, they are extensively used as aqua-feed additives to support gaining weight of fish, improve feed conversion ratio in the fish farms and enhance growth performance (Yeganeh et al., 2017).

Plants are safe and cheap with their numerous benefits in animal nutrition such as supporting antibacterial, antiviral and antioxidant actions, activating appetite and feed intake, improving the secretion of digestive enzyme activation and stimulating immune responses (Citarasu, 2010).

Tribulus terrestris is an herbal supplement that grows in various regions of the word such as Europe, Australia, India and especially South Africa. It is an annual, flowering plant which belongs to a widespread family known as *Zygophyllaceae* family, consisting of about 250 species and 25 genera. *T. terrestris* is a crawling herbal plant that can grow as tall as one meter and usually grows in sandy soils and arid climates. The term Tribulus has its roots in the Greek word "tribolos", referring to a fruit with spiky projections. To treat various health conditions, the fruits of this plant are used in Bulgaria, in Ayurvedic medicine in India, and traditional Chinese medicine (TCM) (Pokrywka et al., 2014).

T. terrestris supplementation contains various compounds that possess both chemical structures and biological properties. These include alkaloids, terpenoids steroidal saponins, polyphenol carboxylic acids, tannins, and flavonoids. Flavonoids are commonly found natural compound providing hepatoprotective and antioxidant benefits (Miller, 1996). Besides, flavonoids are also known for their broad range of biological and chemical functions which include the ability of radical scavenge radicals (Kavitha et al., 2011). Furthermore, people utilize *T. terrestris* to enhance their muscle-building capacities. Also, the dominant saponin in *T. terrestris* is protodioscin which plays an important role in supporting the production of testosterone (Ganzera et al., 2001).

Tribulus terrestris has been found to exhibit antioxidant and hepatoprotective properties in different animals such as *O. mossambicus* (Kavitha et al., 2011). Moreover, as reported that to increase the testosterone hormone level in animals, these natural aromatic plants are used (Cek et al., 2007). In addition to all these, *T. terrestris* also improves sperm motility, stimulates sexual desire and enhances secretion of LH and testosterone (Bucci, 2000; Kavitha et al., 2011).

There are various bioactive components like steroids, alkaloids, flavonoids and phenolic aid in enhancing the antimicrobial activity, appetite, the growth, and the immunity of the cultured fish (Chakraborty et al., 2015). In their research, Cek et al. (2007) claimed that *T. terrestris* positively impacts growth performance, sex reversal and survival rate of various fish species like *Poecilia reticulate*, *Cichlasoma nigrofasciatum* and *Clarias gariepinus*. Besides, the results of the study found out that treated progenies exhibited better growth rates, more successful sex reversal and spermatogenesis compared to untreated ones.

Yet, *T. terrestris* as a natural supplement has been used for different purposes such as improving sexual activities, general health condition, feed utilization and treating male infertility. However, there is no study has been carried out on the impacts of *T. terrestris* on male rainbow trout. Hence, the major goal of this present study is to find out the influences of *T. terrestris* in different amounts on fertilization ability and spermatological parameters of male rainbow trout (*Oncorhynchus mykiss*).

MATERIAL AND METHODS

Study Area and Fish

The experiments were conducted on a commercial trout farm, located on the southwestern part of the Turkey. A total of 300 fish and including 60 replacement fish were selected among brood stock candidates (with a mean weight of 497±5.29 g and a mean length of 33.5±3.4 cm) of the farm in September.

Preparation of Herbal Extracts and Experimental Diets

T. terrestris fruit was obtained from a local market in Turkey. To prepare the extract, 100 g of the fruits were finally ground before mixing 1 liter of 90% ethanol. After heating the mixture at 80°C for 2 hours, through Whatman No. 2 filter paper, they filtered. Under reduced pressure, using a rotary evaporator, the filtrates were evaporated. The fruit extract yield was 20% (wt/wt). To yield 50% (wt/wt) of the crude extract, the aqueous extracts were filtered and then lyophilized. *T. terrestris* fruit extracts are protected and preserved in a refrigerator to use for this study.

The desired (100, 250, 500 mg) concentration of lyophilized dry 10g extract of *T. terrestris* was dissolved in 1 L 90% ethanol and sprayed on a commercial trout pellet (Abalıoğlu fish feed) while mixing the diet continuously for a homogenous distribution of *T. terrestris*. Then, the feeds were dried under vacuum to evaporate alcohol. The feeds were freshly prepared every week and were kept at +4 °C until feeding fish. For the experiment, 30 males were randomly selected for one of the 8 net pens ($2\times2\times2m=8m^3$) and placed into 2 concrete ponds. Each cage was installed one month before the experimental feeding and adaptation to the cages was achieved with standard care.

Experimental feeds were given for 90 days (from October to December). The fish were evaluated in bi-weekly. Before weighing, all fish were anesthetized with 500 ppm phenoxy ethanol. The live weights of the fish were measured in grams (g) with Cas brand precision scales and their lengths were measured in centimeters (cm) with a 1cm precision using a ruler.

Fish were fasted for two days before the feeding to ensure fecal contamination during milking for gamete uptake. Gametes were taken from normal male and female brood stocks by abdominal massage method into glass tubes and plastic containers. After semen was stripped from each cage, sperm samples were kept in plastic containers containing ice at 2-4°C for analysis and transported to the laboratory under controlled conditions. In the laboratory, spermatological characteristics such as sperm quantity, density, total spermatozoa, and percentage of motility, motility duration, osmolality and pH were determined.

Experimental Analysis

A micro pH meter probe (WTW 3110 GmbH, Germany) was used to measure the pH of semen, while within the help of a Gonotec Osmomat 030 cryoscopic osmometer (Gonotec, Berlin, Germany), osmolality measurements were conducted.

Hormonal Analysis

The hormonal levels found in blood serum are measured through various indices such as LH (ng/mL), testosterone (ng/mL), FSH (ng/mL) and were analyzed by centrifugation at 8000 rpm for 5 minutes and stored in liquid nitrogen.

Semen Analysis

Semen analysis was applied according to the method described by Tekin (1994). The following parameters were estimated: motility divided into motility percentage, motility duration and vitality. Sperm motility was determined and recorded in triplicates using a video camera (AxioCam ICc 5, Germany) attached to a phase-contrast microscope (Zeiss Axio Scope A1, Carl Zeiss Microscopy, Germany) at 400× (Rurangwa et al., 2004). The progressive motility (%) and the durations of progressive motility (s) were analyzed. Determination of sperm motility percentages were conducted by measuring the time until forward movement stopped and circular movement began. An arbitrary scale with 10% interval increments was used to assess sperm motility percentages. Non-motility was recorded as 0% (modified from Borges et al., 2005).

Seminal Plasma Composition Analysis

The samples were preserved at a temperature of −20°C to conduct biochemical and ionic analyses. an AbbottAeroset autoanalyzer (Chicago, IL, USA), along with its original kits, was used to measure the parameters of cholesterol, glucose, K^+, Cl^-, Na^+, $Ca2^+$ and $Mg2^+$. Furthermore, the seminal plasma pH level was measured with the standard probe of the WTW pH meter 3110 set2 device. Osmolality values were

determined by Gonotech Osmomat 030 crycoscopic osmometer.

Fertilization Process

To prevent differences that may occur during the fertilization stage and also to prevent contamination of the eggs, it was given importance not to mix urine, blood and feces. After milking process, the ovarian fluid was filtered. Fertilization of eggs was put in 12 cm aluminum containers. Freshly milked eggs were mixed with sperm for one minute using bird feathers. After that 100 ml of activation solution (60 mM $NaHCO_3$; 50 mM Tris; pH=9.5). After that 100ml of activation solution (60 mM $NaHCO_3$; 50 mM Tris; pH=9.5) and waiting for 3 minutes, the eggs were washed with 13°C hatchery water and filled with fresh water again. The eggs were left to swell for one hour then plate in incubation pens with a continuous flow system. The fertilization was performed 3 times for each sperm groups.

Statistical Analysis

Nonparametric Mann–Whitney U tests were used due to the unequal variance and sample size, followed by Kruskal–Wallis tests to show the differences of the variables in terms of the periods of spawning seasons (Mann & Whitney, 1947). One-way analysis of variance (ANOVA) was used to analyze the effect of treatment on different parameters and Tukey's multiple comparison test was used (Kirk, 1968). The effects of treatment on different parameters. Results are shown as mean±SD and statistical significance level was set as $p<0.05$ and all statistical analyses was conducted using SPSS version 11.5.

RESULTS

Hormonal Analysis

The effects of dietary supplementation of *T. terrestris* extract on male rainbow trout's LH, testosterone, FSH are given in Table 1. There were no significant differences ($p<0.05$) among experimental groups for testosterone, FSH and LH. The highest level of testosterone was recorded in TT100 followed by TT250 and TT500, while the lowest value was recorded in CG ($p<0.05$). The highest level of FSH was recorded in TT500 followed by TT250, CG, while the lowest value was recorded in TT100 ($p<0.05$). Finally, the highest level of LH was recorded TT250, followed by TT500 and TT100, while the lowest value was recorded in CG ($p<0.05$).

Semen Analysis

Results of the effect of dietary supplementation of *T. terrestris* extracts on semen parameters of the male rainbow trout are presented in Table 2. It showed significant differences ($p<0.05$) among all experimental groups. The greatest vitality was achieved in TT500 followed by TT250 and TT100, while the lowest vitality was recorded in CG. When the percentage and average duration of motility data were analyzed, it showed that the greatest level of average motility duration was recorded in TT250 followed by in TT500 and TT100, while the lowest value was recorded in CG. Considering all sampling periods, it showed significant differences ($p<0.05$) among all experimental groups. It was observed that all groups and linear motility times were below the literature average, whereas linear motility gained in samples with *T. terrestris* added was better. As a result of the examination of each group's semen parameters, as it is given in Table 2, the duration of the period of highest vitality mean (49.6 s) was recorded in 500 mg/kg, TT-reinforced group, while the lowest one (35.2 s) was saved in CG.

Seminal Plasma Composition Analysis

Results of seminal plasma composition parameters are presented in Table 3. Table 3 shows the presence of no significant differences ($p>0.05$) among experimental groups for osmolality values. There was a clear relationship between seminal plasma osmolality and sperm motility. The osmolality of the seminal plasma and the motility of spermatozoa are mainly affected by the ionic content, pH and osmolality values surrounding the cells.

The suitability of fish sperm to gain motility in the testicles depends on the ion composition, osmolality and pH values of the seminal plasma in the reproductive canals.

Table 1. Effect of oral administration of *T. terrestris* extract on testosterone, FSH and LH of the male rainbow trout

	CG	TT100	TT250	TT500	p-value
Testosterone(ng/ml)	3.19[a]	3.59[a]	3.47[a]	3.44[a]	0.05
FSH (ng/ml)	5.17[a]	5.12[a]	5.37[a]	5.57[a]	0.05
LH (ng/ml)	3.59[a]	3.80[a]	3.95[a]	3.84[a]	0.05

Note: *Significant differences between groups (one-way ANOVA, p<0.05).

Table 2. Effect of oral administration of *T. terrestris* extract on semen parameters of male rainbow trout

	CG	TT100	TT250	TT500	p-value
Mean Motility (%)	85[a]	87[a]	92[ab]	93[ab]	0.05
Mean Motility duration (s)	21.75[a]	24.41[a]	27.16[b]	25.25[ab]	0.05
Mean vitality (s)	35.2 [a]	39.8 [ab]	40.4 [ab]	49.6 [b]	0.05

Note: *Significant differences between groups (one-way ANOVA, p<0.05).

Table3. Effect of oral administration of *T. terrestris* extract on seminal plasma composition of the male rainbow trout

	CG	TT100	TT250	TT500	p-value
Osmolality mOsm/kg	0.269[a]	0.284[a]	0.286[a]	0.288[a]	0.05
pH	7.39[a]	7.47[a]	7.55[a]	7.65[ab]	0.05

Note: *Significant differences between groups (one-way ANOVA, p<0.05).

Table 4. Effect of oral administration of *T. terrestris* extract on reproductive performance of the male rainbow trout

	CG	TT100	TT250	TT500	p-value
Fertilization	92.33[a]	96.11[b]	95.22[b]	94.33[a]	0.05
Incubation	80.55[a]	84.77[ab]	87.11[b]	84.11[ab]	0.05
Hatching	70.37[a]	75.25[ab]	80.25[b]	77.12[ab]	0.05

Note: *Significant differences between groups (one-way ANOVA, p<.0.05).

The pH values among all experimental groups showed significant differences (p<0.05). The highest pH value was recorded in TT500, while the lowest one was observed in CG.

Reproductive Performance

Results of the effect of dietary supplementation of *T. terrestris* extract on reproductive performance parameters (Table 4) showed the presence of significant differences (p<0.05) among experimental groups for fertilization, incubation and hatching. The greatest effect of *T. terrestris* on fertilization values was recorded in TT100 followed by TT250 and TT500, while the lowest value was observed in CG. In addition, while the highest value of incubation in TT250 was recorded, TT100, TT500 and CG followed it respectively. When the hatching values of male rainbow trout were observed, the highest value was recorded in TT250 followed by TT500, TT100 and CG.

DISCUSSION

This study aimed to investigate how different amounts of *T. terrestris* affect male rainbow trout's reproductive efficiency. To achieve this goal, the effects of *T. terrestris* were examined in the diet of *O. mykiss* males. This study revealed that *T. terrestris* extract significantly increased sperm quality, fertilization, incubation and hatching rates of trout.

T. terrestris is rich in bioactive compounds such as protodioscin and steroidal saponins and it was proposed that steroidal saponinsis the primary active constituent stimulating the production of hormone (Kavitha et al., 2011). According to the consequences of this present study, *T. terrestris* extract affects testosterone production and also has positive effect on LH values compared to control group. A variety of studies have been carried out to examine the

influences of *T. terrestris* supplementation causing changes in the hormonal values of living beings.

Moghaddam (2013) studied the effect of *T. terrestris*, a traditional Unani drug used to increase sexual activity, on the gonadotropin levels and sex hormone of dependent male rats fed a diet with the addition of *T. terrestris*. A significant increase in hormones ($p<0.05$) in the treated dependent group was reported and oral consumption of *T. terrestris* also caused to antagonize a significant decrease in sex hormones and gonadotropins. Besides, there are varieties of clinical research supported the findings of this study, has demonstrated that *T. terrestris* extract positively impact the concentration levels of hormones such as estradiol, improves reproductive function, libido and ovulation by minimally affecting testosterone (Dimitrova et al., 2012).

Cek et al. (2007) found a significant correlation between sperm reproduction and motility. It has been stated that increased sperm motility improves the reproductive performance of men; therefore, motility plays a crucial role in assessing the reproductive capacity. In this study, comparative results of spermatological features of motility were examined and accordingly, it was determined that *T. terrestris* supplemented diet with a dosage of 250 mg was the most effective amount for improving semen motility times in rainbow trout. Besides, according to the quantity given in the duration of the period of highest vitality 49.6 s (500 mg/kg, TT-reinforced group) and the lowest 35.2 s (control group) was recorded.

Similarly, Hassona et al. (2020) conducted a study to examine the influences of *T. terrestris* nutritional supplement on reproductive potential and growth performance of male Nile tilapia. In their study, sperm analysis, sperm concentration, viability and motility were investigated. The study revealed that *T. terrestris* had the most significant effect on semen parameters, respectively TT750, TT500 and TT250 as compared to the control group ($p<0.05$). Haghmorad et al. (2019) analyzed the viability and motility of semen in their study on improving fertility parameters by adding *T. terrestris* to the diets of male rats. The viability and motility of the semen was evaluated by the Eosin-Nigrosin method under the light microscope. Accordingly, it was observed that the viability and motility rate of the semen of rats fed with *T. terrestris* extract increased.

Studies have been conducted on the fact that the viability periods of spermatozoa change in various activation solutions (Jayaprakas & Bimal Lal, 1996). Khaleghi et al. (2017) found in laboratory studies on the improvement of sperm parameters in humans by *T. terrestris* extract. 40 volunteer healthy men were divided into 4 groups and incubated at 20, 40, 50 mg/ml *T. terrestris* respectively in three groups other than the control group. *T. terrestris* extract was found to be effective in inhibiting motility of sperm in comparison to control group. Additionally, the extract increased the number of progressive motile sperm in particular and improved the viability of spermatozoa. Grigova et al. (2008) conducted a study to examine the effects *T. terrestris* extract adding in the water of the breed on the quality of sperm of the white Plymouth rock. The results showed that the concentration of spermatozoid, sperm motility and the ejaculate volume of the chickens in the treatment applied by adding *T. terrestris* extract increased.

In this study, besides spermatological parameters, the results of *T. terrestris* extract according to fertilization rates were analyzed. As mentioned above, it was reported that fertilization rates were higher in all male rainbow trout fed with *T. terrestris* extract, but especially in those with 100 mg and 250 mg extract added. It is seen that the fertilization rates of the animals fed with *T. terrestris* extract are higher when compared to the control groups. Haghmorad et al. (2019) studied how *T. terrestris* extract influences the fertility abilities of male rats. It was observed that the hormones affecting fertilization increased significantly in rats fed with TT extract compared to the control group.

Another study conducted to investigate the effects of orally administrating an alcoholic extract of *T. terrestris* on fertility of male rabbits. The study comprised fifteen male rabbits of mixed breed, aged between 4-5 months and weighing 1.5–1.7 kg. It was reported that male rabbits' fertilization parameters were enhanced by the use of alcoholic extract at doses of 75 mg/kg/day, 150 mg/kg/day. The 150 mg/kg/day

dose was observed to be the most effective in improving male rabbits' fertilization level (Meeni, 2016).

The literature review confirmed that many plants are used as fertility regulators around the world (Bhatia et al., 2010). There are several medicinal plants that are commonly utilized as agents to improve fertility (Sumalatha et al., 2010). *T. terrestris* is one of these non-hormonal plant species with tremendous medicinal properties and has been shown to support fertilization by increasing sexual behavior (Gauthaman, 2002).

CONCLUSION

The present results demonstrated that *T. terrestris* supplementation of diet improves spermatological parameters of male rainbow trout. It was revealed that *T. terrestris* extract causes significant increase in the fertilization, sperm quality, and hatching rates of male rainbow trout. Considering the previous experience, it has been observed that it can be effective not only in the breeding season but also in the off-season production.

The results of this study have drawn attention to the importance of *T. terrestris* plant extract, used as an alternative to chemical, leads to increase the reproductive and fertilization performance of cultured fish of rainbow trout. The use of synthetic hormones for the stimulation of the reproduction of farm animals has numerous consequences. The use of hormones is sometimes ineffective and often causes permanent hypo function of the hypothalamic-pituitary-gonadal axis. Apart from this, synthetic hormones and their derivatives accumulate in animal products (milk, meat, etc.) and become dangerous for human health. Therefore, it is necessary to prefer ecologically clean reproductive stimulants in order to obtain high quality and reliable products for human health.

As mentioned, supplementation of phototherapeutic plants such as *T. terrestris* to the diets of cultured fish, which is also the subject of this study, improves gamete quality, supports the increase of fertilization affecting positively production. It was concluded that incorporating medicinal plants into fish can effectively contribute to sustainable, cost-effective and secure fish farming practices.

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Compliance with Ethical Standards

Authors' Contributions

DK: Collected and transported samples to the laboratory, researched and reviewed literature, designed and wrote manuscript, determined and organized sampling field, prepared and designed laboratory experiments, performed analyzed of data, drafted and also edited of manuscript.

BY: Supported literature review and performed drafting.

FÖ: Chose and determined of this study area, managed field sampling, controlled and checked laboratory experiments, performed and managed data analysis.

All authors read, checked and approved the last version of the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

The research has received approval from the relevant institutional and/or national ethics committee and it has been carried out in compliance with the ethical principles outlined in the 1964 Declaration of Helsinki, as well as any subsequent revision or equivalent ethical standards. The authors affirmed that they adhered to all pertinent international, national, and/or institutional protocols concerning the treatment and utilization of animals. It was declared that this study complies with research and publication ethics.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Escherichia coli: Germ Theory, A Bacterial Killer Mechanism, Virulence, Pathogenicity Islands (PAIs), Pathogenesis, Secretion Systems

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ABSTRACT

Why do bacteria damage their hosts? After bacteria bypass the immune system, bacterial virulence enables a host to replicate and propagate within a host in part by demolishing or escaping host defenses. Bacterial pathogens possess an array of specific killer mechanisms that submit virulence and the capacity to intercept host defence mechanisms. Mechanisms of virulence are often mediated by the subversion of normal aspects of host biology. Also, recently, three novels but wide themes have emerged in the field of bacterial virulence: a bacterial killing mechanism, secretion systems and pathogenicity islands. So, pathogen changes the host function so as to support the pathogen's survival or multiplication. Such subversion is often mediated by the specific interaction of bacterial effector molecules with host-encoded proteins and other molecules. Escherichia coli is a considerable and diverse micro alive. E. coli needs only to acquire a mix of mobile genetic elements to become a pathogen capable of causing diseases. The worldwide burden of these diseases is staggering, with hundreds of millions alive affected annually. E. coli strains have been well a bacteria model, and each uses an arsenal of virulence and toxin to subvert host cellular functions to reenforce its virulence. This review focuses on the drastic and different pathogenic mechanisms that are used by various E. coli strains.

INTRODUCTION

A bacterial killer mechanism, virulence, pathogenicity islands, pathogenesis, secretion systems are the pathogenicity of a microbe, that is, its ability to cause disease. The term pathogenicity is used in absolute terms for its ability to cause disease, while virulence is used to express the degree to which a pathogen is capable of causing disease (A Latin Dictionary, 2009). The factors that determine the ability of bacteria to cause disease are the number of infecting bacteria, way of entry into body, effect of defense mechanisms of host organism and unique characteristics of bacteria called virulence. Host-mediated pathogenesis may also be important, as host organism's defense mechanisms can sometimes damage host tissues while preventing infection. Disease mechanisms: Adhesion. First stage of most bacterial diseases is attachment of bacteria to cell surface. Many bacterial and host molecules associated with the attachment of bacteria to host cells have been identified. Receptor to which most bacteria bind is

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essential for the normal functions of the host cell. colonization. Some virulent bacteria secrete special proteins to invade certain parts of the host's body. Infestation (first action to attack and deconstruct the host cell). Some virulent bacteria disrupt the host cell membrane or cause the bacteria to be taken up into host cell by endocytosis. This virulence allows bacteria to enter host cells, so that it is possible for bacteria to enter body through epithelial tissue on body surface. Immune response inhibitors. Bacteria produce virulence that disrupt functioning of host organism's immune system. toxins. Virulence is proteins that poison host cells and cause tissue damage (Levin & Bergstrom, 2000; Freeman et al., 2007).

Escherichia coli is a Gram negative (Gram$^-$) bacillus known to be a part of normal intestinal flora but can also be the cause of intestinal and extraintestinal illness in humans. Its virulence lends to *E. coli*'s ability to evade host defenses and develop resistance to common antibiotics. Actually, 171 somatic (O), 55 flagellar (H) and 80 capsular (K) antigens have been identified, and there are over 160 serological types of *E. coli* (Gambushe et al., 2022).

Bacterial genomes generally consist of stable regions termed core genome, and variable regions that form the so-called flexible gene pool. Flexible part is composed of bacteriophages, plasmids, transposons as well as unstable large regions that have been termed genomic islands. Genomic islands encoding virulence of pathogenic bacteria have been designated pathogenicity islands. Pathogenicity islands were first discovered in uropathogenic *E. coli* and presently more than 30 bacterial species carrying pathogenicity islands have been described (Hacker et al., 2003).

Diseases will be described by the causative *E. coli* subtypes, including enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), which is also known as Shiga toxin-producing *E. coli* (STEC) and will be referred to as *E. coli* EHEC/STEC, enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC) (Manatsathit et al., 2002; Yang et al., 2023).

This review summarizes current knowledge on *E. coli* pathogenesis and their general features, and discusses their putative role in toxicology of *E. coli* in light of virulence of *E. coli*. Also, this review will discuss *E. coli* infections into those causing diseases.

Escherichia coli

Escherichia coli is Gram$^-$ scilicet its cell wall is composed of a thin peptidoglycan layer and an outer membrane. During the staining process, *E. coli* picks up the color of the counterstain safranin and stains pink at Gram-stained smear under microscope (Amanze et al., 2022). Facultative anaerobic, rod-shaped, coliform bacteria that is commonly found in the gut of humans and warm-blooded animals. Most strains of *E. coli* are harmless. However, some serotypes (EPEC, ETEC etc.) can cause severe food poisoning, and are occasionally responsible for contamination (Basavaraju & Gunashree, 2022).

Bacterial conjugation, genetic recombination, operon concepts were first discovered in *E. coli*, many important molecular biology mechanisms (DNA replication, RNA transcription, protein synthesis). At least ten Nobel Prizes are based on research in *E. coli* (Peng et al., 2006). *E. coli* was determined to divide into 2 in 20 minutes (Clark & Maaløe, 1967).

The bacteria are 1-2 μm long and 0.1-0.5 μm in diameter (Yang & Li, 2005). It does not form endospores, it dies by pasteurization and boiling. It reproduces best at body temperature, as it is adapted to grow in the intestines of mammals.

Colonies of *E. coli* on MacConkey agar plate are pink to dark pink, dry and donut-shaped, surrounded by a dark pink area of precipitated bile salts (Onyeberechiya et al., 2021). *E. coli* perform dark pink colony on Flexicult Vet Agar (Cugmas et al., 2021), dark yellow colony on Sorbitol-MacConkey (Schuetz, 2019) and navy blue pigment colony on chromogenic coliform agar (Sawicka et al., 2022). In addition, *E. coli* that is labelled with DsRed-Express2, red fluorescent protein (Smith & Schuster, 2021). *E. coli* O157 on Biosynth™ Agar which contains tellurite at 0.1 μg ml^{-1} (Taylor, 1999). Lactose fermentation produces acids, which lower the pH. This encourages dye absorption by the colonies, which are now colored purple-black. Lactose non-fermenters may increase the pH by deamination of proteins. This ensures that the dye is not absorbed. The colonies will be colorless.

If *E. coli* is grown in Eosin methylene blue (EMB, alos know as ''evine's formulation'') it will give a distinctive metallic green sheen (due to metachromatic properties of the dyes, *E. coli* movement using flagella, and strong acid end-products of fermentation) (Oh & Eom, 2021).

E. coli (STEC/VTEC), which produces Shiga or Vega toxin, is bacteria that can cause serious diseases. *E. coli* STEC is heat sensitive so that be sure to follow basic food hygiene rules when preparing meals. Adhering to WHO "Five keys to safer food" is virtually measure to prevent infections with foodborne pathogens such as *E. coli* STEC (WHO, 2018). *E. coli* is bacteria widespread found in the guts of humans and warm-blooded animals. It is transmitted to humans primarily through the consumption of contaminated foods such as raw or undercooked ground meat products, raw milk, and contaminated raw vegetables and sprouts. *E. coli* STEC grow for optimum temperature is 37°C and so can grow at temperatures between 7°C and 50°C. *E. coli* STEC is destroyed by thoroughly cooking all parts of food until it reaches temperature of 70°C or higher. *E. coli* O157:H7 is virtually health *E. coli* STEC serotype; but other serotypes have often been implicated in sporadic cases and outbreaks (WHO, 2018).

Transmission: most available information on *E. coli* STEC relates to serotype O157:H7, since it is easily differentiated biochemically from other *E. coli* strains. *E. coli* O157:H7 is transmitted to humans primarily through consumption of contaminated foods. Fecal contamination of water and foods, as well as cross-contamination during food preparation, will also lead to infection. Examples of foods implicated in outbreaks of *E. coli* O157:H7 include undercooked hamburgers (WHO, 2018).

E. coli Structure

Flagella is peritrich feature. It is controlled by 20 genes. *hag* gene determines primary sequence of flagellin, *fla* gene encodes other flagella consist of 10 protein, and *mot* gene is responsible for flagella function (Samiei et al., 2023). Cell-wall of *E. coli* consist of lipopolysaccharide (endotoxin) structures. This endotoxin to induce pyrogenic response and stimulate intravascular coagulation is important in symptomatology of *E. coli*-induced septicemic diseases. It also plays role in resistance to phagocytosis. Capsule of *E. coli* produce polysaccharides. Polysaccharide is produced both virulence and *in vivo*. It increases invasiveness, increases resistance to serum neutralization and polymorph phagocytosis (Rathore et al., 2022). Pilus are heat-sensitive, surface-associated proteins that are antigenically unrelated to "O" and "H" antigens which are thin protein filaments. It has adhesive properties. Type 1 battery forms most of pathogenic and apathogenic *E. coli.*

E. coli ETEC has specialized pilus that is antigenically unrelated to common pilus (type 1 pili), which acts as ligand for bacterial cell binding to specific complex carbohydrate receptors on small intestinal epithelial cell surfaces, and this interaction results in colonization of intestine. These pili are called Colonization Factor Antigens (CFA). While most ETEC isolates produce both CFA/I, CFA/II and CFA/IV, CFA/III and a number of other unidentified CFAs occur on other specific serotypes. The CFA-type pili plays an important role in host specificity.

The genes encoding CFA production are found on ETEC virulence plasmids and usually on the same plasmids carrying one or both types of *E. coli* enterotoxin (LT and ST) genes. Most *E. coli* ETEC diarrheas are caused by CFA and *E. coli* with both enterotoxins, less by *E. coli* with CFA and only one toxin (usually LT); and rarely by *E. coli* that do not have CFA but only ST. K99, K88, 987P, F41, colonization factor 1 and 2 are some important pili (Fındık, 2023). Virulence structures of *E. coli* such as flagella, cell wall, colicins, enterotoxins, capsule, cytotoxins, pili, hemolysin and aerobactin etc. have both apparent and potential effects on virulence in the tissues (Berne et al., 2015).

Germ Theory

The germ theory of disease — infectious disease is primarily caused by transmission of an organism from one host to another — is a gross oversimplification (Stewart, 1968). Germ theory of disease is currently accepted scientific theory for many diseases. It states that pathogens or germs can lead to disease. These

small organisms, too small to be seen without magnification, invade living hosts. Their growth and reproduction within their hosts can cause disease. Germ refers to not just bacteria but to any type of microorganism, such as protists or fungi, or even non-living pathogens that can cause disease, such as viruses, prions, or viroids (Oxford Dictionaries, 2016). Population size required by pathogen to infect host cell is called Median Infection Dose (ID). To measure this, method called ID_{50} is used. That is, what is number of bacteria that allows us to see signs of infection in at least 50% of subjects? The answer to this question determines ID_{50} value (Ramesh et al, 2020). In science, Median Lethal Dose (LD) of pathogen is measured by method called LD_{50}, just like ID_{50}. This is known as dose at which pathogen causes at least 50% of subjects to die. A critical point here is size of host organism. Naturally, larger numbers of pathogens may be needed to infect larger organisms. For this reason, sometimes ID_{50} and LD_{50} values are given based on measure known pathogen per fragment. This can be expressed micrograms/milligrams/nanograms per kilogram (LibreTexts, 2023).

Koch's Postulates and Molecular Koch's Postulates

Robert Koch published four criteria postulates that summarized his method for determining whether particular microorganism was cause of particular disease, in 1884. Each of Koch's postulates represents criteria that must be met before disease can be positively linked with pathogen. In order to determine whether criteria are met, tests are performed on laboratory animals and cultures from healthy and diseased animals are compared (LibreTexts, 2023). Koch postulates four criteria for establishing causal relationship between disease and microbe. Criteria, which were first put forward by Robert Koch and Friedrich Loeffler in 1884, were revised and published in 1890 (Koch, 1893).

Koch's Postulates: 1. Suspected pathogen must be found in every case of disease and not be found in healthy individuals. 2. Pathogen should be separated (isolated) from diseased organism and grown in pure culture. 3. Pathogen in culture should cause disease when transplanted into a healthy organism. 4. Pathogen must be re-isolated from vaccinated, diseased experimental host organism and found to be the same as original specific causative agent (Koch, 1893).

In 1988, Stanley Falkow (1934–) proposed revised form of Koch's postulates known as molecular Koch's postulates. The premise for molecular Koch's postulates is not in ability to isolate a particular pathogen but rather to identify gene that may cause organism to be pathogenic (LibreTexts, 2023).

Falkow's modifications to Koch's original postulates explain not only infections caused by intracellular pathogens but also existence of pathogenic strains of organisms that are usually nonpathogenic. Predominant *E. coli* is a member of normal microbiota of host intestine and is generally considered innocuous. However, there are pathogenic strains of *E. coli* such as enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (O157:H7) (EHEC). We now know ETEC and EHEC exist because of acquisition of new genes by once-harmless *E. coli*, which, in form of these pathogenic strains, is now capable of producing toxins and causing disease. Pathogenic state resulted from minor genetic changes (LibreTexts, 2023).

Pathogenicity Islands (PAIs)

Pathogenicity islands (PAIs), as distinct type of genetic element, were described for first time for pathogenic *E. coli* strain (O6:K15:H31) (Blum et al., 1994; Hacker et al., 1983), which is the model organisms of extraintestinal pathogenic *E. coli* (ExPEC) used for studies on ExPEC pathogenesis and evolution of bacterial pathogens. The PAIs type of genetic elements is characterized by large size (>10 kb), presence of virulence-associated genes, frequent association with tRNA-encoding genes or other *att* sites for temperate bacteriophages, and G+C content different from that of rest of chromosome. These elements are frequently flanked by repeat structures and carry many fragments of other mobile and accessory genetic elements, such as Insertion Sequence (IS) elements, bacteriophages, and plasmids. Some PAIs are unstable regions and can spontaneously disappear from chromosome. Therefore, PAIs are considered to have evolved from mobile genetic elements by gene transfer. It can also

be assumed that these DNA regions, since their acquisition, underwent and will continue to undergo further evolutionary changes, resulting in an ongoing evolution of bacterial pathogens (Hacker & Kaper, 1999; Hacker et al., 1999; Dobrindt et al., 2002).

Secretion Systems

Bacterial secretion systems are protein complexes present on cell membranes of bacteria for secretion of substances. Specifically, they are cellular devices used by pathogenic bacteria to secrete their virulence to invade host cells (Trivedi et al., 2022). They can be classified into different types based on their specific structure, composition and activity. Generally, proteins can be secreted through two different processes. One process is one-step mechanism in which proteins from cytoplasm of bacteria are transported and delivered directly through cell membrane into host cell. Another involves two-step activity in which proteins are first transported out of inner cell membrane, then deposited in periplasm, and finally through outer cell membrane into host cell (Bocian-Ostrzycka et al., 2017).

E. coli on lettuce leaves congregate at the stomata. As a result of the consumption of these leaves, the number of bacteria constantly increasing, escapes from the immune system and creates a basis for the disease. Their presence may constitute a health hazard and cause severe food poisoning, particularly if the bacteria are toxigenic (Kotzekidou, 2016). They may get onto the vegetables if the water used for irrigation is contaminated with fecal material. Although thorough washing is recommended, Scanning Electron Microscopy (SEM) shows that the bacteria enter the leaves through the stomata and would resist being removed. Similar bacterial contamination was also found with spinach and green onions (Saldaña et al., 2011).

A Bacterial Killer Mechanism of *E. coli*

There is great diversity within the *E. coli* species, and it has even been shown by modern biotechniques that members of *Shigella* and *Salmonella* families are actually subtypes of *E. coli*. Within the *E. coli* strains have different characteristics. There may be small mutations that make them different from each other, or presence or absence of all gene, or even many genes. These genes are found in bacteriophages, transposons, or plasmids, and they are transmitted from other bacterial species to *E. coli* (Peng et al., 2006).

Among genes that differentiate strains are disease virulence. Shiga toxin gene carried by strain O157:H7 was passed on to *E. coli* from *Shigella*. Some of features that cause *E. coli* to cause disease are listed below. Not all of them go together, certain *E. coli* strains have certain combinations of these factors (Peng et al., 2006).

Pilus or fimbriae are hair-like structures on bacteria that allow it to start on certain surfaces. Although harmless *E. coli* also have pili, specialized pili found in *E. coli* ETEC types enable them to attach to small intestinal epithelial cells. In this way, bacteria are not excreted in stool, but settles in small intestine and can multiply there. That's why these types of pili are called colonization factors (Colonization Factor Antigen, CFA). These factors are specific to the host organism and determine in which animals the bacteria can multiply. Other types of pili connect to urinary tract cells or bladder cells, so they cause urinary tract infections. Exotoxins, ST exotoxin, which causes diarrhea caused by ETEC types, prevents epithelial cells from absorbing water, while LT exotoxin causes cells to secrete water and electrolytes. EHEC type bacteria do not have ST and LT exotoxins, they secrete Shiga toxin, this toxin leads to the death of intestinal epithelial cells, so the intestine loses its ability to absorb water, result is a bloody diarrhea. Capsule is additional protective layer outside cell, preventing body's protection mechanisms from recognizing and destroying the bacteria. Hemolysis enables the breakdown of red blood cells; the released iron is a food source for bacteria. Siderophores serve to collect iron in blood. It provides the iron that bacteria need to grow in bleeding diarrhea and systemic infections. The K1 antigen prevents phagocytosis of bacteria. Endotoxin is glycolipid found in cell membrane, body's strong response to it plays virtually role in inflammation (Peng et al., 2006; Pokharel et al., 2023).

Septicemic *E. coli* (SEPEC) strains are responsible for septicemia in hosts. It has been determined that there are alternative virulence factors in every step of

the disease process and that they can use combinations of these virulence especially for the pathogenicity of invasive strains. The first step in the invasion process is attachment to the intestinal surface. Adhesion may be mediated by fimbrial adhesives. Like F5 in ETEC strains, on the other hand, long polar fimbriae or non-fimbrial attachment is an example. Septicemic strains carry Col V plasmid encoding Colicin V. This plasmid encodes type IV pilus, which has been shown to be important for invasion and attachment in *Salmonella typhi*. In addition, this plasmid regulates serum resistance and the aerobactin iron uptake system, both of which play important roles for the survival of *E. coli* strains (Moulin-Schouleur et al., 2007).

DISCUSSION

These disciplines, in conjunction with advances in molecular biologic techniques, have led to striking advances in our understanding of molecular pathogenesis of infection and role of an ever-widening array of potential bacterial virulence. In their comprehensive review in this topic of Critical Care, Webb and Kahler, 2008 speculate that targeting virulence may be an attractive therapeutic strategy. Leaving aside the issues of drug formulation and delivery, what is required for identification of 'drugable' virulence target?

The observation that important virulence is present in very similar forms in different bacteria may be explained by horizontal gene transfer. Different scenarios can be considered to explain the transfer between bacterial strains and species.

Washing hands, paying attention to cleaning rules while preparing food, choosing well-cooked meat, pasteurized milk and dairy products, well-washed vegetables, drinking clean water, taking a shower before and after entering pool, ensuring that children do not swallow water in pool, these bacteria can on food to grow at 70°C conditions. So, it can be destroyed by cooking at this temperature and above, clean toilets and washing hands with soap at entrance and exit are important in protection from pathogens.

A piece portraits of *E. coli* being infected by T4 bacteriophages. These T4 bacteriophages viruses use a machinery to inject their acid nucleic content into the host, where it will be replicated and transcripted. Eventually, this results in the reproduction of phages and lysis of the cell host. Thus, the host cell structure is disrupted and disease occurs (Hampton et al., 2020).

E. coli plays such an influential role in the world of microbiology that it is a model strain and also a model is on display. A model of *E. coli* at ''The Bacterial World'' exhibition a few years ago at the Oxford Natural History Museum (Figure 1). This 28-metre-long inflatable *E. coli* sculpture was created by artist Luke Jerram in collaboration with researchers from the University of Sheffield, who loaned it to the Museum for the Bacterial World exhibition. Although it has a bad reputation for making people ill, there are millions of *E. coli* living harmlessly in your gut right now, keeping more dangerous bacteria at bay. *E. coli* are also vital in medical research. Described as the 'workhorse' of biomedicine, these bacteria are used by scientists as tiny bio-factories, making useful products for research, medicine and industry (Oumnh.ox.ac.uk 2023).

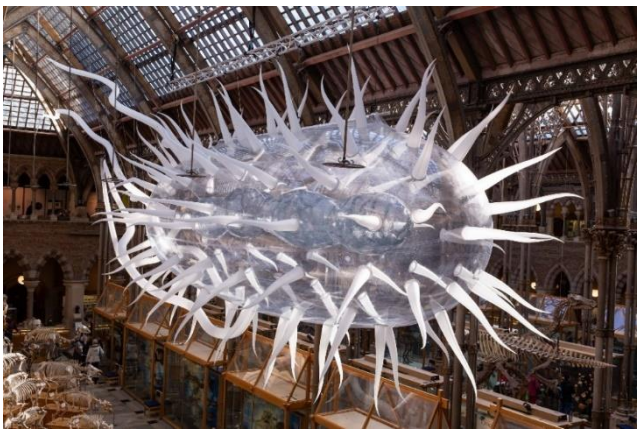

Figure 1. 28-metre-long inflatable *E. coli* sculpture that was created by artist Luke Jerram in collaboration with researchers from the University of Sheffield, who loaned it to the Museum for the Bacterial World exhibition (Oumnh.ox.ac.uk, 2023).

E. coli is one of the three living things that are the top models of science most commonly used in the laboratory (Blount, 2015).

It is essential to know all lethal effect mechanisms of such an effective and strong pathogen and to investigate this pathogen, which is increasing its pathogenicity day by day with genetic instruments, in

terms of reducing its role in infection world. The ability to obtain complex virulence traits in one genetic event, rather than by undergoing natural selection for many generations, provides mechanism for sudden radical changes in bacterial-host interactions. Secretion systems, a bacterial killing mechanism and PIs must have played critical roles in evolution of known pathogens and are likely to lead to emergence of novel infectious diseases in future.

CONCLUSION

E. coli innocuous bacteria can obtain a mixture of wide mobile genetic elements, virulence, toxin, secretion, pathogenic island becoming an emerging living creature pathogen capable of causing a broad spectrum of intestinal and extraintestinal diseases. E. coli pathotypes have been well characterized, causing important diseases. *E. coli* operate many effectors that overthrow cells. Synthesis, specific gene transcription, secretion of diverse micro/macro-molecules and ions, cytoskeleton rearrangement, apoptosis, autophagy, mitochondrial activities, cell division, and signal transduction in epithelial intestinal and extraintestinal host cells are affected by *E. coli*.

Compliance with Ethical Standards

Authors' Contributions

NF: Manuscript design, Writing.

FF: Draft checking, Reading, Editing.

Both authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

Data Availability Statement

Data availability is not applicable to this article as no new data were created or analyzed in this study.

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Impact of Different Nutrient Enrichment Concentrations on the Growth of Microalga *Nannochloropsis* sp. (Monodopsidaceae) Culture

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ABSTRACT

Microalgae consist of unicellular algal species that can produce and accumulate a wide variety of biomolecules. In order to maintain a high cell density in a continuous phototrophic culture of algae, the nutrient can serve as the most important factor in enhancing cell density. In this study, the effect of different concentrations of nutrients on the cell density of microalga *Nannochloropsis* sp. cultured in the mega plastic box (with 50 L capacity) was investigated. Four groups of treatment with four replicates were tested: group A (including 5 g L^{-1} of ferric chloride, ammonium phosphate, and Urea), group B (including 10 g L^{-1} of ferric chloride, ammonium phosphate, and urea), group C (including 15 g L^{-1} of ferric chloride, ammonium phosphate, and urea), and group D (including 15 g L^{-1} of ferric chloride, ammonium phosphate, and urea with 40 g L^{-1} of cow manure). Results revealed that group C and group D achieved maximum density on day three as 86.39×10^6 cell mL^{-1} and 85.59×10^6 cell mL^{-1}, respectively, which were significantly ($p\leq0.05$) higher than the cell density of groups A (58.01×10^6 cell mL^{-1}) and group B (70.67×10^6 cell mL^{-1}). Additionally, the increasing specific growth rate (SGR) of *Nannochloropsis* sp. cultured was obtained in group D at 0.308 day^{-1} after the culture period. From the result of the study, it is concluded that the concentrations of 15 g L^{-1} ferric chloride, ammonium phosphate, and urea (group C) and 15 g L^{-1} ferric chloride, ammonium phosphate, and urea combined with 40 g L^{-1} cow manure (group D) are capable of increasing cell density growth of microalga *Nannochloropsis* sp. cultured in a mega plastic box.

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INTRODUCTION

Microalgae can be used in many biotechnological applications, including health foods, feeds, colorants, bioenergy, and pharmaceuticals (Pulz & Gross, 2004; Mortensen, 2006; Chu, 2012; Dixit & Suseela, 2013). Microalgal compounds are particularly valuable for finding new medicines that treat viral infections, cancers, and bacteria and fungi showing resistance to antibiotic treatments (Skulberg, 2000; Huleihel et al., 2001). In addition, various microalgae contain DHA (docosahexaenoic acid), which contributes to heart health in adults and brain development in babies (Pulz & Gross, 2004). In addition to their role in photosynthesis, microalgal pigments have a wide range of biological properties, including antioxidants (Hejazi et al., 2004; Chidambara-Murthy et al., 2005), anti-obesity, anti-angiogenic, anti-cancer, and neuroprotective properties (Ciccone et al., 2013), as well as an anti-inflammatory (García-González & Ochoa, 1999; Guzman et al., 2001). Growing concern about human nutrition has led to controversy regarding microalgae in poultry and fish (Souza et al., 2021). Furthermore, researchers examined how microalgae can be cultivated rapidly and produced in large quantities (Duong et al., 2012; Leu & Boussiba, 2014; Mohan et al., 2015). Various culturing systems have been used to cultivate microalgae at large scales under controlled conditions, including polyethylene sleeves, outdoor ponds, and tubular bioreactors (Lebeau & Robert, 2003; Benner et al., 2022). Moreover, microalgal growth is influenced by environmental parameters such as temperature, light intensity, and photoperiod in culture systems (George et al., 2014). There are a number of factors that affect microalgae growth and biochemical composition, including nutrients (Juneja et al., 2013; Barkia et al., 2019). The manure of terrestrial animals can have significant effects on plants, including macroalgae (Jjemba, 2002; Bogaard et al., 2007; Giwa, 2017). However, the studies related to the production of microalgae with manure and other inorganic nutrients are very limited. Thus, this study examines the effect of different concentrations of nutrients along with cow manure on the growth of *Nannochloropsis* sp. culture.

MATERIAL AND METHODS

Study Area

The experiment was conducted at the Multi-species Hatchery, situated in the College of Fisheries, Mindanao State University-Tawi-Tawi College of Technology and Oceanography (MSU-TCTO), Sanga-Sanga, Bongao, Tawi-Tawi, Philippines (05°02'3.8" N 199°44'5.0" E) on March 23-27, 2022.

Culture Condition

Mega plastic boxes with 50 L volume were used in *Nannochloropsis* sp. experiment. Chlorinated seawater was used in this study and filtered through a filter bag before being transported to 16 mega boxes containing 45 liters each. After the 16 mega boxes were filled with chlorinated seawater, starter green microalgae of *Nannochloropsis* sp. with 5 L volume inoculated into the culture container. Mega plastic boxes were then mixed with four different nutrient concentrations randomly (Table 1) with four replicates for each treatment. The mega plastic boxes were covered with transparent acetate film to avoid contamination, and fluorescent lamps (Ecolum LED) were used as artificial lighting for the cultures (Figure 1).

Analysis

A daily sample of *Nannochloropsis* sp. culture was taken for cell counting and analysis. The number of cells was counted by using a hemocytometer, and contamination was investigated under a microscope. Specific growth rates (μ) were calculated by following the formula (Durmaz & Erbil, 2017);

$$\mu = \frac{\ln(N_2)-\ln(N_1)}{t_2-t_1} \quad (1)$$

where: N_2 is the biomass cell number at the time (t_2). N_1 is the beginning biomass cell number at the time (t_1).

IBM SPSS software version 20 was used to analyze the significance of differences between the growth using one-way analysis of variance (ANOVA). Post hoc (Duncan) was used to rank the means.

Table 1. Different concentrations of nutrients of ferric chloride, ammonium phosphate, urea, and cow manure that used in four different experimental treatment groups

Treatment	Ferric Chloride (g L^{-1})	Ammonium Phosphate (g L^{-1})	Urea (g L^{-1})	Cow Manure (g L^{-1})
Group A	5	5	5	0
Group B	10	10	10	0
Group C	15	15	15	0
Group D	15	15	15	40

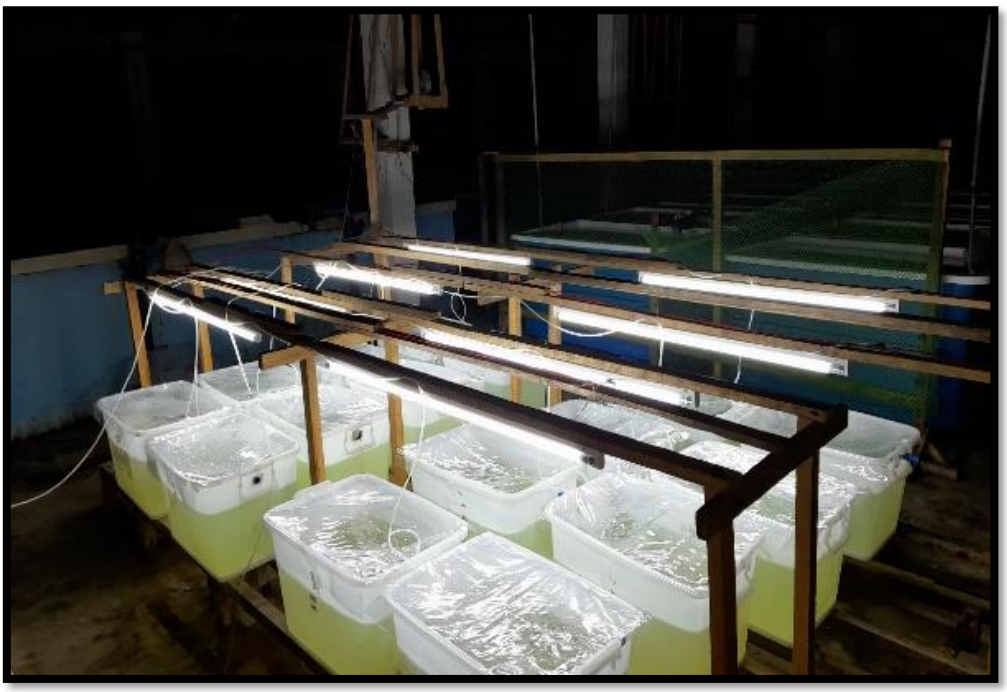

Figure 1. Experimental set-up

RESULTS

The initial cell density of *Nannochloropsis* sp. was arranged as 3.20×10^6 cell mL^{-1} (Figure 2). The microalga *Nannochloropsis* sp. was enriched with different concentrations of nutrients. All of the experimental groups reached the maximum cell density on the 3rd day of culture. The maximum cell density of groups C and D on 3rd day of the culture period was 86.39×10^6 cell mL^{-1} and 85.59×10^6 cell mL^{-1}, respectively, which was significantly ($p\leq0.05$) higher than the cell density of groups A and B at 58.01×10^6 cell mL^{-1} and 70.67×10^6 cell mL^{-1}, respectively. The lag phase was observed on the first day for all the group treatments. After the first day, the cell density of *Nannochloropsis* sp. in group A increased rapidly from 29.55×10^6 cell mL^{-1}, reaching 50.01×10^6 cell mL^{-1}, while in group B, the cell density increased from 27.59×10^6 cell mL^{-1} to 70.67×10^6 cell mL^{-1} by day 3 without any apparent lag phase. Moreover, the cell density in group C was increased from 27.97×10^6 cell mL^{-1} to 86.39×10^6 cell mL^{-1}, while in group D it was increased from 31.05×10^6 cell mL^{-1} to 85.59×10^6 cell mL^{-1} at the 3rd day. Furthermore, the specific growth rate (SGR) of *Nannochloropsis* sp. culture is shown in Figure 3. The SGR of *Nannochloropsis* sp. culture in group D (0.308 ± 0.003 day^{-1}) was increased than the SGR in group A (0.170 ± 0.011 day^{-1}), B (0.173 ± 0.011 day^{-1}), and C (0.188 ± 0.019 day^{-1}) at the end of culture period.

DISCUSSION

The primary aim of producing phototrophic organisms is to maintain a high cell density in a continuous culture. In the present study, green microalga *Nannochloropsis* sp. was investigated in

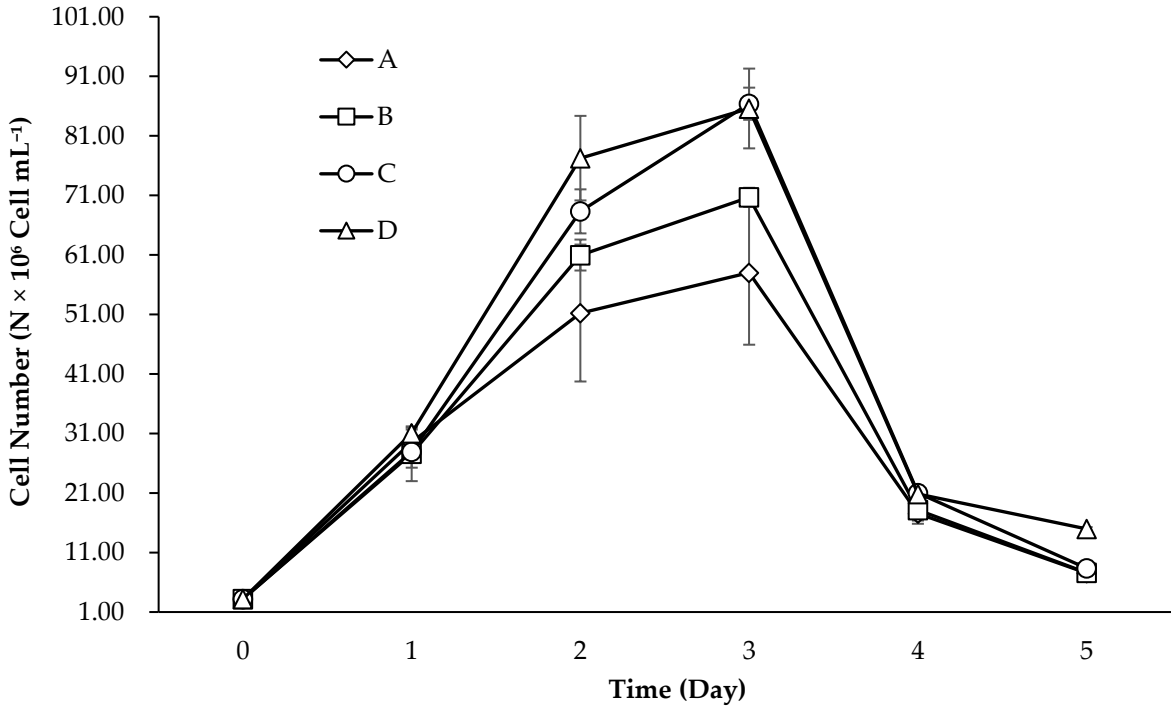

Figure 2. Cell densities of different concentrations of nutrient enrichment. A = (5 g L^{-1} of ferric chloride, ammonium phosphate, and Urea); B = (10 g L^{-1} of ferric chloride, ammonium phosphate, and Urea); C = (15 g L^{-1} of ferric chloride, ammonium phosphate, and Urea); D (15 g L^{-1} of ferric chloride, ammonium phosphate, and Urea with 40 g L^{-1} of cow manure), N=16.

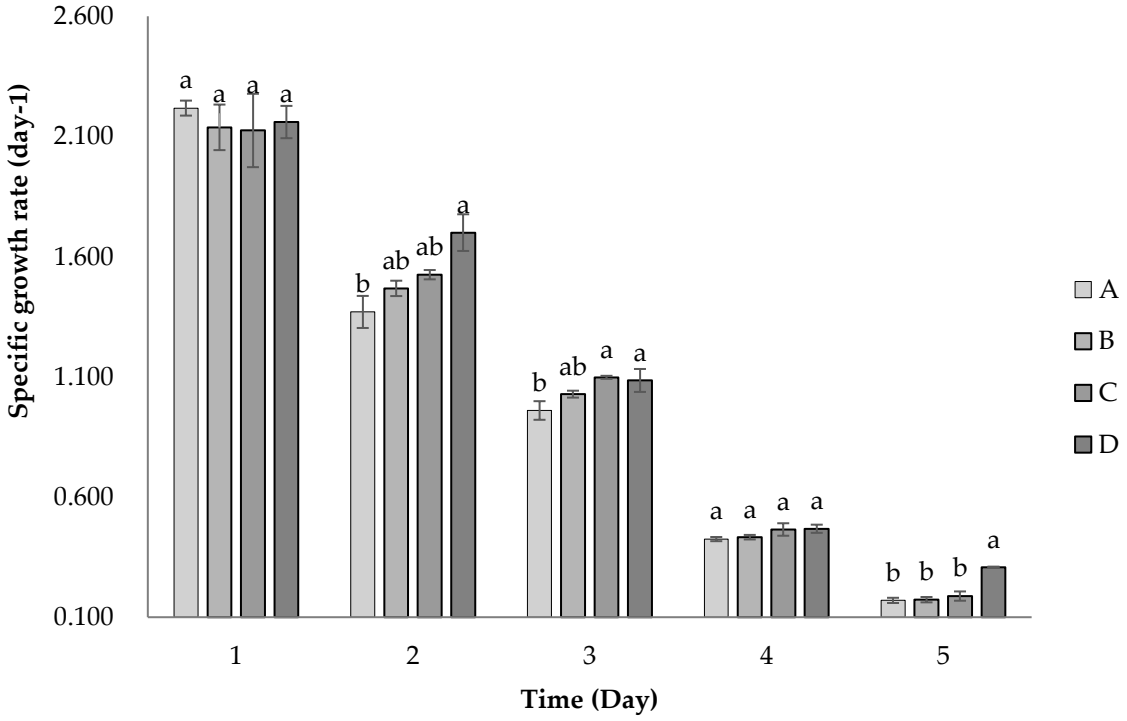

Figure 3. Specific growth rate of different concentrations of nutrient enrichment. A = (5 g L^{-1} of ferric chloride, ammonium phosphate, and Urea); B = (10 g L^{-1} of ferric chloride, ammonium phosphate, and Urea); C = (15 g L^{-1} of ferric chloride, ammonium phosphate, and Urea); D (15 g L^{-1} of ferric chloride, ammonium phosphate, and Urea with 40 g L^{-1} of cow manure). Bar with the same letters is not significantly different ($p \leq 0.05$), Error bars in SEM (standard error mean) N=16.

response to different concentrations of nutrients. Utilizing 15 g L^{-1} ferric chloride, ammonium phosphate, and urea (group C) and 15 g L^{-1} ferric chloride, ammonium phosphate, and urea combined with 40 g L^{-1} cow manure (group D), the maximum cell density reached 86.39×10^6 cell mL^{-1} and 85.59×10^6 cell mL^{-1}, respectively, culture in a mega plastic box (50 L capacity). While increasing SGR of *Nannochloropsis* sp. culture was obtained in group D at 0.308 day^{-1} after the culture period. Another study conducted bag cultivation (50 L) for microalga *Nannochloropsis oculata* cultured in F/2 medium enriched with different concentrations of nitrogen sources such as sodium nitrate ($NaNO_2$) and ammonium chloride (NH_4Cl) where the highest number of cells was given as 52×10^6 cell mL^{-1} and 49×10^6 cell mL^{-1}, respectively, at 881 µmol L^{-1} (Durmaz, 2007). As a result of microalga *N. oculata* culture in a 60 L photobioreactor, harvest cell density in F/2 medium supplemented with 1.76 µmol L^{-1} of nitrogen was 52.8×10^6 cell mL^{-1} (Huang et al., 2013). Low & Toledo (2015) examined that the cell density from 80 L bag culture of microalga *N. oculata* has been harvested at an approximate concentration of 4.55×10^6 cell mL^{-1}. Additionally, fiberglass reinforced plastic panel photobioreactor for microalga *N. oculata* cultured in F/2 medium obtained the highest density of 245×10^6 cell mL^{-1} (Durmaz & Erbil, 2020). It has also been reported that *N. oculata* cultured in 6 L flasks enriched with F/2 medium and supplemented with 100 mg L^{-1} and 500 mg L^{-1} concentrations of myo-inositol achieved maximum densities of 101.00×10^6 cell mL^{-1} and 91.33×10^6 cell mL^{-1}, respectively (Erbil & Durmaz, 2020). Other researchers stated that microalga *Porphyridium cruentum* cultured in flat bottom flasks (1 L) enriched with F/2 medium and combined with 0.585×10^{-5} M ferric chloride achieved a maximum density of 5.39×10^6 cell mL^{-1} (Erbil et al., 2022). Furthermore, it has been demonstrated that high-nutrient manures, such as pig and poultry manures and bi-products of anaerobic digestion, may be viable sources of nutrients for microalgae cultivation (Fenton, 2012; Lu & Xiao, 2022). Our study demonstrated that a combination of cow manure and other inorganic nutrients, such as ferric chloride and ammonium phosphate, significantly increased the microalgae cell density.

CONCLUSION

Nannochloropsis sp. is an important species in aquaculture hatcheries because of its phototrophic nature, enabling a continuous culture of high cell-density organisms. As a result of the present study, the maximum cell density growth of *Nannochloropsis* sp. was significantly increased in both the 15 g L^{-1} ferric chloride, ammonium phosphate, and urea (group C) and the 15 g L^{-1} ferric chloride, ammonium phosphate, and urea combined with 40 g L^{-1} cow manure (group D) at three days of the culture period.

Compliance with Ethical Standards

Authors' Contributions

NBS: Manuscript design, laboratory experiment, and draft checking.

MDH, CTN, RJFR, HAI, JSM, & JHE: Manuscript design and draft checking.

JHS: Statistical analyses, writing, draft checking, reading, and editing.

All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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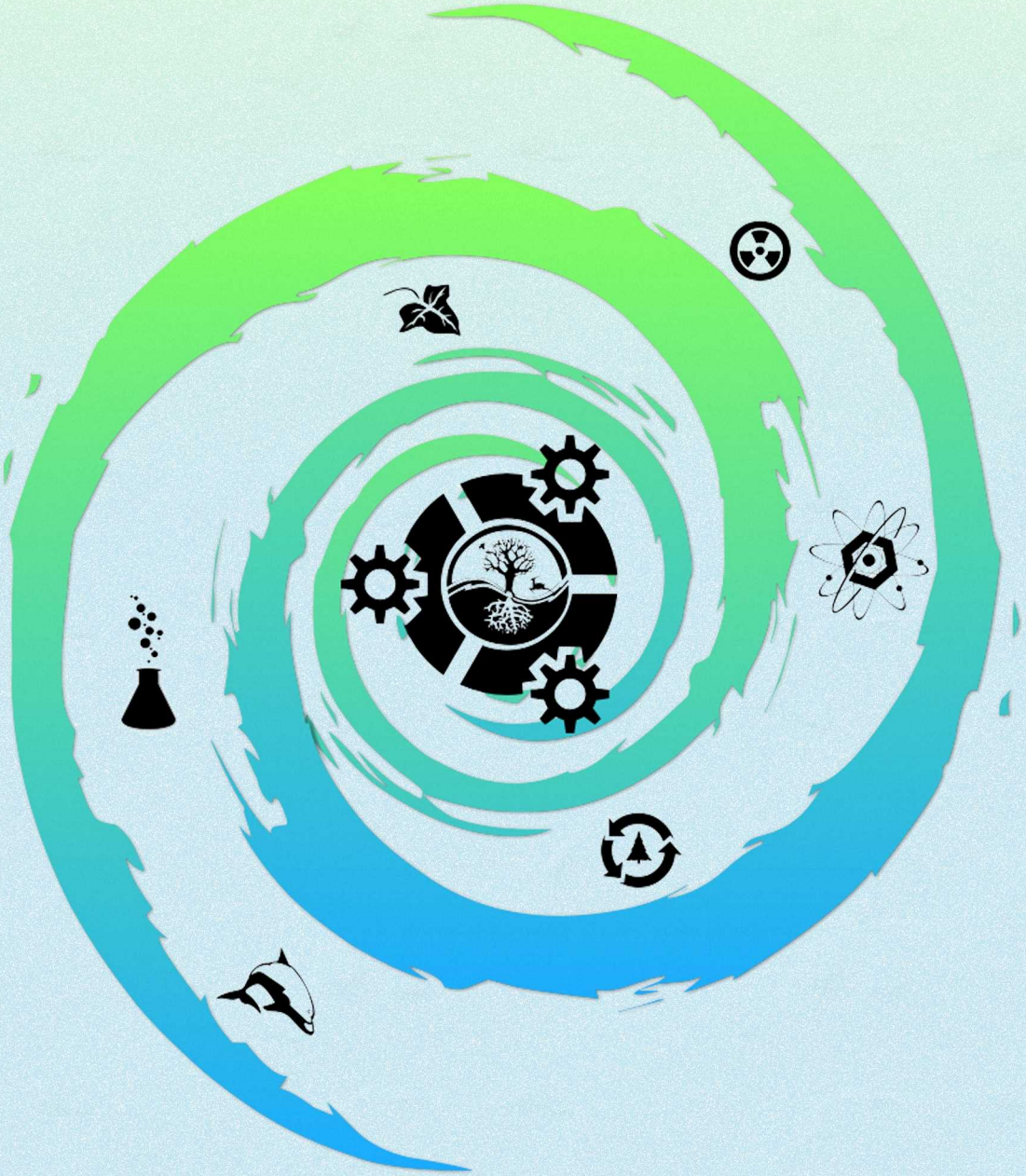

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