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AIMS Microbiology

AIMS Microbiology is an international Open Access journal devoted to publishing peer-reviewed, high quality, original papers in the field of microbiology. We publish the following article types: original research articles, reviews, editorials, letters, and conference reports.

Aim and scope

AIMS Microbology is an international Open Access journal devoted to publishing peer-reviewed, high quality, original papers in the field of microbiology. We publish the following article types: original research articles, reviews, editorials, letters, and conference reports.

AIMS Microbology welcomes, but not limited to, the papers from the following topics:

- Microbial Resources
- Microbial Genomics
- Mycology
- Physiology and Metabolism of Microbial
- Agricultural Microbiology and Biotechnology
- Pathogenic Microorganisms and Immunology
- Environmental Microbiology

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Contents

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Annual Report 2022

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1. Journal summary

AIMS Microbiology is an international Open Access journal devoted to publishing peer-reviewed, high quality, original papers in the field of microbiology. Together with the Editorial Office of AIMSMicrobiology, I wish to testify my sincere gratitude to all authors, members of the editorial board and reviewers for their contribution to AIMS Microbiology in 2022.

In 2022, We received more than 200 manuscripts and 40 of them were accepted and published.These published papers include 23 research articles, 11 review articles, 2 editorials, 2 communications and 1 brief report papers. The authors of the manuscripts are from more than 20 countries. The data shows a significant increase of international collaborations on the research of microbiology.

An important part of our strategy has been preparation of special issues. 2 special issues published more than five papers. AIMS Microbiology have invited 17 experts to join our Editorial Board in 2022.

We will continue to renew Editorial Board in 2022.

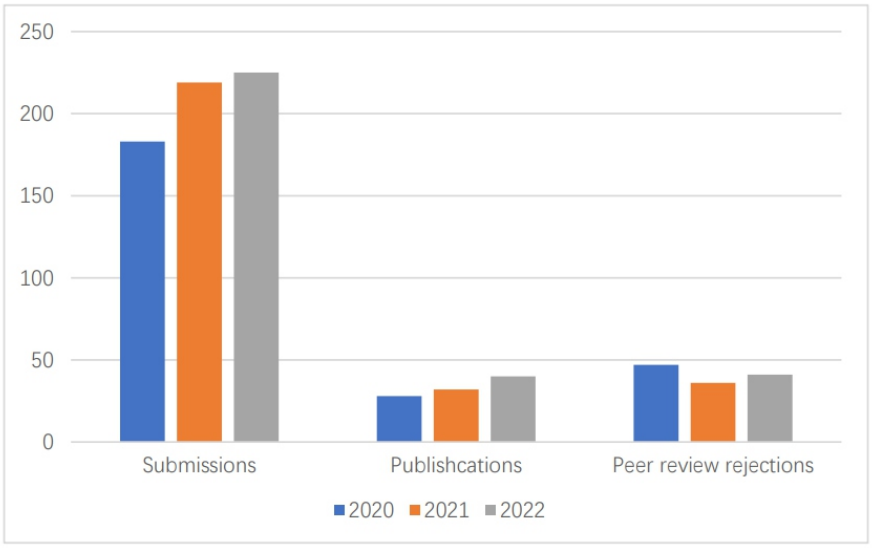
We hope that in 2023, AIMS Microbiology can receive and collect more excellent articles to be able to publish. The journal will dedicate to publishing high quality papers by regular issues as well as special issues organized by the members of the editorial board. We believe that all these efforts will increase the impact and citations of the papers published by AIMS Microbiology.

2. Editorial development

2.1. Manuscript statistics

The submissions of our AIMS Microbiology journal in 2022 increased. In 2022, AIMS Microbiology published 4 issues, a total of 40 articles were published online, and the category of published articles is as follows:

Type	Number
Research	23
Review	11
Editorial	2
Communication	2
Brief report	1



Peer Review Rejection rate: 49%
Publication time (from submission to online): 75 days

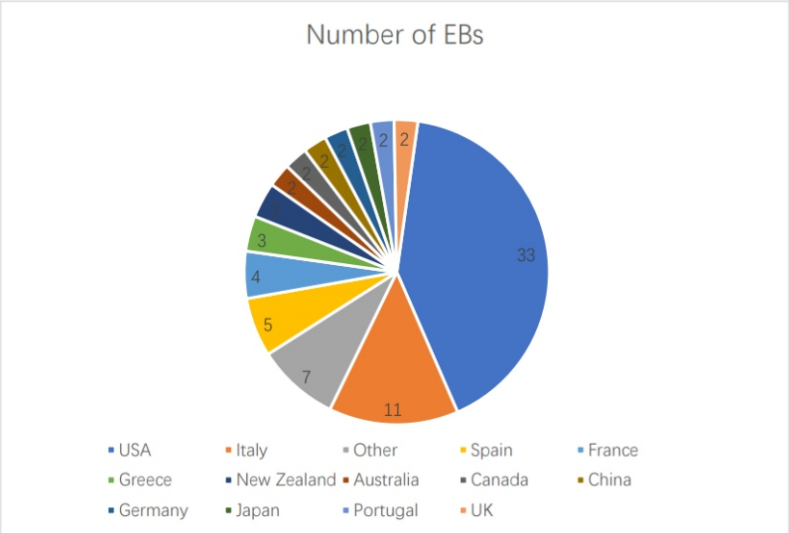
2.2. Some special issues with more than 5 papers

Organizing high-quality special issue is a very important work in 2022. In 2022, 7 special issues were called. Listed below are some examples of issues that have more than 5 papers. We encourage Editorial Board members to propose more potential topics, and to act as editors of special issues.

Special issue	link	Papers
Biotechnological applications of microorganisms in Industry, Agriculture and Environment	https://www.aimspress.com/aimsmicro/article/6262/special-articles	8
Antimicrobials and Resistance	https://www.aimspress.com/aimsmicro/article/6209/special-articles	5

2.3. Editorial Board members

AIMS Microbiology has Editorial Board members representing researchers from 20 countries, which are shown below. We are constantly assembling the editorial board to be representative to a variety of disciplines across the field of microbiology. AIMS Microbiology has 81 members now, and 17 of them joined in 2022. We will continue to invite dedicated experts and researchers in order to renew the Editorial Board in 2022.



2.4. Articles metrics

Top 5 Cited Papers in last 2 years.

No.	Article	Citations
1	Salmonella spp. quorum sensing: an overview from environmental persistence to host cell invasion	10
2	Ways to improve biocides for metalworking fluid	9
3	Yeasts in different types of cheese	7

Continued on next page

No.	Article	Citations
4	Exploring endophytes for in vitro synthesis of bioactive compounds similar to metabolites produced in vivo by host plants	7
5	Listeria monocytogenes isolates from Western Cape, South Africa exhibit resistance to multiple antibiotics and contradicts certain global resistance patterns	6

2.5. Summary & plan

2.5.1. Summary

In the recent two years, our journal has developed much faster than before; Our journal has been indexed in Web of Science, Scopus and PubMed databases. We received more than 200 manuscript submissions and published 40 papers in 2022. We have added 17 new Editorial Board members.

2.5.2. Plan in 2023

In 2023, we expect to publish more articles to enhance the reputation. We will invite more experts in the field of microbiology to publish a review or research article. To set a goal, we would like to publish 40 high-quality articles in 2023. In 2023, we will continue to update our editorial board. We hope that more experts in the field of microbiology can help us review and guest special issues.

Acknowledgments

We really appreciate the time and effort of all our Editorial Board Members and Guest Editors, as well as

our reviewers devoted to our journal in such difficult circumstances. All your excellent professional effort and expertise provided us with very useful and professional suggestions in 2022. Last, but not least, thanks are given to the hard work of the in-house editorial team.

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The use of cellulolytic Aspergillus sp. inoculum to improve the quality of Pineapple compost

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ABSTRACT

Pineapple litter has a complex polymer of cellulose, hemicellulose, and lignin, which makes them difficult to decompose. However, pineapple litter has great potential to be a good organic material source for the soil when completely decomposed. The addition of inoculants can facilitate thecomposting process. This study investigated whether the addition of cellulolytic fungi inoculants to pineapple litters improves the efficiency of the composting processes. The treatments were KP1 = pineapple leaf litter: cow manure (2:1), KP2 = pineapple stem litter: cow manure (2:1), KP3 = pineapple leaf litter: pineapple stem litter: cow manure P1 (leaf litter and 1% inoculum), P2 (stem litter and 1% inoculum), and P3 (leaf + stem litters and 1% inoculum). The result showed that the number of Aspergillus sp. spores on corn media was 5.64 x 107spores/mL, with viability of 98.58%. Aspergillus sp. inoculum improved the quality of pineapple litter compost, based on the enhanced contents of C, N, P, K, and the C/N ratio, during the seven weeks of composting. Moreover, the best treatment observed in this study was P1. The C/N ratios of compost at P1, P2, and P3 were within the recommended range of organic fertilizer which was 15–25%, with a Carbon/Nitrogen proportion of 11.3%, 11.8%, and 12.4% (P1, P2, and P3), respectively.

Keywords: Aspergillus sp; cellulolytic; decomposition inoculum; pineapple litter

1. Introduction

Lignocellulosic biomass is the most abundant biological material on earth. An important fraction of lignocellulosic biomass corresponds to waste from different economic activities, particularly, agricultural and agro-industrial residues such as cereal straws, corn stover, coffee husks, coconut fiber, wood, and forestry waste, palm press fiber, and palm kernel shells, among others [1]. One of the lignocellulosic biomass sources is pineapple plantation residue. Pineapple leaves contain a high fiber content, including cellulose, hemicellulose, and lignin at 43.53%, 21.88%, and 13.88%, respectively [2]. Also, the content of pineapple humps was cellulose, hemicellulose, and lignin at 24.53%, 28.53%, and 5.78%, respectively [2]. These chemical contents are polymers that are difficult to decompose. The common practice of clearing pineapple (Ananas comosus) residues for land preparation for cultivation is by burning and unsustainable agricultural practice that causes environmental pollution. Besides, the disposal of this waste implies high costs and negative environmental impacts. As a source of valuable sugars and polymers, lignocellulosic biomass can be used for the production of a broad spectrum of chemicals and materials such as liquid, gaseous, and solid biofuels, enzymes, organic compounds, synthetic polymers, pharmaceuticals, and food products, among many others [1]. In particular, pineapple wastes may be employed for producing high-quality organic fertilizer [3].

The main obstacle to the natural decomposition of the lignocellulosic residues from agricultural, agro-

-industrial, and forestry activities is their high content of lignin in the lignocellulosic complex bound to cellulose and hemicellulose. Also, in pineapple waste, cellulose components are not easily degraded, either chemically or mechanically, because cellulose has crystalline properties and is insoluble, which comes from its linear structure [4].

Biodegradation of biomass is carried out by different heterotrophic microorganisms, bacteria, fungi, actinomycetes and protozoa [5]. The decomposition process of degradable substrates containing cellulose, hemicellulose and lignin indicates that fungi are the microorganisms playing a major role instead of bacteria [6]. In order to degrade pineapple litters, biological steps are likely to be carried out with the help of enzymes being produced by microorganisms [7,8]. One of the microorganisms speeding up the decomposition process is the fungus group. Fungi excrete enzymes that degrade carbohydrate polymers into simple compounds and release reducing sugars (glucose) as the final product, with reduced glucose being an essential nutrient for the microorganism's survival. Fungi are also the main degrading microorganisms of organic material in the natural environment, as they utilize insoluble compounds, such as cellulose and lignin [7,8].

Applying fungal inoculums in the composting process is intended to accelerate the conversion of complex polymeric of pineapple litter into simple elements, which are returned to the soil as mineral nutrients. The ability of fungi to hydrolyze cellulose is carried out through the cellulase activity it possesses. The fungus groups remodeling cellulose with the help of cellulase enzymes are called cellulolytic fungi [9]. Previous studies reported that some fungal genera containing cellulolytic abilities include genera Aspergillus, Penicillium, and Paecilomyces [10]. Moreover, Helminthosporium sp., Cladosporium sp., Trichoderma sp. and Aspergillus sp. were screened for their highest cellulolytic enzyme activity [11]. Also, some Aspergillus sp. reported to have the potential cellulolytic ability are Aspergillus niger [12], A. fumigatus [13], A. aculeatus [14]. Hypothetically, the addition of a cellulolytic Aspergillus sp. in composting pineapple biomass which is rich in complex organic compounds, will degrade these compounds into simple monomers and release many nutrients for plants.

Based on its contents, pineapple litter has great potential as an organic material, when perfectly decomposed. This research is related to the efforts in accelerating and improving the quality of organic material decomposition. The time and quality of pineapple litter decomposition are also maximized by inoculum fungi, Aspergillus sp. The inoculum containing a pure culture of Aspergillus sp. are likely to be created, using various substrates. Corn has a high cellulose content, which is suitable as a cellulolytic fungi growth substrate, with the inoculum then applied to the pineapple litter. The addition of cellulolytic fungi inocula plays a role in litter composting, due to having the best enzymatic ability to decompose, while also producing high-quality compost [15]. Corn is used as an inoculum medium for Aspergillus sp. because it is rich in cellulose, which is a suitable substrate for the growth of cellulolytic isolates It is expected to increase the spore number and high viability. In this study, we used different parts of pineapple litter and these parts were chopped into a mixture.

In order to discover the quality of Aspergillus sp. as an inducer of cellulose decomposition, the preparation of the cellulolytic fungi (Aspergillus sp.) with corn media and its effect on pineapple litter composting was investigated.

2. Materials and methods

2.1. Materials and analysis

The isolate of Aspergillus sp. (Bioggp 3) was obtained from a previous study in which they were isolated from the isolation process of mixed leaf litter and soil taken from the pineapple plantation of PT. Great Giant Pineapple (PT. GGP) Terbanggi Besar, Central Lampung, Indonesia. The isolate showed

cellulolytic and xylanolytic activity with a cellulolytic index 4.00 ± 0.783 and xylanolytic index 4.20 ± 1.03 respectively [16]. The compost material used is pineapple plant biomass which consists of leaves and stems. The selection of cellulolytic fungi isolates was done by modification of the Congo Red method [17]. Isolates were obtained were cultured in Cellulose Agar (cellulose 5.0, NaNO3 1.0, K2HPO4 1.8, MgSO4.7H2O 0.9, KCl 0.5, 0.5 yeast extract, casein hydrolysat 0.5, agar 20 and distilled water 1L). Confirmation of cellulose-degrading ability of fungal isolates was performed by streaking it on cellulose agar media. Media were 2 layer media (bilayer) with the bottom layer was a PDA of 1/5 recipes, agar 1.5, and distilled water 100 mL. The top layer was Carboxymethyl Cellulose (CMC) 1–2%, agar 1.5 and distilled water 100 mL. Once inoculated with fungi in the middle of the test media, the cultures were then incubated for 4 days [18]. The media were added with 0.1% Congo-Red and allowed to stand for 20 minutes at room temperature. Media was washed with 1 M NaCl. Isolates producing cellulase formed a halo (clear zone) around the colony. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic fungi. Colonies showing decolorization of Congo-Red were taken as positive cellulose degrading fungal colonies [7], and only these were taken for further study. The production of Aspergillus sp. inoculum was carried out in the Microbiology Lab, while composting applications wereconducted at the Green House Botany Laboratory, Department of Biology, Universitas Lampung. Compost chemical analysis was carried out at PT. GGP.

2.2. Procedure

The research used a single factor Completely Randomized Design (CRD), as a treatment that is the difference in the composition of compost materials arranged in 6 levels: KP1; KP2; KP3; P1; P2, and P3. Each treatment was carried out in 3 repetitions; hence it obtained 18 experimental units [19].

The stages involved were:

(1) Inoculum development,

(2) Inoculum application in pineapple litter composting.

Inoculum development was made using modification of Gaind et al. method [20]. Corn grains were used as substitute for fungal strain growth. The corn grains were finely ground and sifted before it was mixed with 4% calcium sulphate, and 2% calcium carbonate (in 1 L distilled water). A loopful of Aspergillus sp. culture was inoculated in each 100 g corn grains added with 25 mLof solutions (sterilized at 15 lb pressure for 1 h) and incubated at 25 °C for 15 days. Each strain's whole growth, including mycelium, spores, and the grains, was used as the inoculum. The inoculum was counted for the number of spores and viability by calculating CFUs [18].

Composting was carried out by modifying the Takakura Home Method (THM) [21], for 7 weeks. The composting process was carried out in a perforated basket with a lid. Basket was lined with cardboard to keep the conditions moist when composting. Next, compost materials were put in the basket and add with Aspergillus sp inoculum.

The composition of raw materials were pineapple leaf, stem litters and mixture of both created into 6 treatments (KP1, KP2, KP3, P1, P2, and P3; K = treatments without inoculum), as the following details,

KP1 = pineapple leaf litter:cow manure (2:1)

KP2 = pineapple stem litter:cow manure (2:1)

KP3 = pineapple leaf litter:pineapple stem litter:cow manure (1:1:1)

P1 = pineapple leaf litter:cow manure (2:1) + 1% inoculum (30 g)

P2 = pineapple stem litter:cow manure (2:1) + 1% inoculum (30 g)

P3 = pineapple leaf litter: pineapple stem litter: cow manure (1:1:1) + 1% inoculum (30 g)

The compost quality testing was carried out by analyzing the levels of carbon ©, nitrogen (N),

phosphorus (P), potassium (K), and C/N ratio. Total organic carbon was determined using wet digestion method [22]. Nitrogen totals were calculated by the Kjeldahl method [23]. Phosphorus was measured by a spectrophotometer using phosphomolybdate blue method [24]. Potassium was measured by a flame photometer.

2.3. Variable observation and data analysis

The parameters measured in this study were the number of spores and values of the Colony Forming Unit (CFU) in the Aspergillus sp. inoculum in corn media, with C, N, P, and P the C/N ratio of pineapple litter compost. The calculations of the number of spores and CFU values were conducted to determine the productivity and viability of the fungi inoculum, respectively. The compost content analysis was also performed in order to observe the quality of pineapple litter compost. The data obtained were analyzed descriptively and presented in graphical form. All the results were statistically analyzed using analysis of variance (ANOVA) test. Treatment means were compared using the least significant difference (CD, $P \leq 0.05$), which allowed the determination of significance between different applications.

3. Results and discussion

3.1. The spore number of inoculum Aspergillus sp. in corn media

There is a relationship between nutrient complexity in the medium and the ability of fungi to grow and sporulate, which is also strain-dependent [25]. For the reproduction and growth of fungi to proceed properly, the microorganism required substrates containing nutrients for their metabolism. Corn has a high crude fiber content of 86.7%, consisting of 67%, 23%, and 0.1% of hemicellulose, cellulose, and lignin, respectively [26]. The high content of cellulose also supported the use of corn as growth media for cellulolytic fungi (Aspergillus sp.).

The results showed that the number of spores on the Aspergillus sp. inoculum was 5.64 x 107spores/mL, which was similar to the rate of the A. parasiticus species from previous studies, with similar media at pH 4–10, which was also in 107[27]. The best spore production in a previous study occurred at pH = 5, with the rate at 8.3 x 107cells/mL [27]. This was presumably due to the pH of the corn media being sufficiently in line with that of the optimum for fungal growth. Spore production was also influenced by environmental factors, including substrate, humidity, temperature, nutrition, and pH [28].

The increase in the number of spores occurred allegedly due to a suitable growth process due to aerobic metabolism. The large particle size of corn provided enough oxygen to have an impact onmycelial propagation, which in turn became easier [28,29]. Also, available nutrients in the environment aid maximum mycelium growth until it reaches the logarithmic phase. Afterward, the nutrients are reduced, triggering the formation of spores as self-adaptation.

3.2. Viability of the Aspergillus sp. in corn media

The viability value of fungi Aspergillus sp. inoculum in the corn media obtained 4.4 x 107CFU/mL (Table 1). According to Mikata [30], isolates showing a high, moderate, low, and very reduced levels of viability, had mean CFU values ≥1 x 107/mL, ≥1 x 106/mL, around 1 x 105/mL, and ≤1 x 104/mL, respectively. Therefore, it was concluded that the viability of Aspergillus sp. in the medium of corn was at a moderate level. Also, Aspergillus sp. had a high life capacity. The inner membrane of fungal spores contained various enzymes and specific protein receptors, which were always quick to repair accumulated damages, while a thick layer protected the outer region.

Moreover, spores contain trehalose compounds, which regulate the osmotic pressure affecting the viability and tolerance of unfavorable environmental stresses. This result is in accordance with the result

reported by Cazorla et al. which stated that the rate of conidia germination was determined by the content of the polyol and trehalose [31]. Also, spores play an essential role in the life cycle of fungi, which acts as a spread or survival. Another research by Garcia-Kirchner et al. [32] shows that corn containing media are suitable for Aspergillus growth. The research mentioned that Aspergillus niger is able to grow on corn containing media and shows its high lignocellulolisic activity, furthermore this corn serves as a carbon source to support its enzymatic activity.

Table 1. Average number of spores and viability of spore Aspergillus sp. inoculum in corn media.

Number of Spores (spores/mL)	Viability Value (CFU/mL)	Number of Spore Logs	Spore Viability Log	Percentage of Spore Viability (%)
5.645×10^7	4.4×10^7	7.75	7.64	98.58

3.3. Carbon content (C) of the pineapple litter compost

The data analysis shows there are no significant differences among treatments within the same week. However, the C level of pineapple litter compost in the 4th to 7th week tended to decrease. The sharpest decrease occurred in P2 (27.58%), with C levels in the 4th and 7th weeks at 29.8% and 21.58% (Figure 1). The activity of degradation and decomposition by microorganisms also caused a decrease in the compost material's C level. The result in this study was consistent with the result reported [33] which stated that C levels decreased according to the maturity of compost. This decrease was caused by the microorganisms' respiration and assimilation activities during the composting process [34]. Pineapple litter contains organic materials, which are energy sources for fungi, and are further converted into simple molecules. This observation was in agreement with the results of Suthar and Gairola, where this activity changed the available organic C, freeing it again in the form of CO2 gas [35]. During the composting process, the carbon elements will be released thereby reducing the amount of carbon. Some carbon- and nitrogen-containing gases are inevitably released during the process of composting due to the different operating conditions, resulting in carbon and nitrogen losses [36].

The absorption of nutrients by fungi was further carried out by remodeling the polymer substrate, using enzymes that were secreted into the environment. The previous report also showed that the genus fungi, Aspergillus sp., produced cellulase enzymes [37]. Cellulase enzymes degraded cellulose enzymatically and produced oligosaccharide and disaccharide compounds with soluble glucose monomers. Afterward, glucose was used as a carbon source for fungal metabolism. Glucose also provided maximum growth for fungi due to being more easily converted into phosphorylation of derivatives, which passes the pathway of the respiratory system [38]. The C content in pineapple litter decomposition fulfilled the Indonesian National Standard (SNI) 19-7030-2004 compost fertilizer quality standards, which is 9.8–32% [39].

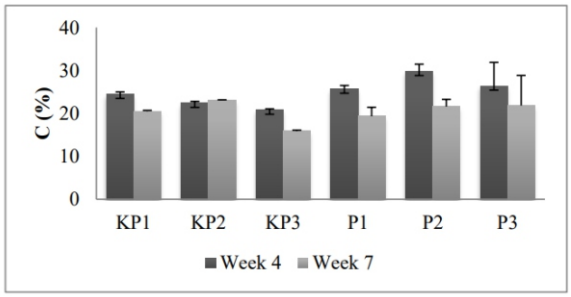

Figure 1. Carbon content of the pineapple litter compost. The test indicates there are no significant differences among treatments within the same week.

3.4. Nitrogen (N) content of the pineapple litter compost

The data analysis shows there are no significant differences among treatments within the same week. However, the highest and lowest N contents of pineapple litter decomposition at 4th week were discovered in P2 and PI composts at 2.55% and 1.77%, respectively (Figure 2). High levels of N were caused by the decomposition process of microorganisms that produced ammonia and nitrogen. However, the decrease in N levels was caused by the loss of nitrogen into the air, during compost mixing. In the 7th week, the highest and lowest N contents were obtained in P2 and KP3 composts at 1.82% and 0.79%, respectively. A decrease in N levels in the 7th week indicated that composting time affected the percentage of nitrogen content. During the composting process, nitrogen mineralization decreased according to the time of fermentation [40]. It was further suspected that microbes still adjusted and metabolized in the initial phase, as their activities only increased cell size, with cells using carbon from the substrate as food and reproduction media for themselves. Afterward, the microorganisms reached an equilibrium amount between living and dead, therefore resulting in the declination of microbial activities, as indicated by the decrease in N levels in the 7th week due to reduced carbon.

The general pattern of pineapple litter compost's N levels in the 4th to 7th week tended to decrease. The sharpest decrease occurred in KP3 (61.46%) (without inoculums), with N levels at the 4th and 7thweek indicating 2.05% and 0.79%, respectively. The sharpest decrease occurred in P2 (28.62 %) in the inoculum applications, with N levels at the 4th and 7th week indicating 2.55% and 1.82%, respectively. The longer composting process caused nitrogen levels to decrease, due to the influence of cell metabolism, which resulted in N being assimilated and lost through volatilization (lost in free air), as ammonia [40,41].

The final result of this mature compost shows promising results and still matches existing standards. Based on SNI: 19-7030-2004 regarding compost specifications of domestic organic waste, the minimum nitrogen yield was 0.4%, while the N contained in the composts was above the standard [36].

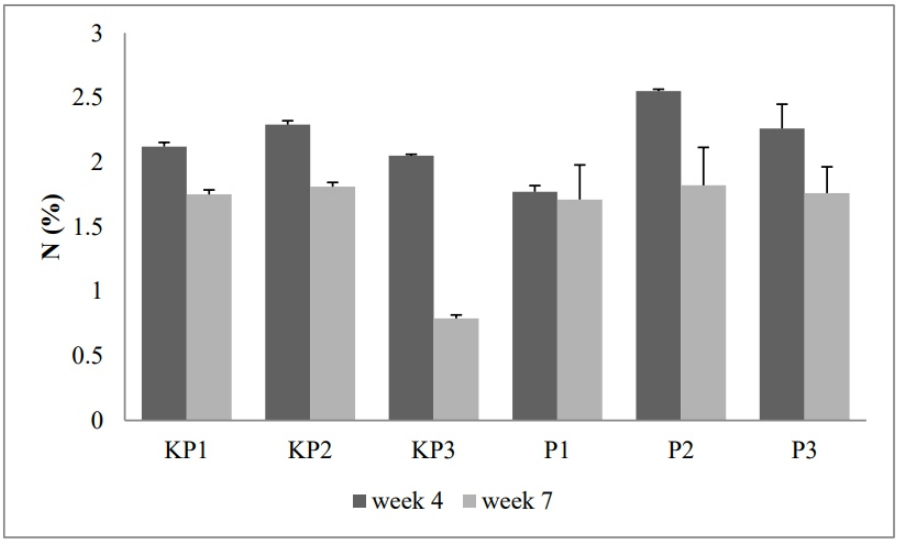

Figure 2. Nitrogen content of the pineapple litter compost. The test indicates there are no significant differences among treatments within the same week.

3.5. Phosphorus (P) content of the pineapple litter compost

Analysis data shows there are significant differences among treatments. The highest and lowest P levels of the pineapple litter in the 4th week were obtained in the P2 and KP3 composts, at 4891.67 and 1813 ppm, respectively (Figure 3). KP3 compost was a treatment without the addition of an inoculum, as the

activity of decomposing microorganisms was low, causing reduced phosphorus synthesis. However, the P2 compost was with the addition of an inoculum, which caused an increase in the number of microorganisms, and total P levels in the compost. In the 7th week, the highest and lowest P levels were obtained in P2 and KP3 composts, at 5148.52 and 1468.71 ppm, respectively. Also, the treatment of KP3 in the 4th and 7th weeks decreased due to the composition of the compost leaving materials (mixture of tubers and pineapple leaves) without the addition of fungi inoculum activator, Aspergillus sp.

Aspergillus sp. inoculum affected the compost quality, as high levels of P were also influenced by an abundance of decomposing microorganisms. The more microorganisms, the quicker the maturity of the compost. Also, phosphorus in the matured stage of the compost was sucked up by the microorganisms. This was the reason phosphorus content in the 7th week increased. [42] reinforced this statement by reporting that phosphorus increased with the number of microorganism cells. The increase in P-level was caused by the multiplicative presence of the microorganisms, which overhauled phosphorus and the process of mineralization by their existence in the formation of P [42]. This causedan accumulation of the phosphorus level contained in the raw material, and the number of microorganisms in the composting process [42,43]. Fungi utilize phosphorus from the environment for metabolism, as their availability increases with increasing levels of P.

The general pattern of P content in pineapple litter compost tended to increase from the 4th to the 7thweek. The sharpest increase occurred in KP2 (47.57%) (without inoculums), with P levels in the 4thand 7th week at 3334 and 4920.03 ppm, respectively. In the inoculum's treatment, the sharpest increase occurred in P3 (40.10%), with P levels in the 4th and 7th week at 2811 and 3937.26 ppm, respectively.

The increase of P is presumably due to the decomposition process of organic materials occurred quite well in line with the increase of compost maturity. The pineapple litter compost's P level in the 4th and 7thweeks showed a significant difference. All compost variations were mature and stable while meeting the minimum P content following SNI 19-7030-2004, i.e., the total P-content was more than 0.1% [39].

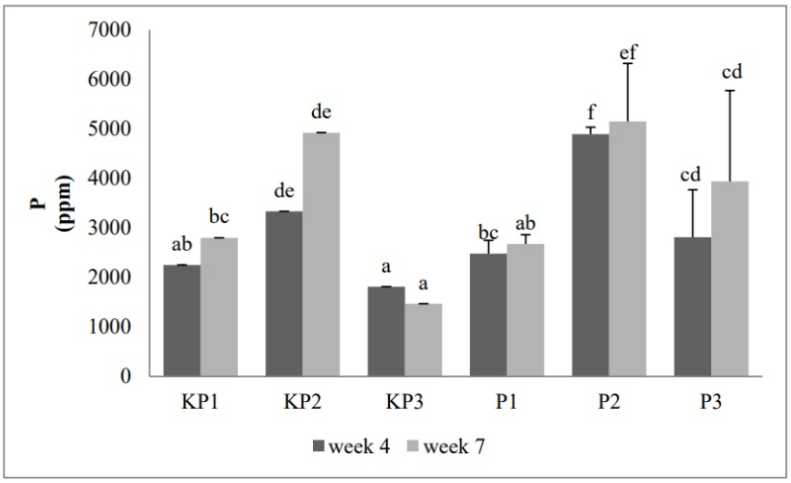

Figure 3. Phosphorus content of the pineapple litter compost. The same letter in the same weeks shows no significant difference among treatments.

3.6. Potassium (K) content of the pineapple litter compost

Analysis data shows there are significant differences among treatments. The highest and lowest K contents of pineapple litter composts at 4th week were obtained in KP1 and KP3 composts, at 1.72% and 1.1%, respectively (Figure 4). In the 7th week, the highest and lowest K contents were further obtained in KP1 and KP3 composts, at 2% and 0.92%, respectively. The KP3 compost had a lower stack height than

other treatments. KP3 is a mixture of compost material with the most complex raw materials; thus, the decomposition of these mixtures was slower compared to the others; therefore, the K released was less. Furthermore, Aspergillus sp. inoculum and composting time were observed to affect the K content of the compost. This was consistent with the opinion of Abubakar [27] which stated that the longer the stirring time, the lower the levels of potassium in fertilizers. This was due to the fact that the potassium bounded was released again. Also, the general pattern of the compost's K levels in the 4th to 7th week tended to be fluctuating. The sharpest decrease occurred in KP3 (16.36%) (without inoculums), with K level at the 4th and the 7th week had 1.1% and 0.92%, respectively. In the inoculums' application,the sharpest decrease occurred in P1 (0.59%) (without inoculums), with K level at the 4th and the 7thweek had 1.69% and 1.68%, respectively. The rest observation shows an increasing pattern. This research was in line with other studies, which stated that potassium levels decreased due to being used as catalysts by microorganisms in the substrate material [34]. However, the K content of compost followed the minimum standard of SNI 19-7030-2004, which was 0.20% [39].

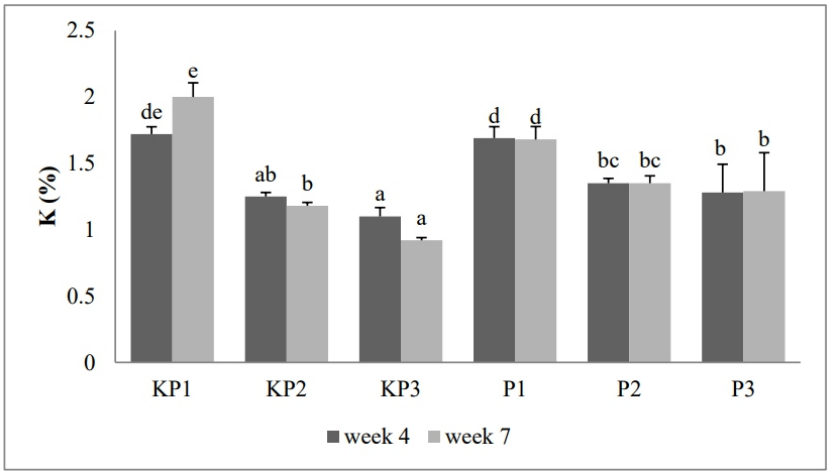

Figure 4. Potassium (K) content of the pineapple litter compost. The same letter in the same weeks shows no significant difference among treatments.

3.7. C/N ratio of the pineapple litter compost

The data analysis shows there are no significant differences among treatments. The highest and lowest C/N ratios of pineapple litter composts in 4th week were obtained in P1 and KP3 composts, at 14.56and 10.15, respectively (Figure 5). The high C/N ratio in the P1 compost was caused by the multiplication of microorganism cells using N levels in compost material, as a decrease does not follow it in C levels. However, the low C/N ratio in the KP3 compost was due to the high heap, as the degradation process acted quickly. The higher the pile, the smaller the porosity, causing the amount of heat generated during the decomposition process to be trapped inside the heap. It is also known that the pile height affected temperature, pH, and Moisture Content (MC), causing decreased microorganism activity [44,45].

The C/N ratio in the KP3 compost was further influenced by the activity of microorganisms, as the decomposition process does not act optimally. Also, the higher C/N ratio indicated that the compost had not been completely decomposed [44]. Probably, the KP3 decomposed slower too than other compost substrates since it contains a more raw material combination. However, matured decomposed waste had physical characteristics changing the physicality of the compost [39], as the color becamebrown/black and odorless, while possessing crumb texture like soil [44]. Moreover, the compost's C/N ratio in the 4th and 7th weeksfluctuated (Figure 5). In the application of inoculums, the sharpest decrease occurred in P3 (16.36%), with C/N ratio level at the 4th and the 7th week had 11.61 and 10.23%, respectively.

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In general, the decomposition process of organic matter in nature involves almost all microorganisms with their respective niches. The Inoculant of Aspergillus sp. is important in this process because it plays a role in initiating the process of cellulose-cell decomposition (the majority in plant cell walls) making it easier for other microorganisms to dominate the next process.

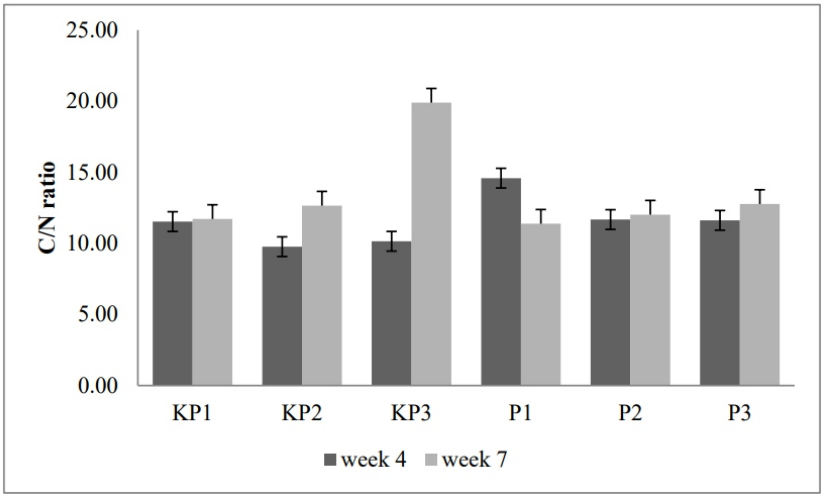

Figure 5. Compost C/N ratio of pineapple litter. The test indicates there are no significant differences among treatments within the same week.

4. Conclusions

Aspergillus sp. inoculum with enrichment of corn media can be used as an element to accelerate the pineapple biomass composting process. Also, the addition of Aspergillus sp. inoculum improved the quality of pineapple litter compost, as observed from the final contents of the decomposition process, in the form of C, N, P, K, and C/N ratio. Compost quality obtained was produced from aninoculum of 1% of pineapple substrate weight and possible to increase to achieve better compost yield. The application of 1% inoculum was able to mature the compost. It caused the sharpest decrease in C levels of 27.6% for pineapple stem litter and cow manure (2:1) compost materials, less effective for N and P changes. With cellulolytic inoculum addition, potassium and C/N ratio changes tend to fluctuate during 7th week of pineapple biomass composting. In future reseach, a study will be developed on the inoculum characters under physical conditions (pH, temperature, salinity, humidity, pesticides) and biology (pathogenicity, mycotoxins, secondary metabolites) before field application.

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Conflict of interest

The authors declare no conflict of interest in this article.

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Antimicrobial resistance genes of Escherichia coli, a bacterium of "One Health" importance in South Africa: Systematic review and meta analysis

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ABSTRACT

This is a systematic review and meta-analysis that evaluated the prevalence of Escherichia coli antibiotic-resistant genes (ARGs) in animals, humans, and the environment in South Africa. This study followed Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines to search and use literature published between 1 January 2000 to 12 December 2021, on the prevalence of South African E. coli isolates' ARGs. Articles were downloaded from African Journals Online, PubMed, ScienceDirect, Scopus, and Google Scholar search engines. A random effects meta-analysis was used to estimate the antibiotic-resistant genes of E. coli in animals, humans, and the environment. Out of 10764 published articles, only 23 studies met the inclusion criteria. The obtained results indicated that the pooled prevalence estimates (PPE) of E. coli ARGs was 36.3%, 34.4%, 32.9%, and 28.8% for blaTEM-M-1, ampC, tetA, and blaTEM, respectively. Eight ARGs (blaCTX-M, blaCTX-M-1, blaTEM, tetA, tetB, sul1, sulII, and aadA) were detected in humans, animals and the environmental samples. Human E. coli isolate samples harboured 38% of the ARGs. Analyzed data from this study highlights the occurrence of ARGs in E. coli isolates from animals, humans, and environmental samples in South Africa. Therefore, there is a necessity to develop a comprehensive "One Health" strategy to assess antibiotics use in order to understand the causes and dynamics of antibiotic resistance development, as such information will enable the formulation of intervention strategies to stop the spread of ARGs in the future

Keywords: Escherichia coli; antibiotic resistance genes; One Health; South Africa

1. Introduction

Escherichia coli is an enteric bacterium that lives in the intestinal tracts of humans and warm blooded animals as part of commensal variations [1]. Animals are important reservoirs for pathogenic E. coli O157:H7 strains, and majority of the illnesses in humans are linked to undercooked meat, contaminated meat, water or raw milk consumption containing these pathogenic strains [2]. There are different pathotypes of E. coli that are related to the pathogenicity potential based on the presence of colonization factors or production of toxins that cause a variety of diseases [3], of which the majority are difficult to treat [4]. Majority of these strains have been isolated in humans and animals [5], however, water sources are regarded as a major public health risk [6,7]. In response to bacteria gaining resistance to commonly used antimicrobial drugs, the expression of antibiotic resistance genes (ARGs) in bacteria is becoming a significant issue for public health [8].

Antibiotic resistant bacteria and their resistance genes have emerged as a critical and growing problem in modern medicine [9]. Additionally, it is a growing global public health concern for both animals and humans [10]. Antimicrobials used in human medicine are also utilized in livestock for growth promotion, disease prevention and disease treatment, thereby increasing selection pressures on bacterial pathogens, as well as the risk of antimicrobial resistance (AMR) onset and dissemination [11].

Different antibiotics have been used to treat E. coli infections in animals and humans [12,13]. Overuse of antibiotics is common in animal husbandry and aquaculture, as they are used as feed additives for disease prevention and growth stimulation [14]. Bacteria develop antibiotic resistance through genetic alterations or the acquisition of ARGs from the host or environment [15].

Surveillance systems are still not well established in many developing nations due to a lack of financial support for sampling, testing, equipment acquisition, and maintenance. In developed countries, antimicrobial resistance surveillance systems implement whole genome sequencing (WGS) as a genotypic tool to supplement phenotypic antimicrobial susceptibility testing [13].

The spread of bacterial antibiotic resistance and pathogenicity imposes a significant health and economic cost [16]. Different bacterial ARGs can become resistant to various antibiotics [17]. Tetracyclines (tet), sulphonamides (sul), β-lactams (bla), macrolides (erm), aminoglycosides (aac), fluoroquinolone (fca), colistin (mcr) and vancomycin (van) are among the classes of antibiotics to which bacterial pathogens can express resistance genes. Key enteric pathogens, such as Klebsiella spp., Salmonella spp. E. coli, Vibrio cholerae and Shigella spp. have demonstrated unfavorable trends in the development of multi-drug resistance (MDR) in the African region to almost all widely available antibiotics [18–20]. Despite the high volume of antibiotics used in South Africa, there is a scarcity of knowledge about the relevant ARGs with regard to humans, animals, and the environment. Therefore, this study was carried out to identify prevalence gaps, analyze, and summarize the pooled prevalence of ARGs from E. coli isolates by carrying out a systematic review and meta-analysis of published studies in South Africa.

2. Materials and methods

2.1. Search strategy

Databases, such as African Journals Online (https://www.ajol.info/index.php/ajol/), PubMed (https://www.ncbi.nlm.nih.gov/pubmed/), ScienceDirect (https://www.sciencedirect.com/),Scopus (https://www.scopus.com/) and Google Scholar (https://scholar.google.com/), were searched for English articles published between January 2000 and December 2021. Relevant articles from each database were imported directly into spreadsheet (Microsoft Excel® 2013). All publications, including antimicrobial resistance genes from E. coli, were searched using the following keywords: Antibiotic resistance AND Antibiotic AND drug resistance AND bacterial resistance AND multi-drug resistance AND antibiotic resistance genes AND Escherichia coli OR E. coli AND Human OR animal [beef OR poultry OR livestock OR cattle OR animal OR cows OR chickens OR pig] AND Environment AND South Africa, with the last search conducted on 18th of December 2021. The articles were screened by their title and abstract, and relevant publications were included in this study.

2.2. Inclusion and exclusion criteria

Studies were included on the basis that they fulfilled the following inclusion criteria; names of authors, location, total number of isolates, availability of the full texts, studies conducted in South Africa, studies that investigated antibiotic resistance genes, and articles published in English only on antibiotic resistance genes in E. coli, conducted from January 2000 to December 2021. Studies were excluded if they were not undertaken in South Africa, were reviews, book chapters, dissertations/thesis and not published in English.

2.3. Data extraction and statistical analysis

To reduce the possibility of bias, one author (TR) extracted the data, and a second author examined and

confirmed it. The data was extracted from all eligible studies following the inclusion and exclusion criteria described above.

To assess the relative risk, we included articles reporting the number of antibiotic resistance genes in this meta-analysis. Studies were grouped based on bacterial species (E. coli). All statistical analyses were carried out using Comprehensive Meta-analysis (CMA) Version 3.0 by Biostat (Englewood, NJ, USA). The 95% confidence interval (CI) and pooled prevalence estimates (PPE) were calculated. The data generated was visualized using forest plots. The Cochrane Q test was used to calculate Cochran's heterogeneity (Q) among the included studies, as well as the percentage inverse variation (I 2). If I2 was ≤ 25%, 50% or ≥ 75%, then heterogeneity was classified as low, moderate or high, respectively. The publication bias was assessed using funnel plots with ocular examination, including the Egger's and Begg's bias indicator tests. A random-effects model was used to generate all pooled estimates.Heterogeneity with a $P < 0.05$ were considered statistically significant.

3. Results

3.1. Literature search and eligible studies

An electronic search of the databases African Journals Online, PubMed, ScienceDirect, Scopus, and Google Scholar yielded a total of 10764 articles (Figure 1). The search for articles related to studies on antibiotic resistance genes of E. coli in South Africa which were conducted throughout until December 2021. Duplication resulted in the removal of 5211 articles. Then, 5498 were excluded after the screening of titles, abstracts and languages. We evaluated 55 full-text papers for eligibility, and 32 of them did not meet our requirements. The exclusion was based on no reporting of the antibiotic resistance genes (n = 27) and incomplete information on resistance genes (n = 5). Only 23 peer-reviewed journal articles met the inclusion criteria. Table 1 summarizes studies that were included in this review with characteristics, such as province, method of detection, source of samples, number of isolates, and screened ARGs.

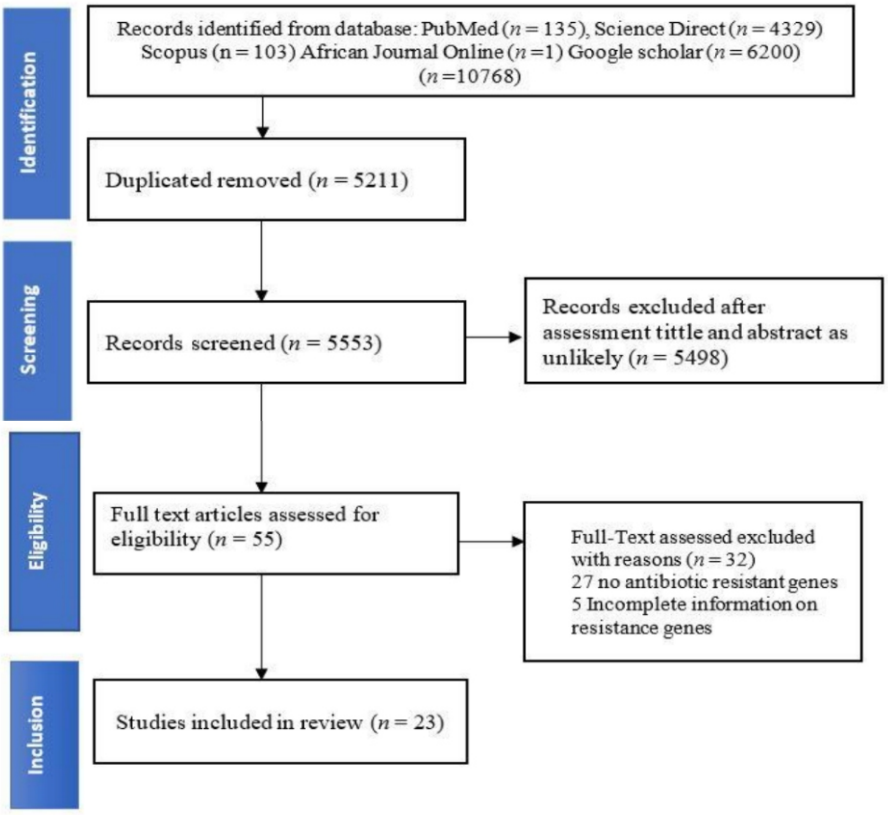

Figure 1. PRISMA flowchart showing selection of eligible articles for inclusion in this systematic review and meta-analysis of Escherichia coli antibiotic resistance genes in South Africa.

Table 1. Characteristics of eligible articles consisting of province, method of detection, source of samples, number of isolates and screened ARGs.

Reference	Province	Method used	Source of samples	One health segment	No. isolates	Antibiotic Resistance Genes
[21]	Eastern Cape	PCR	Wastewater treatment	Environment	223	*str*A, *aad*A, *cat* I, *cmlA*1, bla_{TEM}, *tet*A, *tet*B, *tet*C, *tet*D, *tet*K, and *tet*M.
[22]	KwaZulu-Natal	m-PCR	Wastewater treatment plant	Environment	75	bla_{CTX-M}, bla_{TEM}, bla_{KPC}-2, bla_{OXA}-1, bla_{NDM}-1
[23]	North West	PCR	Humans, cattle, and pigs	Human and animal	76	*tet*B
[24]	Gauteng	PCR	Apples, carrots, tomatoes, spinach, and cabbage	Environment	56	bla_{TEM}, *tetA*, *tetB*, *tetL*, *sulI*, *sulII*, *aadA1a*, *strAB*
[25]	North West	WGS	Faecal (beef and/or dairy)	Animal	80	*tet*A, *tet*B
[26]	North West	PCR	Stool samples from Human and water	Environment and human	212	bla_{CTX-M}, bla_{DHA}, bla_{SHV}
[27]	KwaZulu-Natal	PCR	Wastewater treatment plants	Environment	80	bla_{CTX}-*M*, bla_{TEM}, bla_{SHV}
[28]	Eastern Cape	PCR	Wastewater treatment plants	Environment	111	*mcr*-1, *erm*A
[29]	Eastern Cape	PCR	Faecal samples from dairy cattle	Animal	95	*blaampC*, bla_{CMY}, bla_{CTX}-*M*, bla_{TEM}, *tet*A, *str*A
[30]	Eastern Cape	PCR	Irrigation water and agricultural soil	Environment	46	*tet*A, *tet*B, *tet*C, *cat*II, *cat*III, *sul*I
[31]	Eastern Cape	PCR	Carcasses	Animal	264	*aadA*, *strA*, *ampC*, *catI*, *tetB*, sul1.
[32]	KwaZulu-Natal	PCR	Urinary tract (Human)	Human	26	bla_{CTX}-M, *gyr*A, *qnr*A, *qnr*B, *qnr*S, *qep*A, *aac (6')-Ib-cr*
[33]	Gauteng	WGS	Human (blood, urine, and unknown sources)	Human	20	bla_{CTX}-M, bla_{TEM}-1B, bla_{OXA}, bla_{CTX}-*M-15*, bla_{OXA}, bla_{CTX-M}-*14*, bla_{CTX}-*M-27* (E013), bla_{OXA}-*10*, *blaCMY-2*
[34]	KwaZulu-Natal	PCR	Chickens (slaughter and final retail product)	Animal	266	bla_{CTX}-*M*, *sul*1, *tet*A, *tet*B
[35]	Eastern Cape	PCR	Human (stool)	Human	265	*sul*II, *amp*C, bla_{TEM}, *tet*A
[36]	North West	PCR	Cattle faeces	Animal	73	*aad*A, *str*A, *str*B, *erm*B, *tet*A
[37]	Eastern Cape	PCR	Stool samples from Human	Human	324	*ampC*, bla_{TEM}, *sul*I, *sul*II, *aad*A, *tet*A.
[38]	Western Cape	PCR	Human	Human	12	*mcr*-1
[39]	Western Cape	PCR	Water from the river	Environment	171	*aadA*, *Bla*
[40]	KwaZulu-Natal	m-PCR	Wastewater treatment plant	Environment	146	bla_{TEM}, bla_{CTX}-*M*
[41]	Eastern Cape	PCR	Stool samples from Human	Human	106	*cat*A1, *tet*A
[42]	Western Cape	PCR	Humans	Human	22	bla_{CTX}-*M*, bla_{CTX}-M-15, bla_{CTXM}-14, bla_{CTX}-*M*-3.
[43]	Western Cape	PCR	Wildlife and livestock species	Animal	35	bla_{CMY}, *sul*1, *sul*2, *aad*A1, *tet*A, *tet*B.

WGS = Whole Genome Sequencing, m-PCR = Multiplex PCR

Of the 23 included studies, 7 were environmental samples, 6 were samples from animal sources, 8 were from human and 1 included both human and environmental samples. All the studies included in this review were derived from five provinces in South Africa. Eastern Cape (n = 8) had majority of the studies, followed by KwaZulu-Natal (n = 5), North West (n = 4), Western Cape (n = 2) and Gauteng (n = 1) with the least number of studies (Table 1). The most common method for determining the antibiotics resistance genes of E. coli isolated from all articles included in this systematic review and metaanalysis was PCR (19/23:82.6%), followed by multiplex PCR (2/23:8.7%) and WGS (2/23:8.7%).

3.2. Pooled prevalence estimates (PPE) of antibiotic resistance genes

The blaTEM-M-1 gene was detected from E. coli isolates with a PPE of 36.3% (95% CI: 18.7–58.5), followed by ampC gene 34.4% (95% CI: 16.6–58.1), tetA 32.9% (95% CI: 17.1–53.7), blaTEM 28.8% (95% CI: 18.8–41.5), blaTEM-M 23.3% (95% CI: 7.6–44.1), blaSHV 22.6% (95% CI: 3.3–71.7), strA 21.7% (95% CI: 4.2–63.3), aad 19.4% (95% CI: 9.1–36.8), sul1 15.8% (95% CI: 5.6–37.4), tetB 14.7% (95% CI: 8.5–24.2), cat1 14.0% (95% CI: 0.1–94.8) and sulII 11.9% (95% CI: 4.1–30.3). The rest of the PPE ofARGs is shown in Table 2. However, genes such as blaOXA-1, cat2, tetD, tetK, tetG, tetM, blaCMY-2, dfrA7, strA, bla pse1, bla ampC, ant (3")-la, qnr-B, qnr-S, ermB, blaCTX-M, blaCTX-M-15, blaCTX-M-3 and blaSHV-2were not included for meta-analysis due to the low number of studies. The forest plot depicts the point estimate for individual studies, reporting the presence of ampC, aadA, blaTEM and tetA (Figure S1).

Table 2. Pooled prevalence rate and 95% CI of antibiotic resistance genes of E. coli species based on meta-analysis.

Antimicrobial agents	Number of studies	Number of isolates	% Prevalence (95% CI)	I^2 (95% CI)	Begg and Mazumdar rank *P*-value
strA	4	126	21.7	(4.2–63.3)	0.49691
catI	3	109	14.0	(0.1–94.8)	0.60151
blaCTX-M	5	85	23.3	(7.6–44.1)	1.0000
blaSHV	4	201	22.6	(5.6–37.4)	1.0000
tetB	7	147	14.7	(8.5–24.2)	0.65230
ampC	3	104	34.4	(16.6–58.1)	0.60151
sulII	5	87	11.9	(4.1–30.3)	1.0000
blaCTX-M-1	6	167	36.3	(18.7–58.5)	0.85098
blaTEM	9	323	28.8	(18.8–41.5)	0.53161
tetA	10	401	32.9	(17.1–53.7)	0.17971
sul1	6	111	15.8	(5.6–37.4)	0.57303
aadA	5	172	19.4	(9.1–36.8)	0.14164

A total of 6 animal studies with 813 isolates were included in the meta-analysis, and they had a PPE of 25.4% (95% CI: 13.7–42.3) and 41.2% (95% CI: 10.1–81.4) for the blaTEM and tetA genes, respectively. For humans, 8 studies with 738 isolates were included in this review. The strA gene had a PPE of 30.2% (95% CI: 4.2–81.1), followed by tetA 22.1% (95% CI: 9.1–44.7), Sul1 8.5% (95% CI: 6.5–11.1), Sul11 5.8 % (95% CI: 2.9–11.4), and tetB 13.4 % (95% CI: 10.9–16.2). While 7 studies from the environment were included in this review, only the blaTEM gene was reported, with a PPE of 45.7% (95% CI: 22.5–70.9) from 685 isolates (Figure 2).

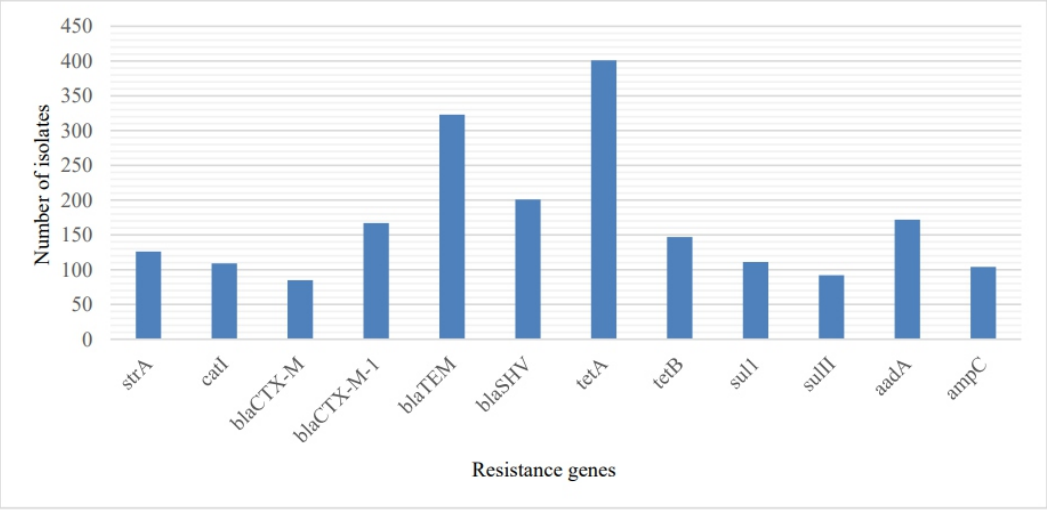

Figure 2. Antibiotic resistance genes detected in South African E. coli isolates from animals, humans, and the environment.

3.3. One health perspective

Of the 23 studies, 29 ARGs from humans, 26 from animals, and 19 from the environment were detected. Eight ARGs were detected in both humans, animals and in the environmental samples, whereas 9 were

detected from humans and animals, 3 from animals and the environment and 2 from humans and the environment, as shown in Table 3.

Table 3. The antimicrobial-resistant genes (ARGs) detected between environmental, humans and animals.

Human & animal	Human & environment	Animal & environment	Animals, human & environment
*amp*C	bla_{SHV}	*aad*A1a	bla_{CTX-M}
*str*A	bla_{OXA-1}	*aad*A1	$bla_{CTX-M-1}$
*cat*I	*qnr*B	*erm*B	bla_{TEM}
*cat*II			*tet*A
*cml*A1			*tet*B
*tet*C			*sul*1
*tet*D			*sul*II
*tet*M			*aad*A
*qnr*B			

3.4. Publication bias

The Begg and Mazumdar rank correlation test demonstrated no significant publishing bias for all parameters.

4. Discussion

Most of the studies included in this review were conducted on humans (34.8%). Out of the nine provinces, only five (55%) provinces, that is, North West, Eastern Cape, KwaZulu-Natal, Gauteng and Western Cape, were represented in this study. However, the Free State, Limpopo, Mpumalanga, and Northern Cape were not represented in the data sets, which may be due to a lack of research facilities in these provinces and/or a scarcity of researchers in the infectious microbiology field. The other reason might be that there are no Medical Research Council (MRC) institutes in those provinces.

AMR continues to increase internationally as a result of the widespread and unchecked use of antibiotics in veterinary and medical procedures [44]. Bacterial antibiotic resistance can spread to unaffected bacteria via DNA or other genetic components like integrons, bacteriophages and transposons [45]. Bacteria expressing ARGs are on the rise as a result of widespread agricultural practices, and the excessive and uncontrolled use of antibiotics to treat human illnesses [45]. Humans, animals, and the environmental components interact, and either directly or indirectly contribute to the spread of antimicrobial resistance [46,44]. In this study, a high prevalence of ARGs in E. coli was found in both human and animal samples.

Twelve resistance genes, namely streptomycin (strA), chloramphenicol (catI), β-lactams (blaTEM, blaCTX-M, blaCTX-M-1, blaSHV), sulphonamides (sul1 and sulII), aminoglycosides (aadA), ampicillin (ampC) and tetracycline (tetA and tetB) were the most detected resistant genes, based on data obtained from studies analyzed in the current review. Infections brought on by pathogenic E. colihave been successfully treated with β-lactam antibiotics. However, a vast number of hydrolytic enzymes, namely the β-lactamases produced by bacteria, are currently seriously impairing the usefulness of β-lactams [47]. The tet (A, B, and C) gene is amongst detected genes in E. coli in this study from both animals, humans, and the environment. Tetracyclines are the most often used or overused antibiotics in livestock production in South Africa [48,44]. Furthermore, Eagar et al. [49] indicated that tetracyclines were the most commonly used antibiotics in animals in South Africa between the years 2002 and 2004, hence, it is not surprising that most bacteria have a high level of tetracycline (tet) resistance [45,50,51].

Therefore, the excessive continued use of this antibiotic has led to the development of resistance. The chloramphenicol, catI gene, was also detected in humans and animals. This is surprising because chloramphenicol has been removed from standard prescription lists due to the side effect of bone marrow aplasia. Gene cassettes of the aadA have been widely found inthe environment and in animal production. The aadA group of genes encodes resistance to streptomycin and spectinomycin [14].

The quinolones, qnr gene, was also found in E. coli isolates of humans, animals and the environment in this study. DNA gyrase and topoisomerase IV are protected from quinolone chemicals by the genes (qnr) expressing proteins that are members of the pentapeptide repeat family, which mediates quinolone resistance in plasmids [52,53]. According to this study, environmental organismsmay have been the source of the circulating qnr genes [54]. Fluoroquinolone resistance is significant since it can spread rapidly among bacterial species that threaten human health. The cross-species and cross-genus transfers of resistance determinants are also possible [55].

In this review, three major molecular approaches were utilized to detect ARGs, such as PCR, multiplex PCR, and whole genome sequencing (WGS). Eighty-eight percent of the articles used traditional PCR techniques, most likely due to easy access to PCR cyclers and the reduced costs involved with PCR. Despite the fact that WGS offers a number of benefits, it was only utilized twice in all of the studies analyzed. More than 70 genes that may be related to drug resistance have been found in many recent large WGS investigations [56]. The WGS analysis has demonstrated the capacity to eliminate phenotypic and genotypic inconsistencies [56–58]. Due to its ability to quickly identify resistance pathways, WGS has become a crucial tool for profiling ARGs and has also played a role in measuring the rate at which resistance emerges [56]. WGS and other high-throughput diagnostic technologies have shown significant promise in medical diagnostics, and have proven to be essential in the control of antibiotic resistance [59].

Using the “One Health” approach, multiple disciplines work locally, nationally, and internationally to achieve optimal human, animal, and environmental health, realizing that the three are interconnected [60]. Since humans, animals, plants, food, and the environment are the main sources of antimicrobial resistance, the necessity of a “One Health” control strategy is highlighted in combating this problem [15]. The presence of similar zoonotic E. coli isolates, in animals, humans and the environment must be taken into consideration in South Africa. Food safety, zoonotic disease control,laboratory services, neglected tropical diseases, environmental health, and antimicrobial resistance are among the areas of work where a “One Health” approach is particularly relevant, according to the World Health Organization (WHO) (https://www.euro.who.int/en/home). The WHO recommends using a “One Health” approach to address health threats at all three interfaces [10,62]. There is a dynamic interaction between human, animal, and environmental components that contribute to the rapid emergence and spread of antimicrobial resistance, either directly or indirectly [44]. This concept emphasizes the importance of balance and interconnectedness across the human-animal-environment sectors.

Even though we have organized data on the prevalence of antibiotic resistance genes in E. coli, the following limitations apply to our study: PPE of some resistant genes were not calculated because there are few reports on each. With respect to provinces, Limpopo, Free State, Mpumalanga, and Northern Cape are underrepresented.

5. Conclusions

This systematic review and meta-analysis gave an overview of scientific data on E. coli antibiotic resistance genes in human, animal, and environmental samples from South Africa. There are significant gaps in surveillance and a lack of published studies on the prevalence of E. coli resistance genes in some provinces like Limpopo, Free State, Mpumalanga, and Northern Cape. This studyrevealed the highest

PPE of E. coli resistance genes to ampC, tetA, blaTEM, blaTEM-M, blaSHV, strA, aad, sul1, tetB and cat1, while eight genes (blaCTX-M, blaCTX-M-1, blaTEM, tetA, tetB, sul1, sulII and aadA) were detected in E. coli isolates from animals, humans, and the environment. This finding calls for the restricted use of this group of antibiotics. There is also a need for detailed studies that document the relationships between the phenotypic and genotypic occurrences of antibiotic resistance, as well as the presence of virulence genes. The fact that resistance genes have been detected in humans, animals, and environmental samples means there is a need for consolidated "One Health" approaches from the ecological, human, and animal health sectors in terms of epidemiological, therapeutics, and policy formulation research,

Conflict of interest

We declare that there are no conflicts of interest.

Author contributions

TR, KEL and OT conceived and designed the study. TR performed the literature review and extraction of data. TR and MT analyzed and interpreted the data, created figures and tables and drafted the manuscript. OT and KEL offered mentorship and guidance on antimicrobial resistance, as well as reviewing the manuscript. All authors read, commented and approved the final manuscript.

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Study of the antibacterial effects of the starch-based zinc oxide nanoparticles on methicillin resistance Staphylococcus aureus isolates

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A B S T R A C T

This study aimed to assess the efficacy of starch-based zinc oxide nanoparticles (ZnO-NPs) against methicillin-resistant Staphylococcus aureus (MRSA) isolates from clinical specimens in Basrah, Iraq. In this cross-sectional study, 61 MRSA were collected from different clinical specimens of patients in Basrah city, Iraq. MRSA isolates were identified using standard microbiology tests, cefoxitin disc diffusion and oxacillin salt agar. ZnO-NPs were synthesized in three different concentrations (0.1 M, 0.05 M, 0.02 M) by the chemical method using starch as the stabilizer. Starch based ZnO-NPs were characterized using ultraviolet–visible spectroscopy (UV-Vis), X-raydiffraction (XRD), field emission scanning electron microscopy (FE-SEM), energy dispersive X-ray spectroscopy (EDS), and transmission electron microscopy (TEM). The antibacterial effects of particles were investigated by the disc diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the most effective starch-based ZnO-NPs were determined using a broth microdilution assay. The UV-Vis of all concentrations of starch-based ZnO NPs exhibited a strong absorption band at 360 nm which was characteristic of the ZnO-NPs. XRD assay confirmed the representative hexagonal wurtzite phase of the starch-based ZnO-NPs, and their purity and high crystallinity. The spherical shape with a diameter of 21.56 ± 3.42 and 22.87 ± 3.91 was revealed for the particles by FE-SEM and TEM, respectively. EDS analysis confirmed the presence of zinc (Zn) (61.4 ± 0.54%) and oxygen (O) (36 ± 0.14%). The 0.1 M concentration had the highest antibacterial effects (mean ± SD of inhibition zone = 17.62 ± 2.65 mm) followed by the 0.05 M concentration (16.03 ± 2.24 mm) and the 0.02 M concentration (12.7 ± 2.57 mm). The MIC and the MBC of the 0.1 M concentration were in the range of 25–50 µg/mL and 50–100 µg/mL, respectively. Infections caused by MRSA can be treated with biopolymer-based ZnO-NPs as effective antimicrobials.

Keywords: antibiotic resistance; antimicrobial; Iraq; MRSA; ZnO-NPs

1. Introduction

Staphylococcus aureus is a Gram-positive bacterium and commensal microorganism that colonizes about 30% of the anterior nares of human individuals [1]. This bacterium plays a considerable role in causing both nosocomial and community-acquired infections including skin infections, endocarditis, osteomyelitis, bacteremia, necrotizing pneumonia, toxic shock syndrome, infections associated with foreign bodies, post-operative surgical infections, and food poisoning [2–4]. During the past few decades, treating infections caused by S. aureus has become challenging due to the emergence of multidrug-resistant, particularly methicillin-resistant Staphylococcus aureus(MRSA) [5].

The antimicrobial resistance including methicillin resistance in the MRSA strains is correlated with the acquisition of a mobile genetic element called staphylococcal chromosomal cassette mec (SCCmec),

which harbors both the mecA or mecC genes, which are responsible for the production of proteins with low binding affinity for beta-lactam antibiotics such as PBP2a [5,6]. Today, the emergence of MRSA strains resistant to linezolid, vancomycin, and daptomycin has been reported [7].

Since antimicrobial resistance has emerged, spread, and endured in MRSA strains, it has become imperative to develop new and effective alternatives to traditional antibiotics to treat the infections caused by these pathogens [5]. In this regard, nanotechnology can be used to develop antimicrobial nanomaterials with more effective properties compared with traditional antibiotics [5]. It is largely due to their nanoscale size and distinct structures that nanomaterials including inorganic nanoparticles have demonstrated a novel and developed biological functions [8]. Recent studies have found that zinc oxide nanoparticles (ZnO-NPs) possess safe and stable properties that make them one of the ideal antibacterial agents [5–8]. There has been speculation that the antimicrobial activity of ZnO-NPscomes from a free radical formation on the surface of metal oxide, which destroys bacterial cell walls and inhibits their growth [8]. Since the experiments conducted in this field are rarely seen in Iraq, this study aimed to assess the efficacy of starch-based synthesized ZnO-NPs against MRSA isolates collected from clinical specimens in Basrah city, Iraq

2. Materials and methods

2.1. Ethics statement

This study was approved by the University of Basrah, Basrah, Iraq according to the Declaration of Helsinki. All methods were performed in accordance with the relevant guidelines and regulations of the University of Basrah, Basrah, Iraq. The clinical samples were collected as routine clinical care for referred and admitted patients and not for this study. Hence, the written informed consent was waived by the University of Basrah, Basrah, Iraq.

2.2. Study design and sample collection

A total of 150 clinical specimens including wound swabs, sputum, throat swabs, nasal swabs, pus,and urine were collected from patients suffering of urinary tract infection (UTI), wound infection, and upper respiratory tract infection. These patients attended the outpatients and inpatients clinics of Alsadr Teaching Hospital and Al-Shefa General Hospital, Basrah, Iraq for a seven-month period from 1 January to 30 July 2022. All samples were collected in sterile conditions with sterile containers andtransmitted to the microbiology laboratory of the College of Medicine, University of Basrah for isolation and identification of MRSA isolates.

2.3. Bacterial isolation and identification

Each clinical sample was directly inoculated into plates of mannitol salt agar (MSA, Merck, Darmstadt, Germany) and sheep blood agar (SBA, Merck, Darmstadt, Germany) and incubated at 37 °Cfor 24–48 h. Then, all colonies from primary cultures were identified depending on the morphological features in culture media as beta hemolytic on blood agar and fermentation of the mannitol sugar on MSA. In addition, a panel of standard microbiology and biochemical tests including Gram staining, catalase, DNase, slide and tube coagulase were performed to confirm the S. aureus isolates [9–11].S. aureus ATCC® 29213™ was used as a quality control strain.

2.4. Identification of MRSA

In vitro detection of MRSA strains were applied by cefoxitin (30 µg) disk diffusion test and oxacillin salt agar that compromised of Mueller-Hinton agar (MHA, Merck, Darmstadt, Germany) containing 6

μg/mL of oxacillin (Sigma, USA) supplemented with 4% NaCl following the Clinical and Laboratory Standards Institute (CLSI) instructions [12]. The plates were inoculated with S. aureusisolates at a concentration of 1.5 ×108 CFU/mL equal to the 0.5 McFarland tube and incubated at 35 °Cfor 16–18 h for cefoxitin (30 μg) disk diffusion and 24 h for oxacillin salt agar, respectively. In the cefoxitin (30 μg) disk diffusion, the isolates were considered MRSA if the inhibition zone around the disks was recorded ≤ 21 mm [12]. In the oxacillin salt agar, the existence of > 1 colony or light film of growth was considered as MRSA [12]. S. aureus ATCC® 29213™ and S. aureus ATCC® 43300™were used as negative and positive quality control strains, respectively.

2.5. Antibiotic susceptibility testing of MRSA isolates

Antibiotic susceptibility testing of MRSA isolates were investigated by disk diffusion test on MHA medium according to the CLSI instructions [12]. The following antibiotic disks (Mast, UK) were used: azithromycin (15 μg), norfloxacin (10 μg), erythromycin (15 μg), rifampin (5 μg),chloramphenicol (30 μg), tetracycline (30 μg), and clindamycin (2 μg).

2.6. Preparation of starch-based ZnO-NPs

The previously described method was used to prepare the ZnO-NPs with minor modifications [13]. We examined different parameters to obtain an optimum synthesized ZnO-NPs. ZnO-NPs were prepared by the wet chemical precipitation method using the zinc nitrate 6-hydrate (Zn(NO3)2.6H2O) (Sigma Aldrich, USA) in three concentrations (0.1 M, 0.05 M, 0.02 M) and sodium hydroxide (NaOH) (Sigma Aldrich, USA) in concentrations of 0.2 M, 0.1 M, and 0.04 M as a precipitating agent in ratio 2:1. Also, in this method the soluble starch (Sigma-Aldrich, USA) was used as a stabilizing agent in concentrations (0.5%, 0.25%, 0.1%) for each prepared concentration of precursors mention above. The different concentrations of the primary precursors and stabilizer were prepared with deionized water and stirred vigorously using a magnetic stirrer till complete dissolution. The zinc nitrate solutions were kept under constant stirring at room temperature using a magnetic stirrer for 1 hour. Next, the starch solution was added and the mixture was magnetically stirred to obtain a homogeneous solution. Then, the NaOH solution was slowly added drop by drop at room temperature under vigorous stirring, which resulted in the formation of a white precipitate of the nanoparticles. The solution was allowed to settle overnight. Then, the precipitate was separated by centrifugation (10000 g for 10 min). The produced nanoparticles were washed three times with distilled water to remove the byproducts and the excessive starch particles that were bound to the formed nanoparticles. Finally, the nanoparticles were dried at 60–80 °C for overnight [13].

2.7. Characterization of starch-based ZnO-NPs

2.7.1. Ultraviolet–visible (UV-Vis) spectroscopy analysis

The presence of nanoparticles was proved by UV-Vis analysis using the Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer (Shimadzu, Kyoto, Japan) in the Department of Physics,University of Basrah. This device detected the surface plasmon resonance (SPR) peak of the prepared starch-based ZnO-NPs in the scanning range of 200–800 nm. An absorbance test was conducted on 1 cm quartz cells using starch-based ZnO-NPs dispersed in deionized water [13].

2.7.2. X-ray diffraction (XRD)

Crystalline structure, nature of the phase, lattice parameters, and crystalline grain size of the starch-based ZnO-NPs were evaluated using XRD type Xpert MPD (Empyrean, Malvern Panalytical, Malvern,

Panalytical, Malvern, United Kingdom) in the Department of Physics, University of Basrah. The parameters were as follows: Cu-K 1 radiation (λ = 1.5406 Å) at 40 kV and 40 mA to work in Bragg–Brentano geometry with 2θ = (20–80)°, a speed of 2 sec/step and 0.02° step, and extract analysis 2θ = (0–80)°[14].

2.7.3. Field emission scanning electron microscopy (FE-SEM) and energy dispersive X-ray spectroscopy (EDS)

The surface morphology and structure (mean particle size) of starch-based ZnO-NPs were evaluated using the FEI Nova NanoSEM 450 (FEI, Hillsboro, OR, USA) equipped with energy dispersive X-ray spectroscopy (EDS) in the Department of Physics University of Basrah. Starch-based ZnO-NPs were mixed with acetone and small drops of each sample were placed on a glass slide and allowed to dry. The samples were coated with thin layers of platinum to be conductive. The device was operated at a vacuum of the order 5–10 Torr. The acceleration voltage of the device was kept in the range of 10–20 kV. In the next step, the compositional analysis of the samples was carried out by EDS attached to the FE-SEM device. EDS analysis was used to determine the elemental compositions of the synthesized ZnO-NPs.

2.7.4. Transmission electron microscopy (TEM)
The prepared solutions of starch-based ZnO-NPs in distilled water (Milli-Q®, Millipore Corporation, Bedford, MA, USA) were placed on carbon-coated copper grid and allowed to dry under ambient conditions. The particle size and the shape of starch-based ZnO-NPs were observed by a TEM microscope (Tecnai G2 200 kV TEM, FEI Electron Optics) with an accelerating voltage of 200 kV [15].

2.8. In vitro antibacterial assay of starch-based ZnO-NPs
Qualitative and quantitative assays were performed to evaluate the antibacterial effects of starch based ZnO-NPs. The qualitative antibacterial effect of starch-based ZnO-NPs against clinical MRSA isolates was performed by standard disc diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were then determined using a broth microdilution assayas a quantitative assay.

2.8.1. Disc diffusion method
For the disc diffusion method, the bacterial isolates were grown aerobically in nutrient broth for 24 hrs at 37 °C. Then, 100 µL of the bacterial suspensions (concentration equal to 2 ×108 CFU/mL)was spread on sterile MHA plates. Sterile Whatman filter paper discs (5 mm) (Sigma-Aldrich, USA) saturated with 50 mg/L of prepared starch-based ZnO-NPs in distilled water (Milli-Q®, Millipore Corporation, Bedford, MA, USA) were placed on each inoculated plate. The cultured agar plates were incubated at 37 °C for 24 h. Finally, the zones of inhibition were recorded. Distilled water was used as the negative control [15]. The experiments were performed in triplicate. Results were estimated as mean ± the standard deviations (SD) of three replicates.

2.8.2. Broth microdilution assay
MIC and MBC were determined for the concentration that showed the highest antibacterial effects with the disc diffusion method. About, 100 µL of starch-based ZnO-NPs was added into a sterile 96 well microtiter plate containing 100 µL of Mueller-Hinton broth (MHB, Merck, Darmstadt, Germany)to reach serially diluted concentrations of 200 to 0.2 µg/mL. Then, 100 µL of the bacterial suspensions (concentration equal to 2 × 106 CFU/mL) was inoculated in each well to reach the concentration of 2 ×

105 CFU/mL. The microplate was incubated at 37 ℃ for 24 h. The MIC was described as the smallest amount of starch-based ZnO-NPs that prevented MRSA growth. Re-culturing (10 µL) of wells with no visible growth was performed on the MHA medium to determine the MBC. Incubation of the MHA plates was conducted aerobically at 37 ℃ for 24 h. The MBC for the examined strains was based on the starch-based ZnO-NP concentration at which bacterial growth was not detected. MHB inoculated with MRSA suspension and MHB alone were used as positive and negative controls, respectively. These experiments were repeated three times and the best observation was recorded as the final result [16,17]. An MIC90/MBC90 was defined as a MIC/MBC that inhibits/kills 90% of MRSA isolates, while the MIC50/MBC50 was the MIC/MBC value that inhibits/kills 50% of isolates [18].

2.9. Data analysis

Statistical analysis of the data was performed using GraphPad Prism 9 (GraphPad Software, USA) and repeated measures ANOVA test. Data were presented as mean ± standard deviation (SD). The significant differences were considered based on the P-value < 0.05.

3. Results

3.1. S. aureus and MRSA isolates

In this study, a total of 61 (40.7%) S. aureus were isolated and identified from 150 clinical samples during the survey period. The isolates that showed round Gram-positive cocci with aggregate in clusters (irregular grapes) phenotype, positive catalase test, positive slide or tube coagulase test, fermentation of mannitol on MSA medium (changing the color of MSA from pink to yellow), and beta hemolysis on SBA were selected as S. aureus isolates. The results of cefoxitin (30 µg) disk diffusion and oxacillin salt agar showed that all 61 isolates were resistant to methicillin and were considered as MRSA. The most prevalence of MRSA isolates was found in ear pus samples (32.8%, 20/61), followed by sputum (29.5%, 18/61), urine (18.0, 11/61), nasopharynx (9.8%, 6/61), throat (6.6%, 4/61), and wound samples (3.3%, 2/61) (Table 1).

Table 1. Prevalence of methicillin-resistant Staphylococcus aureus (MRSA) isolates based on the clinical specimens.

Type of clinical specimens	Methicillin-resistant/Number	*Staphylococcus aureus* isolates/%
Wound	2	3.3
Sputum	18	29.5
Throat	4	6.6
Nasopharynx	6	9.8
Ear pus	20	32.8
Urine	11	18.0
Total	61	100.0

3.2. Antibiotic resistance patterns of MRSA isolates

The antibiotic resistance patterns of the MRSA isolates were shown in the Table 2. Accordingly, the most and the less resistance rates were against rifampin (60.7%) and chloramphenicol (11.5%), respectively

Table 2. Antibiotic susceptibility testing of 61 methicillin-resistant Staphylococcus aureus (MRSA) isolates.

Antibiotics	Resistance/ N (%)	Susceptible/ N (%)
Oxacillin (1 µg)	61 (100)	—
Cefoxitin (30 µg)	61 (100)	—
Clindamycin (2 µg)	12 (19.7)	49 (80.3)
Tetracycline (5 µg)	18 (29.5)	43 (70.5)
Chloramphenicol (30 µg)	7 (11.5)	54 (88.5)
Rifampin (5 µg)	37 (60.7)	24 (39.3)
Erythromycin (15 µg)	21 (34.4)	40 (65.6)
Norfloxacin (10 µg)	21 (34.4)	40 (65.6)
Azithromycin (15 µg)	29 (47.5)	32 (52.5)

3.3. Characterization of starch-based ZnO-NPs

The pour and dried white powder of 3 different concentrations (0.1 M, 0.05 M, 0.02 M) of the starch-based ZnO-NPs is shown in Figure 1.

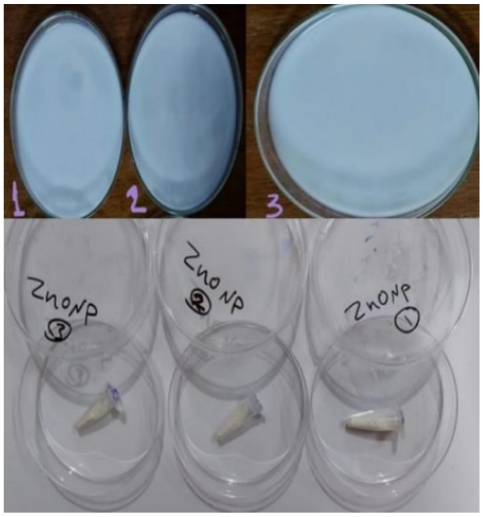
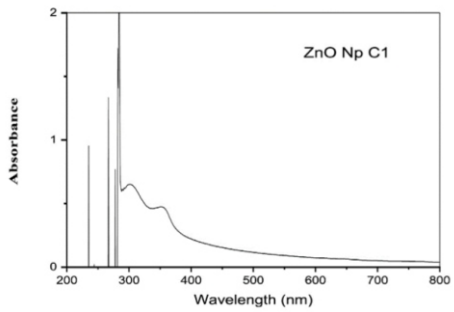


Figure 1. The pour and dried white powder of 3 different concentrations (1: 0.1 M, 2: 0.05 M, 3: 0.02 M) of the synthesized zinc oxide nanoparticles (ZnO-NPs).

3.3.1. UV-Vis analysis

The UV-Vis spectra of the different three concentrations of starch-based ZnO-NPs (0.1 M, 0.05 M, 0.02 M) that prepared with 0.5%, 0.25% and 0.1% of soluble starch were shown in Figure 2.

The three different concentrations of starch-based ZnO-NPs exhibited a strong absorption band in the region below 400 nm (at 360 nm) that was the characteristic for the ZnO-NPs (Figure 2).



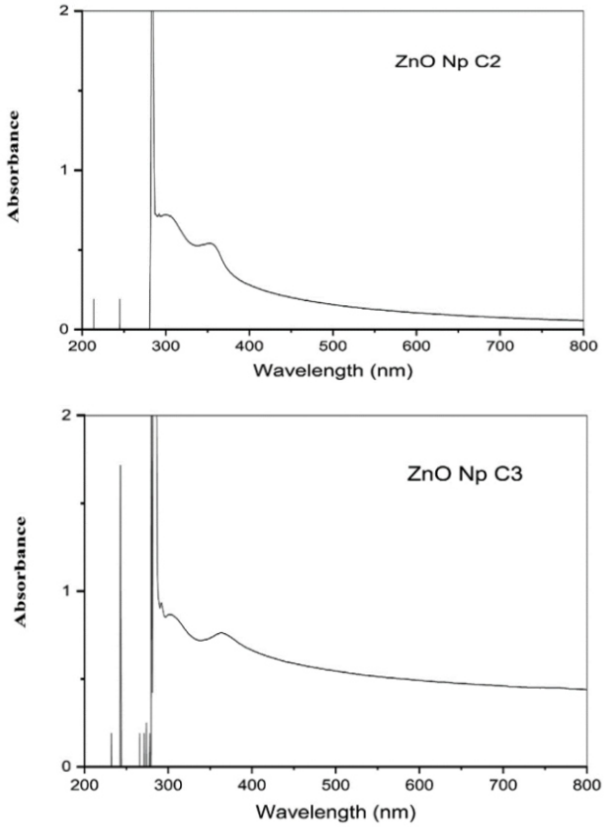
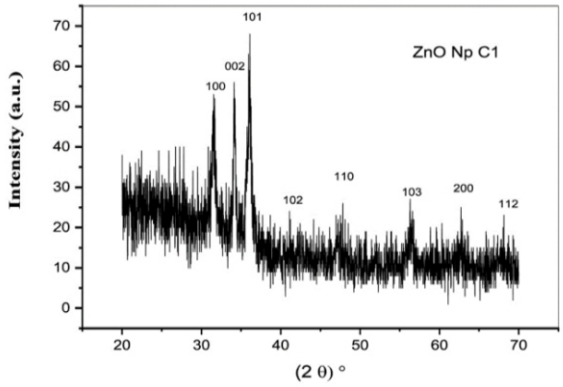


Figure 2. Ultraviolet–visible (UV-Vis) spectroscopy analysis of the three different concentrations (C1: 0.1 M, C2: 0.05 M, C3: 0.02 M) of the zinc oxide nanoparticles (ZnO NPs) that prepared with 0.5%, 0.25% and 0.1% of soluble starch, respectively.

3.3.2. XRD analysis

XRD patterns of the three concentrations of starch-based ZnO-NPs were shown in Figure 3. All diffraction peaks were obtained at 2θ values of 31.7°, 34.4°, 36.2°, 47.5°, 56.6°, 62.8°, 66.3°, 67.9° and 72.5°corresponding to (100), (002), (101), (102), (110), (103), (200) and (112) orientation planes, confirming the representative hexagonal wurtzite phase of the ZnO-NPs. The XRD spectra did not exhibit additional peaks associated with impurities, suggesting the high purity of the starch-based ZnO NPs. Also, as evident from Figure 3, the signal sharpness indicated the high crystallinity of the starch based ZnO-NPs. There were no differences in XDR patterns of different concentrations of ZnO-NPsas all of them showed the diffraction peaks at 2θ values of 31.7°, 34.4°, 36.2°, 47.5°, 56.6°, 62.8°,66.3°, 67.9° and 72.5°.



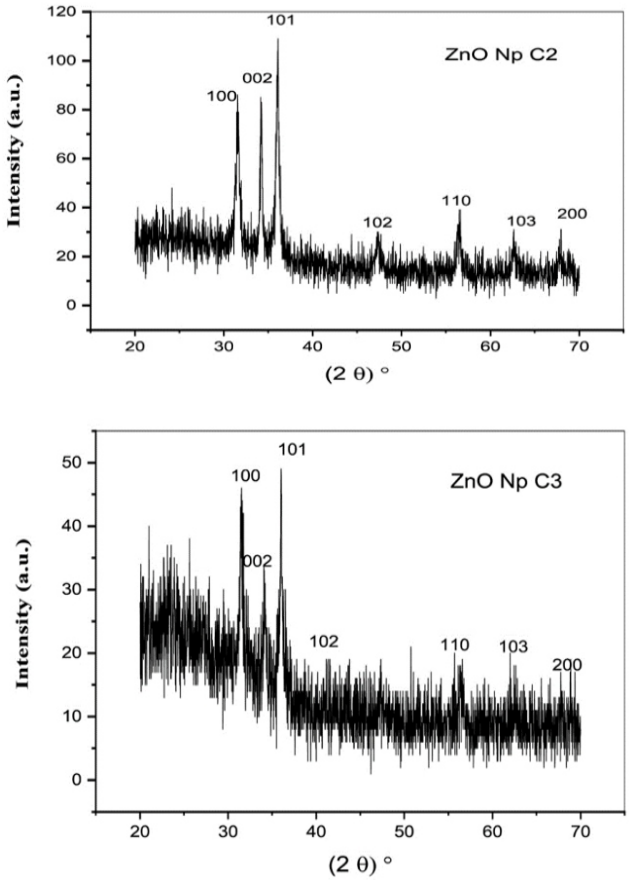


Figure 3. X-ray diffraction (XRD) of the three different concentrations (C1: 0.1 M, C2: 0.05 M, C3: 0.02 M) of the zinc oxide nanoparticles (ZnO-NPs) that prepared with 0.5%, 0.25% and 0.1% of soluble starch, respectively.

3.3.3. FE-SEM and TEM analysis

The morphology of the starch-based ZnO-NPs was investigated by the FE-SEM and TEM as shown in Figure 4 A and B, respectively. The diameter of the ZnO-NPs was in the range of 18.47 to 25.19 (mean ± SD = 21.56 ± 3.09 and 22.87 ± 2.32 nm by FE-SEM and TEM, respectively). Starch based ZnO-NPs displayed spherical morphology as shown by FE-SEM and TEM images. Also, a smooth surface was generally present on the particles, with uniform sizes and shapes. There were no significant differences (P-value > 0.05) in the mean ± SD of the size of three concentrations of the starch-based ZnO-NPs confirming the similarity of their size. Also, the shape of all synthesized ZnO NPs showed spherical morphology confirming their shape similarity.

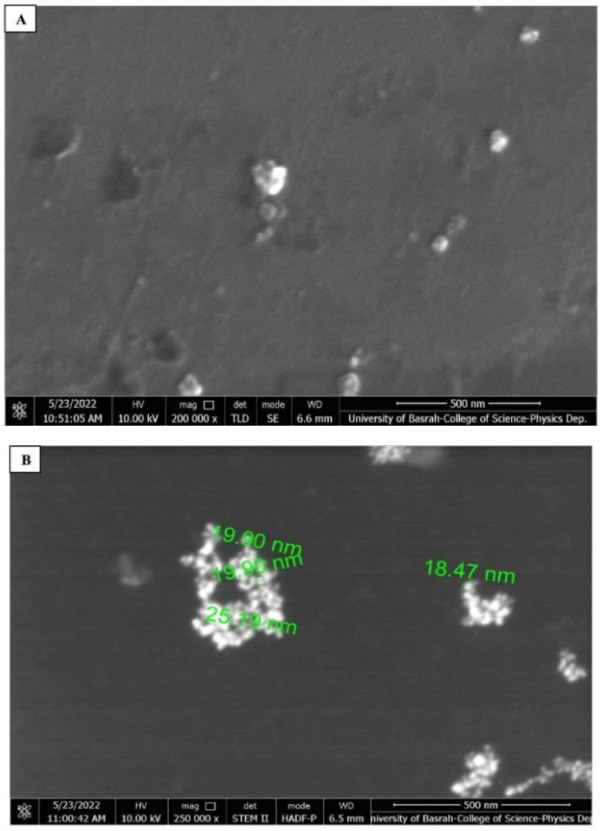
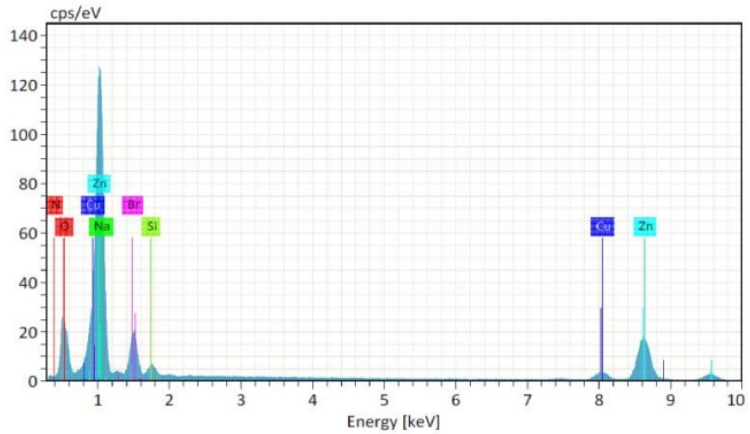


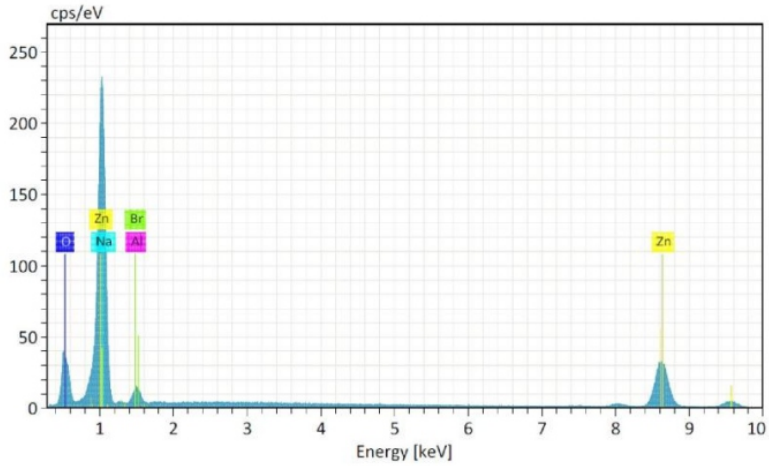
Figure 4. A: Field emission scanning electron microscopy (FE-SEM) morphology of the zinc oxide nanoparticles (ZnO-NPs). B: Transmission electron microscopy (TEM) morphology of the zinc oxide nanoparticles (ZnO-NPs).

EDS plots of the FE-SEM of three concentrations of starch-based ZnO-NPs were presented in Figures 5, 6, and 7. EDS analysis confirmed the presence of zinc (Zn) (54.22%) and oxygen (O) (9.68%) in C1 (0.1 M) concentration (Figure 5). Non-intentional dopants including Na (19.65%), Si (0.81%), Br (7.43%), Cu (6.32%), and N (1.89%) elements were also detected (Figure 5). This was probably due to the presence of substrate over which the ZnO-NPs samples were held for analysis. Also the zinc (Zn) (63.47%) and oxygen (O) (8.98%) were found in C2 (0.05 M) concentration (Figure 6). Non-intentional dopants including Na (24.25%), Alu (0.21%), and Br (3.08%) elements were also detected (Figure 6). Meanwhile, the zinc (Zn) (27.4%) and oxygen (O) (5.39%) were found in C3 (0.02 M) concentration (Figure 7). Non-intentional dopants including Cu (62.86%) as the major element,Alu (3.61%), and N (1.11%) elements were also detected (Figure 7). The carbon was not detected in any synthesized nanoparticles because the precipitates were produced from the reaction to obtain ZnONPs nanoparticles were separated by centrifugation at 10000 g for 10 min. Then, the produced nanoparticles were washed three times with distilled water to remove the byproducts and starch particles that were bound to the formed nanoparticles because the starch used in this method was a stabilizing agent and when the reaction was complete, we exclude it from the formed nanoparticles.



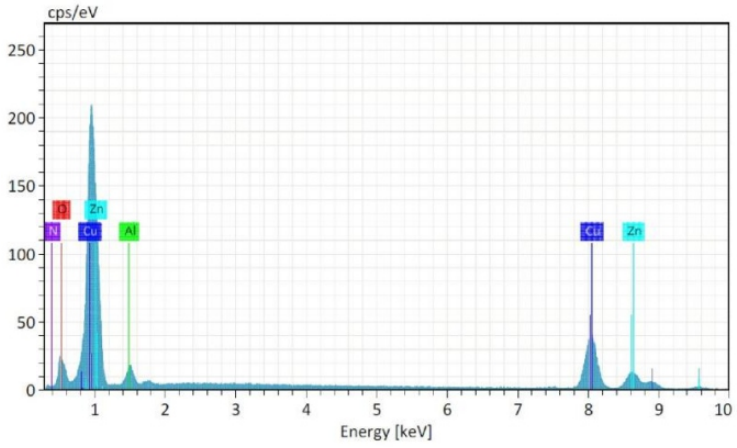
Element	At. No.	Netto	Mass [%]	Mass Norm. [%]	Atom [%]	abs. error [%] (1 sigma)	rel. error [%] (1 sigma)
Nitrogen	7	2510	4.82	1.89	5.09	0.89	18.50
Oxygen	8	31161	24.72	9.68	22.88	3.06	12.37
Sodium	11	93366	50.16	19.65	32.31	3.14	6.25
Silicon	14	5217	2.06	0.81	1.09	0.12	5.79
Copper	29	7986	16.15	6.32	3.76	0.58	3.58
Zinc	30	45478	138.43	54.22	31.35	4.63	3.34
Bromine	35	26411	18.98	7.43	3.52	0.92	4.86
		Sum	**255.30**	**100.00**	**100.00**		

Figure 5. Energy dispersive X-ray spectroscopy (EDS) plot of the field emission scanning electron microscopy (FE-SEM) of C1 (0.1 M) starch-based ZnO-NPs.



Element	At. No.	Netto	Mass [%]	Mass Norm. [%]	Atom [%]	abs. error [%] (1 sigma)	rel. error [%] (1 sigma)
Oxygen	8	27342	14.65	8.98	21.31	1.84	12.59
Sodium	11	92280	39.59	24.25	40.07	2.48	6.27
Aluminium	13	871	0.34	0.21	0.30	0.05	13.85
Zinc	30	51019	103.61	63.47	36.86	3.47	3.34
Bromine	35	8130	5.04	3.08	1.47	0.27	5.42
		Sum	**163.24**	**100.00**	**100.00**		

Figure 6. Energy dispersive X-ray spectroscopy (EDS) plot of the field emission scanning electron microscopy (FE-SEM) of C2 (0.05 M) starch-based ZnO-NPs.



Element	At. No.	Netto	Mass [%]	Mass Norm. [%]	Atom [%]	abs. error [%] (1 sigma)	rel. error [%] (1 sigma)
Nitrogen	7	1172	1.57	1.11	4.06	0.37	23.28
Oxygen	8	13610	7.62	5.39	17.25	1.05	13.76
Aluminium	13	11382	5.11	3.61	6.85	0.27	5.32
Copper	29	50940	88.96	62.86	50.67	2.86	3.21
Zinc	30	16203	38.27	27.04	21.18	1.33	3.49
		Sum	**141.53**	**100.00**	**100.00**		

Figure 7. Energy dispersive X-ray spectroscopy (EDS) plot of the field emission scanning electron microscopy (FE-SEM) of C3 (0.02 M) starch-based ZnO-NPs.

3.4. In vitro antibacterial effects of the starch-based ZnO-NPs

The results of the disc diffusion method showed that all concentrations of the starch-based ZnONPs had inhibitory effects on MRSA isolates. The 0.1 M concentration had the highest antibacterial effects with the mean ± SD of the inhibition zone of 17.62 ± 2.65 mm followed by the 0.05 M concentration with an inhibition zone of 16.03 ± 2.24 mm and the 0.02 M concentration with aninhibition zone of 12.7 ± 2.57 mm (Table 3). The MIC of the 0.1 M concentration was in the range of 25–50 µg/mL, while the MBC was in the range of 50–100 µg/mL. Also, the MIC90/MBC90 and the MIC50/MBC50 were 50/100 µg/mL and 25/50 µg/mL, respectively (Table 3).

Table 3. Antibacterial effects of the synthesized ZnO-NPs against methicillin-resistant Staphylococcus aureus (MRSA) isolates.

Antibacterial effects	Concentrations of ZnO-NPs		
	C1 (0.1 Mol)	C2 (0.05 Mol)	C3 (0.02 Mol.)
Inhibition zones of synthesized ZnO-NPs (Mean ± SD) (mm)	2.65 ± 17.62	16.03 ± 2.24	12.7 ± 2.57
MIC of 0.1 M concentration	25–50 µg/mL		
MBC of 0.1 M concentration	50–100 µg/mL		
MIC_{90}/MBC_{90} of 0.1 M concentration	50/100 µg/mL		
MIC_{50}/MBC_{50} of 0.1 M concentration	25/50 µg/mL		

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

The 0.1 M and 0.05 M concentrations of the starch-based ZnO-NPs showed significantly greater inhibition zones against MRSA isolates compared to 0.02 M concentration (P-value = 0.0001). Likewise, the inhibition zones of the 0.1 M concentration were significantly greater than those of 0.05 M

ZnO-NPs (Figure 8).

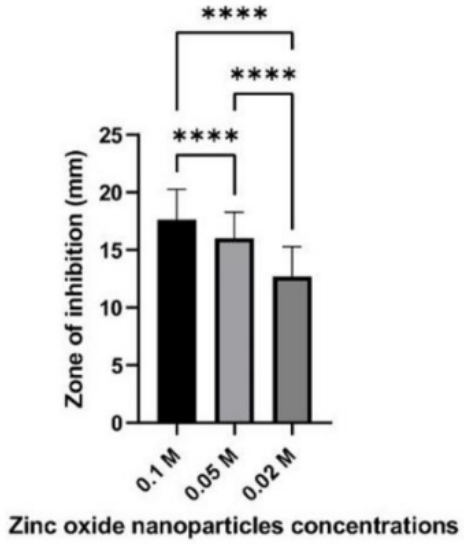


Figure 8. Inhibition zones of different concentrations (0.1 M, 0.05 M, 0.02 M) of zinc oxide nanoparticles against methicillin resistance Staphylococcus aureus isolates. The statistically significant differences were according to repeated measures ANOVA test (Pvalue < 0.05), **** = P < 0.0001.

4. Discussion

Various microbes have been prevented from growing on humans due to the use of zinc salt for decades. Also, there are extensive studies demonstrating the effectiveness of ZnO-NPs against pathogenic bacteria including E. coli and S. aureus [5,8,19]. However, the antibacterial effects of starch-based ZnO-NPs on MRSA isolates from Iraq is lacking. In this study, 61 MRSA isolates were collected from 150 different clinical samples of Iraqi patients, confirming the prevalence of 40.7%. This prevalence rate of MRSA was lower than previous reports from Iran (78.9%) [11] and Iraq (53.1%) [20],and was higher than studies from Italy (1.1%) [21] and Ghana (17.1%) [22]. Differences in prevalence rates may be explained by the differences in bacteria detection methods, examined populations, and studied sample types and sizes in various countries. The MRSA isolates showed relatively high resistance rates against azithromycin and rifampin (more than 40.0%), while the other antibiotics including chloramphenicol, tetracycline, clindamycin, norfloxacin, and erythromycin were more effective with resistance rates below 35.0%. In comparison to this study, previous research from Iran [11], found a higher resistance rate to azithromycin (100%) and erythromycin (98.3%) among MRSA isolates.However, in a previous study from Fiji [23], MRSA isolates showed a significantly lower resistance rate against clindamycin (0.0%), rifampicin (0.0%), and tetracycline (12.0%) that was in contrast to this study. These differences may be explained by the variations in the patients' demographics and geographical location that influence the resistance rates.

In this study, the qualitative and quantitative antibacterial assays showed promising effects of all synthesized starch-based ZnO-NPs against all MRSA isolates. These observations were in line with the previous studies from Egypt [5], Iraq [8], and Iran [16], in which the strong inhibitory effects of ZnO-NPs were found on multidrug-resistant S. aureus. The highest rate of inhibition was found at 0.1 M concentration with the mean ± SD of the inhibition zone of 17.62 ± 2.65 mm followed by the 0.05 M concentration (16.03 ± 2.24 mm) and the 0.02 M concentration (12.7 ± 2.57 mm). In previous studies,ZnO-NPs showed inhibition zones of 73.95 ± 2.17% at 10 mg/mL against vancomycin-resistant S. aureus (VRSA) and 16–21 mm against various Gram-negative and Gram-positive bacteria [8,17]. In

another study by Kamarajan et al. [24] from India, ZnO-NPs at a concentration of 10 µg/mL showed inhibitory effects against Escherichia coli (25 mm), Pseudomonas aeruginosa (23 mm), S. aureus(22 mm), and Bacillus subtilis (21 mm). The discrepancies in the inhibitory zone size in different studies may be due to the bacteria studied, shape, size, concentrations of the synthesized ZnO-NPs, and method used to synthesize ZnO-NPs.

Previous studies have found that the shape, size, concentrations of the synthesized ZnO-NPs, and the method to synthesize ZnO-NPs affect the antibacterial properties of the nanoparticles [24–27]. In this study, the highest concentrations of the starch-based ZnO-NPs exhibited significantly greater inhibition zones compared to the lowest concentrations. These results were consistent with the previous studies in which higher concentrations of ZnO-NPs showed stronger antimicrobial effects [8,25,26].

However, some studies showed that the inhibition zone of nanoparticles starts to shrink beyond an optimal concentration [28,29]. One of the possible reasons may be due to the accumulation of nanoparticles in high concentrations and the inability to penetrate into bacterial cells [28,29].

ZnO-NPs are believed to act in four distinct ways including releasing Zn2+ ions, damaging the cell wall, producing reactive oxygen species (ROS), and by ZnO-NPs internalizing [25]. The antibacterial activity of ZnO-NPs depends on their penetration into bacterial cells. Thus, the antibacterial effects of ZnO-NPs can be evaluated by the broth dilution method as a precise and confirmative assay [5]. In this study, the broth microdilution assay revealed the MIC of the starchbased ZnO-NPs in the range of 25 to 50 µg/mL at the 0.1 M concentration. Also, the MBC was in the range of 50 to 100 µg/mL. These values were lower than a previous study that reported ZnO-NP MICs ranging from 128 to 2048 µg/mL against S. aureus isolates [5]. MIC values in this study were also lower than those reported by Jasim et al. [8] against VRSA isolates (625 µg/mL). However, in a previous study by Tănase et al. [14] from Romania, the chemical and Saponaria officinalis extractmediated ZnO-NPs showed lower MICs (<20 µg/mL) against standard strains of S. aureus, P. aeruginosa, E. coli, and Candida albicans. The differences among studies may be due to the used methodology, the antibiotic resistance patterns of examined bacteria, and the structural nature of the synthesized ZnO-NPs.

In this study, the structural nature of the starch-based ZnO-NPs were investigated by various methods.The UV-Vis analysis showed that three concentrations of starch-based ZnO-NPs (0.1 M, 0.05 M, 0.02 M) exhibited a strong absorption band at 360 nm which was characteristic of the ZnO-NPs. This observation was in good parallel with the previous studies from Egypt [13], Romania [14], and Jordan [30] which showed the absorption peaks of ZnO-NPs below 400 nm. The shape, size, and method of fabrication of ZnO-NPs are all factors influencing the absorption peak. In general, ZnO NPs exhibit a UV-Vis spectroscopic peak between 350 and 390 nm [30]. Moreover, the XRD analysis of the three concentrations of the starch-based ZnO-NPs confirmed the representative hexagonal wurtzite phase, high purity, and high crystallinity of the starch-based ZnO-NPs. These results were consistent with the previous observations from Malaysia [15] and Jordan [30]. Another observation of this study was the spherical morphology of the starch-based ZnO-NPs with a diameter of 21.56 ± 3.42 and 22.87 ± 3.91 nm by FE-SEM and TEM, respectively. There was no any significant difference among three concentrations of the starch-based ZnO-NPs in terms of size and shape. In contrast to this study, Saleemi et al. [15], showed rod-shaped morphology of standard ZnO-NPs with the diameter of 49.39 ± 22.54 nm [15]. Alshraiedeh et al. [30] reported spherical ZnO-NPs with a size of 100 nm in their study.

From a future perspective, it is recommended to examine the synergistic effects of the synthesized starch-based ZnO-NPs in combination with standard antibiotics or other chemical or plant-based materials against different pathogens and cancer cell lines. Previous studies have shown the significant effects of combining nanoparticles with other materials against microorganisms [31,32]. Although several studies have investigated the antibacterial effects of ZnO-NPs against MRSA isolates, but in

each of them, the different researchers looked forward to finding more effective nanoparticles in terms of their shape, quality, and antibacterial effects. The novelty of this study was the synthetization of relatively smaller nanoparticles in comparison to previous studies. Also, the synthesized nanoparticles showed promising antibacterial effects in low concentrations (0.02 Mol). However, this study had several limitations as follows: lack of investigation of starch-based ZnO-NPs against other Gram positive and Gram-negative bacteria, lack of in vivo experiment, and lack of time-kill kinetics assay.

5. Conclusion

UV-Vis, XRD, FE-SEM, and TEM analysis showed the crystalline organization, spherical shape, and smooth surface of the starch-based ZnO-NPs with a size below 27 nm. Qualitative and quantitative antimicrobial assays showed the promising effects of the starch-based ZnO-NPs against clinical MRSA isolates with MIC ranging from 25–50 µg/mL. Further in vivo experiment is needed to reveal the mechanism of action of the synthesized starch-based ZnO-NPs.

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Conflicts of interest

The authors declare no conflicts of interest.

Authors' contributions

RMAM and HAJ were involved in the conception and design of the work, data analysis and drafting of the article. RMAM and AH were involved in experimental works and data collection. HAJ and AH revised and approved the final version of the manuscript.

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Minimally processed fruits as vehicles for foodborne pathogens

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ABSTRACT

The consumption of minimally processed fruit (MPF) has increased over the last decade due to a novel trend in the food market along with the raising consumers demand for fresh, organic, convenient foods and the search for healthier lifestyles. Although represented by one of the most expanded sectors in recent years, the microbiological safety of MPF and its role as an emergent foodborne vehicle has caused great concern to the food industry and public health authorities. Such food products may expose consumers to a risk of foodborne infection as they are not subjected to prior microbial lethal methods to ensure the removal or destruction of pathogens before consumption. A considerable number of foodborne disease cases linked to MPF have been reported and pathogenic strains of Salmonella enterica, Escherichia coli, Listeria monocytogenes, as well as Norovirusaccounted for the majority of cases. Microbial spoilage is also an issue of concern as it may result in huge economic losses among the various stakeholders involved in the manufacturing and commercialization of MPF. Contamination can take place at any step of production/manufacturing and identifying the nature and sources of microbial growth in the farm-to-fork chain is crucial to ensure appropriate handling practices for producers, retailers, and consumers. This review aims to summarizeinformation about the microbiological hazards associated with the consumption of MPF and also highlight the importance of establishing effective control measures and developing coordinated strategies in order to enhance their safety.

Keywords: fresh-cut fruit; foodborne pathogens; cross contamination; microbiological quality; control measures

1. Introduction

Fruits comprise a large group of plant foods that represent an important source of essential nutrients for a balanced diet. They also provide bioactive phytochemicals, such as flavonoids and phenolic compounds, associated with several health-promoting benefits [1]. In recent years the European Union (EU) health institutions have run campaigns recommending the daily consumption of at least “5 a day” fruit and vegetable portions. In addition, the World Health Organization (WHO) recommends a minimum of 400 g per capita [2]. These campaigns have been strongly supported by the increasing evidence of an enriched fruit diet associated with a lower risk of cardiovascular diseases and several types of cancer [3]. In fact, a daily fruit intake seems to have a positive impact on the prevention of a great number of chronic diseases [4].

Worldwide significant changes in lifestyles and major shifts in consumer trends have taken place.Such changes reflect the demand for a new and wider range of fresh products, which along with ashorter available time for home cooking, led the food industry to an emerging market of ready-to-eat fresh products. As a response to a growing demand for convenient, healthy, and easy-to-prepare fresh products, a wide range of minimally processed fruit (MPF) has been developed [5]. These products seem to represent a good alternative to today’s lifestyle as they provide a safe handling and a rich source of

nutrients, along with an attractive presentation [6]. They can also allow the consumer to reduce waste, since only the edible part of the product is taken home.

This mini-review aims to gather information about the microbiological and safety issues associated with the consumption of processed fruit taking into consideration their highly perishable nature, as well as provide some insight into prevention measures.

2. Processing of fruits

MPF are products that have undergone physical changes but retain the freshness and the natural properties of the original fruit. Also called as "ready-to-eat", "pre-cut" and "fresh-cut", these fruits are submitted to unit operations, which include selection, cleaning, washing, trimming, peeling, cutting/shredding/mashing, sanitizing, and finally packing. During production they are not submitted to microbial lethal techniques (e.g., pasteurization) that might reduce microbiological risks, so these foods can be potential vehicles for the transmission of pathogenic bacteria, viruses, toxins or spore forming microorganisms [7]. As a result, fresh-cut fruit must be stored, distributed and marketed under refrigeration to achieve a satisfactory shelf-life, which can last between 7 to 20 days if an adequate cooling temperature is maintained. However, the tendency to extend the shelf-life of refrigerated foods may facilitate the proliferation of psychrotrophic microbial contaminants, pathogenic or spoilers. Concern about the microbiological safety of MPF has increased due to the emergence of foodborne infections connected to their consumption and to the increase of vulnerable populations (seniors, weakened immune systems individuals). A number of disease cases have been reported in the United States of America (USA) and EU countries representing a public health hazard and a negative impact on this food industry sector. Contamination can occur at any step of production, processing, distribution, and also as a result of consumer practices. Therefore, understanding the key factors in the transmission chain will be a valuable contribution to establishing best practices and prevention measures. After harvesting the fruits are processed through a series of operations, summarized in Table 1

Table 1. Main steps/operations of industrial processing of minimally processed fruit (MPF) [7,88–90].

Selecting	o Selection of good quality fruits with adequate color, acidity, Brix o Advanced maturation stages limit the shelf-life of MPF o Early maturation stages impact negatively the sensorial characteristics of MPF
Washing	o Removal of field residues (dust, pesticides, insects, etc.) o Cooling immediately after washing to lower internal temperature and delay the biological processes that accelerate the maturation
Peeling	o Removal of the fruits outer layer when it is not edible or when the final presentation requires it o Manual, mechanical, enzymatic. Hot water or high-pressure steam may be used o All peeling should be done in the least abrasive way to prevent the invasion of internal tissues by microorganisms (internalization) and avoid darkening
Cutting	o Dimension reduction operations: slicing, chopping, grating, cutting into cubes or into sections o Manual or mechanical o Good sharp cutting tools should be used to reduce physical damage to the cells
Washing (2^{nd}) and disinfection	o Done by spraying with water or immersing the fruit for a pre-established period of time in chilled water in tanks (1–10 °C) containing an adequate concentration of disinfectants o Most common disinfectant: chlorine (50–200 ppm) o Step that reduces the microbial load during MPF processing o Microbiological and chemical quality of the water should be regularly monitored to avoid cross-contaminations

Rinsing	○ Removal of excess surface water and disinfectants residues from MPF ○ Residual surface drops of moisture and surface exudation of freshly cut fruits may stimulate the growth of fungi and bacteria ○ Avoid damage to the fruit tissues
Packaging	○ MPF weighing ○ MPF packaging ○ Metal control ○ Bags, boxes or trays and different types of protective films ○ Refrigerating temperatures

3. Microbial hazards

MPF are raw foods ready to consume, characterized by cut, non-sterilized, physiologically active surfaces rich in nutrients and water, and not thermal or chemically preserved. The minimal processing (peeling and dimension reducing) contributes to the increase of the tissue respiration rate as well as other biochemical reactions, such as the production of ethylene in the cells, which generates heat and accelerates degradation. As a result, in addition to spoilers and pathogens growth, these products are also susceptible to several visual changes during shelf-life (color, flavor, texture, and surface desiccation) (Figure 1).

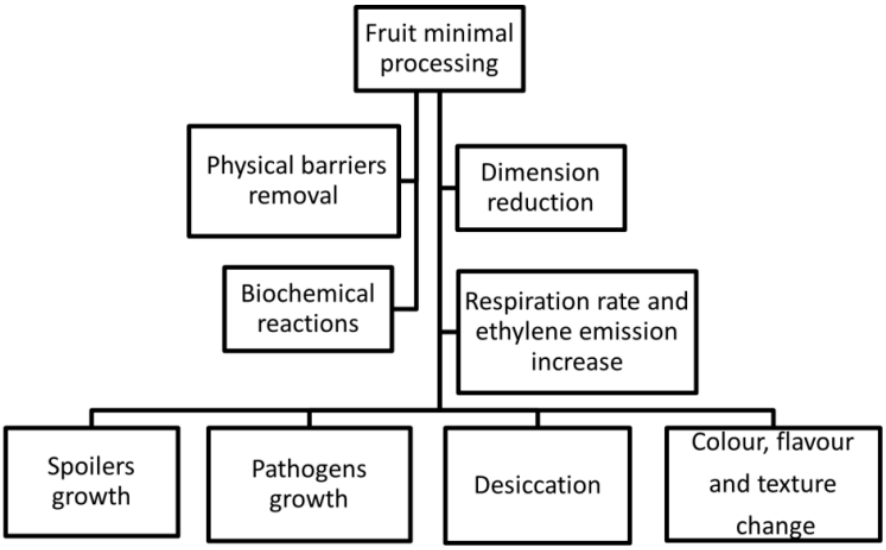

Figure 1. Impact of minimally processing on the quality of the final product.

Due to the survival and growth of pathogenic microorganisms on MPF, their consumption has been connected with infections caused by bacteria, viruses and parasites [8,9]. Nowadays, consumers have a great choice of fruits in the retail markets, which are available in several forms: fresh, minimally processed, canned, frozen, or dried. Processing of these kinds of fruit products consists of a series of operations where contamination and cross-contamination can take place at both the industry or home level. Therefore, as previously mentioned, these commodities are not submitted to any surface pasteurization or cooking, which make them a significant route for foodborne pathogens representing a threat to consumers' health. The major pathogenic microorganisms associated with the consumption of fruit are Salmonella enterica, Escherichia coli, Listeria monocytogenes, as well as the Hepatitis A and Norovirus viruses [7,10,11].

3.1. Salmonella enterica

Salmonella enterica is one of the leading agents of foodborne illnesses and is responsible for thousands of deaths every year. Infections caused by this pathogen are a major concern to public health and the food industry worldwide, and both animal and non-animal food sources are potential vehicles of disease transmission. In the last two decades a great number of S. enterica outbreaks have been traced to fresh, fresh-cut and frozen fruits consumption, which were associated to a high diversity of serovars. A very high number of serotypes/serovars of S. enterica are distinguished on the basis of somatic, flagellin and capsular antigens, resulting in over 2,500 antigen combinations [12].

Depending on the host and serotype, S. enterica is the etiologic agent of enteric fever (typhoid fever), enterocolitis/diarrhea and bacteremia. Serotypes S. Typhi and S. Paratyphi are particularly adapted to humans, causing typhoid fever. Non-typhoid Salmonella serotypes are the most frequent salmonellosis agents in developed countries. Several studies have demonstrated the ability of Salmonella spp. to multiply on the leaf surface of young plants suggesting that plants may constitute alternative hosts for Salmonella and play an important role in their transmission to animals [13–15].

The common reservoir of S. enterica is the human, domestic and wild animal gastrointestinal tract. S. enterica enters the soil and agricultural environments through animal feces and can directly contaminate plants and surface waters used for irrigation and pesticide/fertilizer preparation.

Salmonellosis occurs after ingestion of contaminated food or water or as a result of contact with symptomatic or asymptomatic carriers. Bacteria survive the acidity of the stomach and colonize the intestine where they invade the epithelial cells and are trapped in vacuoles. Vacuoles containing the bacteria can be destroyed, releasing the microorganisms into the cytosol of host cells and replication can occur allowing Salmonella to display an intracellular lifestyle [16,17].

According to the microbiological criteria of the EU [18] Salmonella must be absent in various food categories including vegetables and MPF. If there is fecal food contamination the transmission of the microorganisms will occur, particularly under growth promoting conditions when food is stored at inadequate temperatures. Numerous studies have demonstrated the survival and growth of Salmonella spp. in fruit. Strawn and Danyluk [19] observed an increase in Salmonella cells on cut mangos and papayas, stored at 12 ºC, and survival for 28 days when storage was at 4 ºC. They also reported the survival of Salmonella on cut mangos and papayas after 180 days of freezing. An increase in Salmonella populations in fresh-cut peaches stored at 20 and 25 ºC was also addressed by Alegre et al. [20]. Ukuku et al. [21] observed the survival of Salmonella population in sliced Cantaloupe melon stored at 5 ºC for 7 days and an increase in its growth rate in samples stored at 10, 15 and 20 ºC. Palekar et al. [22] reported the survival of Salmonella Poona for 21 days in Cantaloupe melon slices stored at 5 ºC. In 'Rocha' pear, Salmonella grew at 12 and 20 ºC, reaching a population of more than 8 log cfu/g, in a period of 24 hours. However, in three days at 8 ºC, it only increased about 1 log cfu/g and at 4 ºC it did not multiply [23].

3.2. Escherichia coli O157:H7

The pathogenic strain of Escherichia coli, namely E. coli O157:H7, is a relevant pathogen related to MPF safety since it has been implicated in some outbreaks and sporadic cases. E. coli strains are members of the gastrointestinal microbiota of man and mammals. Fecal matter can contaminate food and water, including irrigation and recreational waters. Human infections by pathogenic E. coli strains occur after the consumption of contaminated food such as undercooked meat, contaminated fresh vegetable and fruit or through contact with contaminated animals [24]. Disease transmission through person-to-person contact may also occur when proper hygiene care is not ensured. Fruit, like any other food of plant origin, can be contaminated by cross-contamination through contact with raw materials and meat. Food handlers (symptomatic or asymptomatic carriers) may also be responsible for the

transmission of the bacteria [25,26].

The ability of E. coli strains to survive and grow in environments other than the gastrointestinal tract represents a public health threat. E. coli has been isolated from environments such as soil, manure and irrigation water. However, it has also been found in the internal tissues of lettuce [27] and plant roots [28]. MPF permit E. coli dissemination in food industry processing and packaging environments, contributing to the transmission through the food chain when good manufacturing practices are not complied. E. coli O157:H7 can survive and multiply outside the gut and has been implicated in various disease outbreaks related to the intake of fresh-cut fruits and vegetables [7]. Abadias et al. [29] found the survival of the E. coli O157:H7 population inoculated in fresh-cut pineapple and melon, packed in a modified atmosphere, and stored at 5 °C for 15 days. In peaches an increase of 2 log cfu/g was observed in the E. coli O157:H7 population at 20 and 25 ºC [20]. Strawn and Danyluk [19] reported that E. coli O157:H7 was able to grow in fresh-cut mango and papaya stored at 23 ºC, surviving for 28 days when samples were stored at 4 °C and 180 days when frozen at -20 ºC. In 'Rocha' pear E. colishowed significant growth at 12 and 20 ºC, reaching high populations (>8 log cfu/g, in 24 h). At 8 ºC the microorganism increased more than 1 log cfu/g in 3 days, although at 4 °C no proliferation occurred [23].

3.3. Listeria monocytogenes

Listeria monocytogenes is a robust pathogen with relevance to public health and the food industry as it can lead to listeriosis, a severe foodborne disease affecting mostly risk groups, such as children, pregnant women, elderly and immunocompromised people. Although relatively rare compared to other foodborne infections, listeriosis is associated with a high mortality rate and clinical cases can lead to 20–30% deaths [30]. This bacterium is widely found in the environment (soil, water, manure, decaying vegetation), can persist in mammalian and avian feces, and also in multiple food processing environments. Its ability to survive and grow in multiple niches is supported by a complex system of tolerance responses [31]. Those responses help the pathogen to survive to several inhibitors frequently found in the food industry, such as disinfectants, sanitizers, low-pH conditions, osmotic pressure and eventually allowing it to remain in this environment for long periods [32]. Strains that are involved in foodborne diseases seem to be also linked to biofilm formation, which makes them even more difficult to eliminate from industrial food facilities once they are established [33]. As a psychrotrophic, L. monocytogenes can survive and multiply at low temperatures and may reach dangerous levels in foods kept under refrigeration. The ability of L. monocytogenes to grow under temperature abuse conditions (3 days at 4 ºC followed by 5 days at 8 ºC) was also reported on freshcut 'Conference' pears packed under modified atmosphere during their shelf-life [34]. Furthermore, this tolerance response system permits the pathogen to have a successful transition from food to the gastrointestinal host system, leading to cell invasion and the establishment of infection [35].This pathogen has been associated with a great variety of foods including dairy, poultry, meat, seafood, fruits, and vegetables [36]. In the last decade, the importance of the consumption of non-animal foods, as a cause of listeriosis has been increasingly recognized in fresh, fresh-cut, and frozen fruits [37,38].

Along with S. enterica and E. coli O157:H7, L. monocytogenes became a microorganism of great concern to fruit and vegetable safety [39]. In 2011, the consumption of contaminated cantaloupe was responsible for a multistate outbreak in the USA resulting in 147 people infected and 33 deaths. In 2014, another multistate listeriosis outbreak associated with the ingestion of caramelized apples cross contaminated at the packing facility caused 35 hospitalizations and three deaths [40]. Environmental samples collected at the apple packinghouse and clinical isolates revealed the presence of similar strains [41]. In the same year, an outbreak linked to stone fruits (peaches, plums and nectarines) contaminated with L. monocytogenes was also reported [42].

For a long-term period, it had been generally assumed that intact fruits were only contaminated by microorganisms at the external surface and the internal acidic environment of most fruits would prevent bacterial contamination. However, the whole fruit can also serve as a vehicle for foodborne disease transmission. Caramel apples were assumed to be a minimal risk food for listeriosis due to the internal fruit acidity and to the low water activity of caramel, but growth of Listeria had already been reported in fresh apples by Conway [43]. The survival, growth, and internalization of L. monocytogenes in fresh and processed fruits have been well documented and they are now recognized as one of the emergent food products at risk of listeriosis transmission [42]. Graça et al. [23] studied the growth of Listeria spp. on fresh-cut 'Rocha' pear and reported the bacteria capacity to multiply at 20, 12, 8, and 4 °C, despite needing adaptation periods inferior to 24 h at 8 and 4 °C. According to Zeller et al. [44] the growth of L. monocytogenes on MPF correlated significantly with the pH. None of the tested MPF with a pH below 4 showed a significant proliferation of this bacterium.

L. monocytogenes is usually killed by cooking and high-temperature methods, so food products eaten raw, such as fresh, fresh-cut and frozen fruits, are at the highest risk. According to the EU regulation, the absence of L. monocytogenes is not required in all ready-to-eat products. In food products that support growth, the levels of L. monocytogenes cannot increase higher than 100 cfu/g over the shelf-life [18]. However, in the USA there is zero tolerance for this pathogen.

Taking into consideration the characteristics of L. monocytogenes, it becomes a difficult, if not impossible task, to completely eliminate it from processing environments. Furthermore, the last reported outbreaks, sporadic cases and several recalls of fresh and fresh-cut fruit due to the presence of L. monocytogenes have highlighted the serious hazard of this food products safety and the urgent need for improving control measures in the fruit supply chain [45].

3.4. Norovirus

Food-borne viral infections have been increasingly recognized as one of the major causes of human diseases. Norovirus (NoV) has emerged worldwide as a leading agent of acute gastroenteritis outbreaks, causing million cases annually. Although characterized as mild infections, in risk groups, such as children under five, elderly or immunocompromised people, these diseases can be responsible for severe outcomes with a high rate of hospitalizations and deaths [46]. NoV are enteric pathogens, non-enveloped single-strand positive-sense RNA viruses, classified into the Caliciviridae family, which comprises a genetically and antigenically diverse group, having at least seven known genotypes (GI-GVII).Three genotypes (GI, GII and GIV) can cause disease in humans, being the GII the most frequently reported [47].

NoV and hepatitis A virus(HAV) are the main foodborne viruses associated with the consumption of fresh, MPF, and frozen berries [2], as well as the pathogenic bacteria mentioned before. Fresh and frozen fruits, such as grapes, raspberries, blueberries and strawberries have been reported as vehicles of an increasing number of NoV outbreaks [48–50]. One of the most severe NoV outbreaks occurred in Germany in 2012, when 11,000 people were affected by the consumption of frozen berries imported from China [51]. In March 2009 a massive outbreak associated with frozen raspberry ingestion caused 500 cases in a primary school in Finland [52]. Fruits pose a significant risk to foodborne viral transmission as they are consumed raw and are not submitted to lethal treatments. Contamination can occur at any step of the production chain. Even an endophyte contamination of fruit has been suggested [53]. Water is a critical vehicle in the virus's infectious cycle. Non-enveloped RNA viruses are usually present in human sewage in high loads. They are neither completely removed or inactivated by conventional sewage treatment processes and can be discharged into the environment. The use of contaminated water in agriculture or in processing operations (washing steps) are major vehicles of viral

transmission. The shedding of virus particles by symptomatic or asymptomatic carriers can spread the disease through handlers and surfaces/equipment contributing to the viral transmission [54].Although the majority of noroviruses outbreaks were caused by frozen berries the consumption of minimally processed fruits can also represent a potential risk for the transmission of this foodborne disease etiological agent [55–57].

NoV shows high resistance to environmental stressors, such as heat, high/low pH, drying, light and UV exposure and also to chemical and physical disinfection treatments [58]. This persistence allows them to remain infective in foods for periods from 2 days to 4 weeks. As most foodborne viruses they are supposed to have very low infectious doses and fewer than 10 viral particles are required to cause disease [59]. NoV infections usually have a 12–48 h incubation period followed by symptoms, such as vomiting, diarrhea, abdominal cramps, and low-grade fever.

NoV has also shown to be resistant to the most common food preservation methods and can survive chilling, freezing, acidification, reduced water activity and modified atmosphere packaging [60] Minimally processing can induce cross-contamination of uncontaminated fruit during all post-harvest operations/utensils, in addition to the involved workers' health condition [61]. The long-term persistence of NoV on surfaces of food preparation plays a significant role in viruses dissemination [62–64].

Screening food products for the presence of foodborne viruses is challenging due to the physical and chemical properties of the food matrices. On the other hand, cultivating foodborne viruses in cell cultures is yet not possible. Therefore, the detection methods of these microorganisms rely upon the molecular techniques that are crucial in the investigation and prevention of outbreaks. The last decade has shown significant development and optimization of new and sensitive methods of NoV detection and the RT-PCR assay has become the gold standard for food products. Rapid laboratory diagnosis can be an important tool in controlling NoV outbreaks and guiding the choice of prevention measures such as cleaning/disinfection protocols, isolation of infected food handlers and surveillance of the pathogen in the food processing environment.

4. Sources of contamination

MPF are susceptible to microbiological contamination at any step of the production chain and eventually in the consumers' kitchen. The contamination can originate from human, animal or environmental sources and take place during the pre-harvesting, harvesting, and post-harvesting stages.

4.1. Pre-harvest

One of the pre-harvesting sources of contamination is the soil where plants are cultivated since soil is a natural habitat of various microorganisms and crop production is often fertilized by untreated manure/human biosolids, which can harbor high levels of pathogenic microorganisms [65]. Low quality water sources used for irrigation, such as reutilized or waste waters, can as well contribute to significant contamination. Meteorological conditions also matter and the occurrence of rain or drought, the presence of dust, aerosol, and feces may have a great influence on microbiota proliferation [66].

4.2. Post-harvest

During the post-harvest all the steps are susceptible to cross-contamination and can eventually lead to an increase of the microbial population on the final products. Fruit washing may not remove microorganisms as they can remain attached to plant surfaces or become internalized in the edible parts of the fruit [67]. The efficacy of antimicrobials used in the disinfection never reaches 100% as their active principles may be consumed in chemical reactions with organic matter or microorganisms may be

protected in biofilms or internalized in the fruit tissues. The methods used to decontaminate the fruits are based on physical or chemical processes or a combination of both. The most widely used disinfecting agent in the fresh food processing industry is chlorine. However, the levels of bacterial reductions obtained when using chlorine solutions in MPF, at the concentrations (50–200 ppm) and contact times allowed (1–2 minutes), are around 1 to 2 log cfu/g [68]. The most active form of chlorine is hypochlorous acid whose highest activity occurred between pH values of 5.0 and 6.0 as measured by Marin et al. [69]. In addition, the same authors showed that the most adequate pH regulators were the non-organic ones, such as phosphoric acid when compared to the organic pH regulators (for example citric acid). The microbiological characteristics of the water used in the washing procedures are crucial to avoid cross contaminations. For example, Penteado et al. [70] observed that mangoes ('Tommy Atkins' variety) can get contaminated with S. enterica when contaminated water at 47 °C was used in the washing.

The post-harvest microbial contamination of MPF is also associated with a great number of sources: the use of contaminated containers/utensils, unhygienic conditions of food handling, unhygienic surfaces/equipment, packaging material, transport vehicles, and inadequate storing temperature. For example, the peeling and size reduction operations as well as food contact surfaces also present the risk of contamination and recontamination due to the contact of contaminated equipment with fruits (Figure 2).

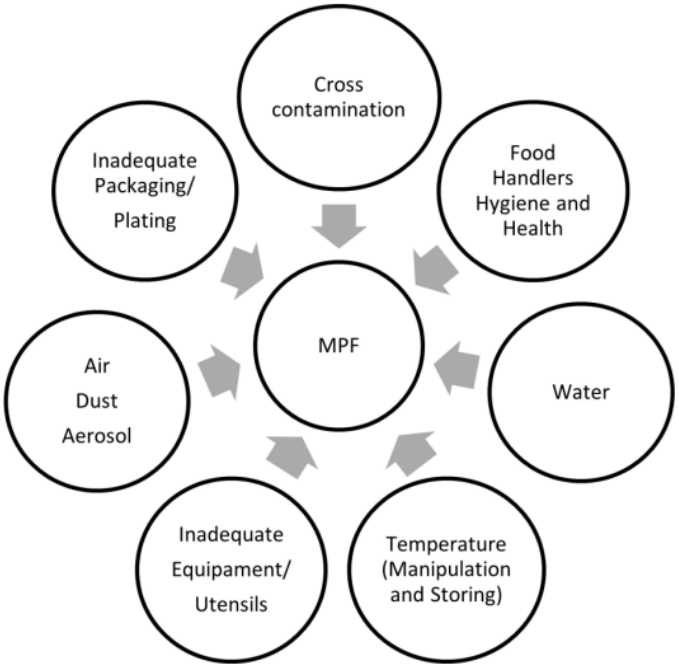

Figure 2. Main sources of MPF microbial contamination at the post-harvest level.

A study conducted in industrial plants revealed high levels of total aerobic microorganisms, higher than 20 cfu/cm2, on all food contact surfaces, namely in peeling equipment, knives, and cutting boards, among others [71]. The same study reported high counts of the Enterobacteriaceae family (β Glucuronidase positive E. coli) on cutters (90 cfu/cm2) and the chopping boards (76 cfu/cm2). In fact, once introduced, some microorganisms are able to persist and form biofilms if the design, compositionand topography of surfaces allow. Poor design characteristics, such as horizontal surfaces, right angles, welds/joins, and container/tank corners are critical regarding hygiene, being more difficult to cleanand, more susceptible to the development of biofilms, as studied in fresh-cut washing containers [72].Another example of the importance of well-designed equipment was the one related to the

listeriosis outbreak caused by cantaloupe consumption, in 2011, which indicated a postharvest contamination of fresh melons. Changes in the equipment used for the washing and drying process of these fruit products created favorable environmental conditions for microbial growth. This equipment, not designed for an efficient sanitization, allowed the contact of its components (such as brushes and felt rollers) with the fruits enabling pathogen adhesion and colonization, which may have spread to other food contact surfaces and promoted microbial contamination [73].

Packaging is a critical step in MPF processing and should be carried out with maximum hygiene. The modified atmosphere packaging (MAP) is one of the available techniques at this level to reduce the microbial load and extend the shelf-life of fresh fruit and MPF. This method consists of the modification of the internal atmosphere of a package, replacing the oxygen content with carbon dioxide or nitrogen. The process decreases the respiration rate, ethylene production, and enzymatic browning, providing a delay of ripening and suppressing the growth of indigenous aerobic microbiota, then maintaining the appearance of the fruit. Atmospheres with low O2 increase the anaerobic metabolism of fruits and fermentation which may produce acetaldehyde and taste-altering compounds. Concerning microbial growth, atmospheres with low O2 concentrations inhibit the growth of most aerobic degrading microorganisms, such as Gram-negative bacteria or filamentous fungi. However, under certain conditions, the growth of spoilage facultative anaerobic yeasts and anaerobic or microaerophilic psychrotrophic pathogenic microorganisms, such as Clostridium spp. and L.monocytogenes may be stimulated. The growth and toxin production of Clostridium botulinum is of particular concern. On the other hand, the extension of shelf-life may increase the available time and the possibility of pathogens, if present in these food products, to grow [7].

The use of MAP for fresh-cut fruits requires careful selection of the film and package type for each product. Temperature control at storage and distribution is also an important factor for an effective MAP system. However, the effect of MAP on microorganisms can vary depending mainly on the storage conditions and the type of product. According to Corbo et al. [74], MAP containing 5% O2 and 30% CO2had no effect on L. monocytogenes growth on cactus-pear; the pathogen survived and also grew at refrigeration temperatures (4 and 8 °C). Furthermore, Abadias et al. [29] observed that high levels of CO2 (11, 25, 39 %) under 1, 2 and 3 days of storage had little or no inhibitory effect on E. coli growth on fresh-cut melon kept at 25 °C. On the other hand, Salmonella spp. showed high variability in response to MAP conditions in different studies. Raybaudi-Massilia et al. [75] reported a slight decrease in Salmonella Enteriditis population on fresh-cut apples and pears at 5 °C, under MAP conditions. On the contrary, an increase in Salmonella Michigan population on fresh-cut peaches was registered when samples were stored at 25 °C with a CO2 level higher than 20% [20].

However, it is important to highlight that some of the mentioned hazards are predictable but manyof them may be unexpected, particularly in cases of endophytes, when microorganisms produce biofilms, or internalize in the fruit tissues.

5. Internalization of pathogens

The ability of human pathogens to internalize plant tissues has been addressed in a great number of studies. Human pathogens can survive the harsh soil environment, adhere to, and actively invade plants. They can enter plant tissues either through natural elements (stomata, roots junctions, flowers), as well as through damaged tissues (cut surfaces, wounds) [76,77]. The contact with pathogens leads to infiltration and colonization of the plant tissues and this process can take place both at pre-harvesting and post-harvesting phases.

The presence of human enteric pathogens in crop fields can be a result of contaminated irrigation water, climate conditions (rain and wind), insect and nematode vectors [78]. Soils and plants can be

contaminated by contact with raw manure or sewage and the enteric pathogens persistence in this kind of environment has been observed [79]. E. coli O157:H7 and S. enterica have been isolated from the feces of birds and domestic animals and transmission to soil and plants has been well documented [80].MPF surfaces are especially favorable to the entrance of pathogens since current sanitizing practices are not effective to remove or inactivate internalized bacteria. Surface bacteria may not be totally removed by washing procedures with hypochlorite at the standard concentrations during industrial processing [81].
Multiple studies have addressed the potential for the systemic transfer of internalized bacterial cells within plant tissues. Holden et al. [82] were able to show that internalized E. coli O157:H7 and S. enterica remained viable and cultivable in the leaves of lettuce, spinach and tomato. Deering et al. [83]also observed the viability and persistence of those pathogens after a period of three weeks on the same vegetables, which is relevant to the harvesting time of those plants. In addition, the pathogenicity and virulence of those bacteria are not affected by the plant colonization [84].
To be able to colonize the surface or interior of a plant human pathogenic bacteria must compete with the naturally present microbiota. To utilize plant nutrients and ensure their persistence in the tissues those microorganisms may depend on the presence of natural endophytes (bacteria or fungal populations), which can provide carbon and energy sources (via degradation of cell wall polymers or induced secretion of sugars) that otherwise would be inaccessible to the pathogens [85,86]. Successful pathogens probably accept nutrient efflux mechanisms of the host to redirect nutrient flux. However, nutrient acquisition used by bacterial pathogens and the mechanisms they use to alter host physiology, notably the efflux of sugars to support growth, are poorly understood [56]. Future research is needed on the identification of bacterial effectors and their target genes in plant cells that facilitate pathogen nutrition [56,87]. A better understanding of the dynamic interactions between the plant endophytes and soil microbiome as sources of contamination during plant growth are relevant to the fresh produce production chain. The internalization of pathogenic microorganisms and the occurrence of endophytism in vegetables make microbial contamination increasingly unpredictable and need to be considered in risk assessment.

6. Preventive measures during processing

It is crucial to apply preventive measures to reduce foodborne pathogens throughout the farm-to fork chain of fresh-cut fruit production and ensure safety and quality, decreasing the risk of any potential outbreak due to their consumption (Table 2).

Table 2. Preventive measures to reduce microbiota contamination and growth (pathogens and spoilers) during post-harvest stages of MPF fruits (HACCP- Hazard Analysis and Critical Control Point).

Preventive measures	Operations/actions
Use good quality water in:	Cleaning Washing Disinfecting
Disinfection:	Chemical (chlorine, organic acids, electrolyzed water) or physical (irradiation) processes or a combination of both Most common disinfectant: chlorine
Select refrigeration temperatures during: (Avoid temperature abuse)	Storage Transportation Distribution Exhibition/Marketing In restaurants, hotels, at home

Avoid moisture in the processing environment and on fruits surface:	Drying after washing and rinsing (Avoid condensation in packages)
Use proper and sharp cutting equipment:	Reduction of fruit tissue destruction
Segregation is a mandatory rule to decrease cross-contamination:	Segregate processed from unprocessed fruit Segregate animal from plant origin food
Select hygienic-designed equipment and infrastructures:	Choice of containers without corners, welds, right angles
Select cleaning and disinfection plans of food plants, food contact surfaces and equipment with the appropriate frequency:	Elect processing facilities with a hygienic architecture Choose hygiene-designed equipment Disinfectants are not 100% efficient Avoid biofilm production Prevent aerosol formation
Adopt good manufacturing practices:	Prevent fecal contamination Stimulate the adoption of strict personal hygiene Provide education for food handlers Implement HACCP
Be aware that microorganisms can:	Express virulence Evolve and mutate Adapt to disinfectants/biocides Grow during shelf-life Produce biofilms Internalize the tissues of the fruit Possess an endophytic profile

7. Conclusions

The safety of MPF is a relevant issue since these food products can act as vehicles for foodborne infections and diseases associated with their consumption are of concern to public health services and to the food industry. Controlling them requires specific guidelines aimed at reducing the risks of contamination, from pre to post-harvest stages until the consumers' table. Implementation of good and hygienic agricultural and manufacturing practices prior, during and after the processing of MPF, including an efficient cold chain, along with compliance to HACCP principles by all the involved stakeholders, are key measures to mitigate outbreak risks and minimize the economic impact of MPF spoilage. Regular monitoring and surveillance from food safety authorities and regulatory agencies are also crucial strategies to increase the quality, safety, and shelf-life of MPF. Nevertheless,epidemiological traceability of these products as human pathogens carriers is difficult to achieve.Further studies are required on microbial internalization, endophytism, and microbiological survival and growth on MPF at different stages of processing. Professionals, from production to commercialization, should be made aware that microorganisms evolve, and adapt to biocides/disinfectants and various stressful conditions, in addition to their ability to internalize, which makes microbial contamination highly unpredictable. Control measures should focus on prevention and be strongly robust to deal with the growing unpredictability of microbial risks.

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Conflict of interest

The authors declare no conflicts of interest.

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