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# Editorial

## From Climate COP to Biodiversity COP

**Prof. Dr. Dr. hc. mult. Angelika Ploeger,** Head of the Editorial Board FoFJ

*This year's UN Climate Conference (nov.22), hyped as the "Implementation COP" where world leaders were to finally turn words into action, failed in Sharm El Sheikh, Egypt.* The last - minute agreement on a proposed funding mechanism for loss and damage because of climate changes, especially for countries from the South, was all that could be salvaged. As the two-week long protests of civil society showed, COP discussed false solutions, with carbon markets clinging to the climate solution bandwagon polluting climate conversations inside the negotiation rooms. Across governments, in both the Global North and South, it seems that the corporations dominate climate policymaking.

Now, COP15 in Montreal expected to deliver new global commitments to biodiversity protection and conservation. United Nations Member States must shake off corporate lobbying and commit to full divestment e.g. from intensive livestock farming and other harmful industries if we are to have any hope of addressing the intertwined threats of climate change and biodiversity loss. COP 15 is set to open on December 7 with negotiations aimed at reaching a consensus on a Global Biodiversity Framework to guide global policy on biodiversity conservation for the coming decades. Biodiversity1 is fundamental to human well-being and a healthy planet as well as economic prosperity for people living in harmony with nature. It underpins every part of our lives. People depend on it for food, medicine, energy, clean air and water, security from natural disasters as well as recreation and cultural inspiration and identity, and it supports - among others - all systems of life on earth. The global biodiversity framework seeks to respond to the Global Assessment Report of Biodiversity and Ecosystem Services issued by the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) in 2019.

Climate change and biodiversity loss are intertwined crises that require immediate action to heal "Mother Earth". The framework is based (see figure 1) and recognizes that urgent action at global, regional, subregional, national level is required, and assumes that transformative actions are taken to (a) address the drivers of biodiversity loss, (b) put in place tools and solutions for implementation, (c) reduce the threats to biodiversity and , (d) ensure that biodiversity is used sustainably for the joint benefit of people and the planet, and (e) recognize the rights of "Mother Earth" and that these actions are supported by the

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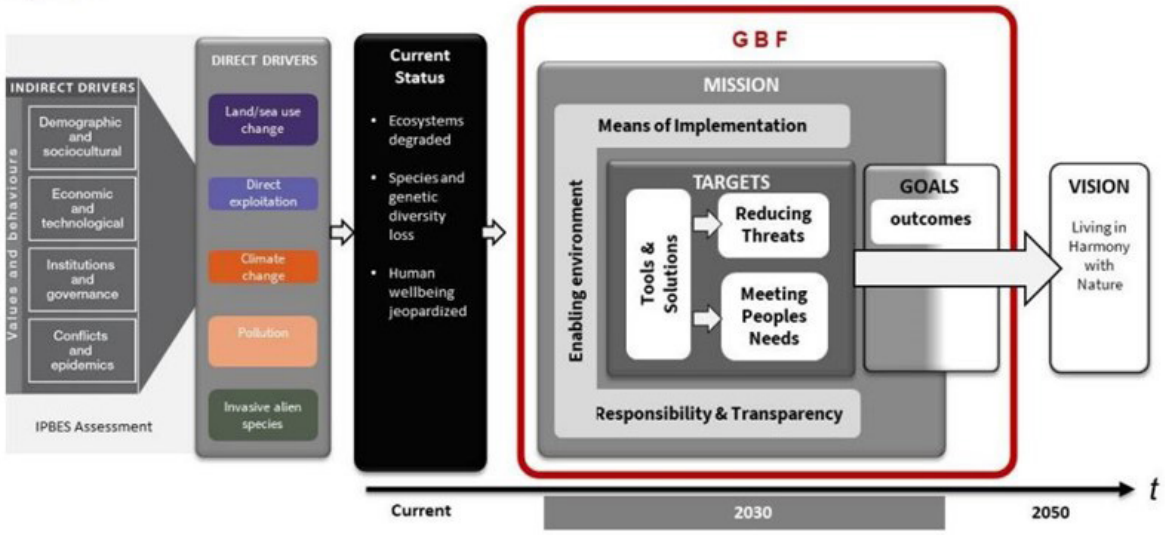
Figure 1




**Resource:** WG2020-5 – CG-6 Non-paper on item 4 Date: 4 December 2022 – 5.30 a.m. Version 1 page 3

necessary enabling conditions, and -in particular in developing countries- including financial resources, capacity building, scientific cooperation, technology transfer, knowledge, and effective responsibility and transparency mechanisms. It also assumes that progress is monitored in a transparent and responsible manner to ensure that, by 2030, the world is on a path to reach the 2050 Vision for biodiversity (1, page 2].

The vision for 2050 of the framework is a world of living in harmony with nature where: "By 2050, biodiversity is valued, conserved, restored and wisely used, maintaining ecosystem services, sustaining a healthy planet and delivering benefits essential for all people."

The mission of the framework for the period up to 2030, towards the 2050 vision is:

"**Option 1.** To take urgent action and provide the necessary resources to halt and reverse biodiversity loss to achieve a nature positive world and to put nature on a path to recovery for the benefit of the planet/ Mother Earth and people;

**Option 2.** To take urgent action to halt and reverse biodiversity loss to achieve a nature positive world/ to put nature on a path to recovery, in a fair and equitable way, for the benefit of the planet/Mother Earth and people by conserving and sustainably using biodiversity, and ensuring the fair and equitable sharing of benefits from the use of genetic resources, while providing the necessary means of implementation (1, page 3)"

**It is up to all of us to support this mission by changing our lifestyle to live in harmony with nature!**

1 WG2020-5 – CG-6 Non-paper on item 4 Date: 4 December 2022 – 5.30 a.m. Version 1

2 CBD COP15: Biodiversity's 'Paris' or 'Copenhagen'? written by Simone Lovera Heinrich- Böll Stiftung Nov. 25nd 2022: https://www.boell.de/en/2022/11/25/cbd-cop15-biodiversitys-paris-or-copenhagen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Research Paper

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10 (5) November 2022

# A scoping review on the presence of antibiotic residues in milk and the government strategies to control the use of antibiotics in the milk industry in India

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Antibiotic residues; Milk; India; Dairy industry; Health risks; Tetracyclines

In dairy farms, antibiotics are administered for therapeutic and prophylactic purposes. After antibiotic administration, lack of adherence to withdrawal periods and irrational use of antibiotics in the feed may lead to antibiotic residues (AR) in milk. The issue is predominantly concerning in India, as it has the most extensive consumer base in the world. It is a matter of grave concern, as numerous studies have identified that consuming milk contaminated with antibiotics may pose a myriad of health hazards. Therefore, this scoping review was conducted to collate all the available information on AR in milk, its associated health risks, and the government initiatives in place to combat inappropriate antibiotic use in the Indian dairy industry. The review was conducted systematically using Joanna Briggs Institute (JBI) guidelines, 2020 as its framework. A comprehensive search was performed using databases such as PubMed, Scopus, Google Scholar, Web of Science, and Google. A total of 19 articles were retrieved for the AR in milk, and 11 grey literature were identified for the government initiatives to control the use of antibiotics. Analysis of the collated information revealed that tetracyclines were the most commonly occurring AR in milk. Additionally, AMR was identified as the most common health hazard that may arise due to AR in milk, followed by allergic reactions. Finally, the review concluded that there is a requirement for more stringent regulations to curb AR in the dairy industry in India.

## 1. Introduction

Misuse of antibiotics is the driving agent responsible for Antibiotic resistance (AMR) in humans. In 2011, Jaipur Declaration highlighted that antibiotics' irrational use among food animals is another major contributor to AMR along with the human sector (Jadhav, Lata Chauhan, and Garg, 2019a). Some of the animal-derived food products are meat, fish, honey, milk, and eggs. Milk and its derivatives form an integral component of the entire global populace. It is a highly consumed commodity that has proven human health benefits (Sachi et al., 2019). However, it has been established that milk is subjected to adulterants either intentionally or unintentionally, diminishing the health benefits of milk. Generally, milk is adulterated by products such as water, sugar, pond water, detergent, and other synthetic compounds (Singh and Gandhi, 2015). Milk also gets contaminated due to residues from veterinary drugs such as antibiotics following the treatment of infectious diseases in cattle (Sachi et al., 2019. The prevalence of antibiotic residues (AR) in samples of milk has been documented since the 1960s and thus is not a new issue (Sachi et al., 2019). Globally, many studies have reported the presence of AR in milk.

The Food Safety and Standards Act (FSSA), 2006, define veterinary drug residues as "the parent compounds or their metabolites or both in any edible portion of any animal product and include residues of associated impurities of the veterinary drugs concerned" (FSSAI). The most pertinent cause of antibiotic residues in milk is its injudicious use.

### 1.1. Antibiotic use in the dairy industry

In dairy farms, antimicrobial drugs are used for therapeutic and prophylactic purposes (Panigrahi, Singh Sheoran, and Ganguly, 2017). Antibiotics are administered mostly for clinical mastitis and as "dry cow therapy" to treat any intra-mammary infections (Kumar and Gupta, 2018). Penicillin, tetracyclines, sulphonamides, and aminoglycosides are frequently used medicines among veterinary drugs (Panigrahi et al., 2017). The residues in the milk are mostly found because of extensive use as growth regulators by mixing in their feed, failure to observe the withdrawal period, lack of medical records, injudicious use for treating diseases, and utilization of unapproved drugs (Panigrahi et al., 2017; Pawar N, 2012).

### 1.2. Global Scenario

The number of antibiotics used in livestock was estimated to be 63,151 ± 1,560 tons worldwide (Van Boeckel et al., 2015). Its indiscriminate use in the dairy industry can have debilitating effects on humans as the global consumption of milk is projected to increase in the coming years due to a surge in demand (Anon, 2020). A review that was done at the global level found that the majority of the work was done in Asia, followed by Europe. In Asia, China accounted for the major proportion of the studies, whereas India had a comparatively lesser number of published works (Sachi et al., 2019). Moreover, the risk of AR in milk is believed to be higher in developing countries antithesis to developed countries. The lack of infrastructure and regulatory authorities for detecting and controlling drug residue levels in food products generated from animals may be one of the reasons. (Jayalakshmi et al., 2017).

### 1.3. Indian Scenario

India stands among the top five countries with sizable shares of global consumption of antimicrobials in food animals (Sivaraman and Yann, 2018). Consumption of milk infiltrated with AR may steer potential health hazards in consumers. It is a rising issue of prime concern as the milk industry in India has the second-largest consumer market in the world (FICCI, 2020). Additionally, India's dairy industry is the world's major and fastest-growing industry, and antibiotic-contaminated milk can have grave economic consequences. (FAO, 2014). Studies on mastitis have exhibited that milk contaminated with antibiotics causes significant economic losses to the farmers, as the milk infiltrated with such residues must be discarded (FAO, 2014). A loss of 1390 INR per lactation was recorded in a study due to mastitis, in which 48.53% of the loss was incurred because of discarded milk (MK and NN, 2013). Apart from financial losses, inappropriate utilization of antibiotics in cattle can predispose the emergence of resistant bacteria, ultimately leading to antimicrobial resistance in humans along with other long-term health effects (Moudgil, Jasbir Singh Bedi, et al., 2019). WHO posited that Antimicrobial resistance (AMR) develops when bacteria, viruses, fungi, and parasites evolve and become unresponsive to antibiotics, making infections more difficult to treat and raising the chances of disease transmission. With this increased drug resistance, antibiotics are becoming alarmingly ineffective, threatening the ability to treat common infections in humans (WHO, 2020).
Recognizing the gravitas of the situation in India, this scoping review was conducted with the dual objective of mapping the evidence on the occurrence of antibiotics in milk, its public health-associated risks, and the government strategies that have been implemented so far to limit the use of antibiotics in India's dairy industry.

## 2. Methodology

The scoping review followed the framework prescribed by Joanna Briggs Institute (JBI) Reviewer's Manual 2020 guidelines (Peters et al., 2020). This framework was primarily used to systematically synthesize the evidence available for AR in milk samples and the health hazards linked with it. A priori protocol was developed before commencing the study. The study systematically followed the six steps mentioned in the JBI framework.


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### 2.1 Study Location

The geographical context chosen for this study was India, a South-East Asian country as per WHO regions (WHO). India is divided into distinct regions such as North, South, Central India, East, West, and North-east region. India was selected for this study, considering the widespread prevalence of antibiotic use among livestock animals(Sivaraman and Yann, 2018).

### 2.2. Identifying the research question

For this study, the research questions were set in accordance with the objectives of the study. The research questions identified were: (1) What are the various types of AR found in milk? (2) What is the various health hazards linked with the presence of AR milk? (3) What government policies/ regulations were formulated and implemented to combat the indiscriminate usage of antibiotics in the dairy industry in India?

### 2.3. Search Strategy

Based on the inclusion criteria, a comprehensive search method was developed to identify relevant studies. Initially, limited searches were conducted in PubMed to identify suitable keywords. Based on the determined keywords, an extensive search was conducted from 25th January 2021 to 12th February 2021 on databases for mapping evidence on AR in milk: (1) PubMed (2) Scopus (3) Web of Science, and (4) Google Scholar. The research articles were limited to those from January 2011 to January 2021 and published in the English language. The articles were restricted to quantitative studies and contained the following terms in their title and abstract: Antibiotic residues, Milk, and India. Various synonyms used for Antibiotics were Oxytetracycline/ Tetracycline/ Penicillin/ Sulfonamide/ Veterinary Antibiotic; Traces was used as a synonym for Residues. Whereas, for milk, milk samples/ dairy milk/ cow milk/ buffalo milk was used. Multiple combinations of keywords were used in conjunction with Boolean operators like AND/ OR/ NOT to form the search string. The terms were also modified in each database to find the maximum number of articles (Appendix 2).

Whereas the preferred type of document to understand the government initiatives to control the use of antibiotics in India was literature in the form of government policies and regulations. A comprehensive search was conducted from 13th February 2021 to 5th March 2021 using the Google search engine. The timeline chosen for the grey literature was also limited to that published from 2011 to 2021 and in English.

### 2.4. Study Selection

Following the search, all identified citations were collected and imported into the reference manager. Duplicate articles were removed, and subsequently, the search results were stepwise screened based on titles and abstracts by the first reviewer. The studies that met the inclusion criteria were retrieved and further screened for full text. Any ambiguity over the inclusion or exclusion of the articles was discussed with the second reviewer and based on the consensus between the two reviewers; the articles were included/ excluded from the review. The study selection process was later charted with the help of the Preferred Reporting Items for Systematic Reviews and Meta-analyses for Scoping Reviews (PRISMA-ScR) flow diagram, and the results of the selection process were elaborated in the results section.

### 2.5. Charting the data

All the included studies were reviewed and charted using a data extraction tool devised and aligned to the objectives and the research questions of this scoping review. The domains under which the data extraction for AR in milk (Appendix 3) were:

1. Distribution of study as per location in India
2. Type of Antibiotics found
3. Type of risk assessment used for the milk residue, and
4. Public health risks identified.

For the government initiatives, the finalized grey literature articles were categorized into three domains and charted using a table. The three domains were:

1. Initiatives on Quality and Control of Antibiotic Use
2. Initiatives Related to Dairy Animal Health
3. Initiative to Control AMR Arising Due to Food-Animals

### 2.6. Data Analysis

Following data charting for the retrieved articles and grey literature, all the collated data were exported to Microsoft Excel Spread sheet, and subsequently, data analysis was conducted. The extracted data were subsequently summarised in the form of narrative synthesis.

## 3. Results

### 3.1. Identified Studies

The comprehensive search yielded a total of 1027 (PubMed-24, Scopus-20, Web of Science-11, Google Scholar-972) articles for the first objective. After importing the retrieved articles from EndNote, a duplication removal was done from the search results and 287 articles were excluded from the study, resulting in 740 articles. After duplication removal, based on title and abstract screening, 36 articles were selected, and 704 were excluded. Subsequently, full-text screening yielded 17 articles. Additionally, two articles were selected after thoroughly screening the references of the 17 eligible articles, which were later added to the study. The reasons for excluding the articles were:

1) Country setting (other than India)
2) Posters, Conference proceedings, and Review articles
3) Articles that only demonstrate the method of antibiotic residue extraction using spiked milk samples, and
4) Full-text not available.

Ultimately, 19 articles were included in the scoping review. The selection process was later charted in the PRISMA flow diagram (Appendix 1).

### 3.2. Characteristics of Included studies

#### 3.2.1. Geographical distribution of study

In terms of the geographical distribution of the studies, the gradated map shows that most of the studies were published in Punjab and Haryana, located in Northern India, and Kerala, which is a southern state (Fig. 1). Out of the top 10 major milk-producing states in India (NDDB, 2019), studies have been conducted

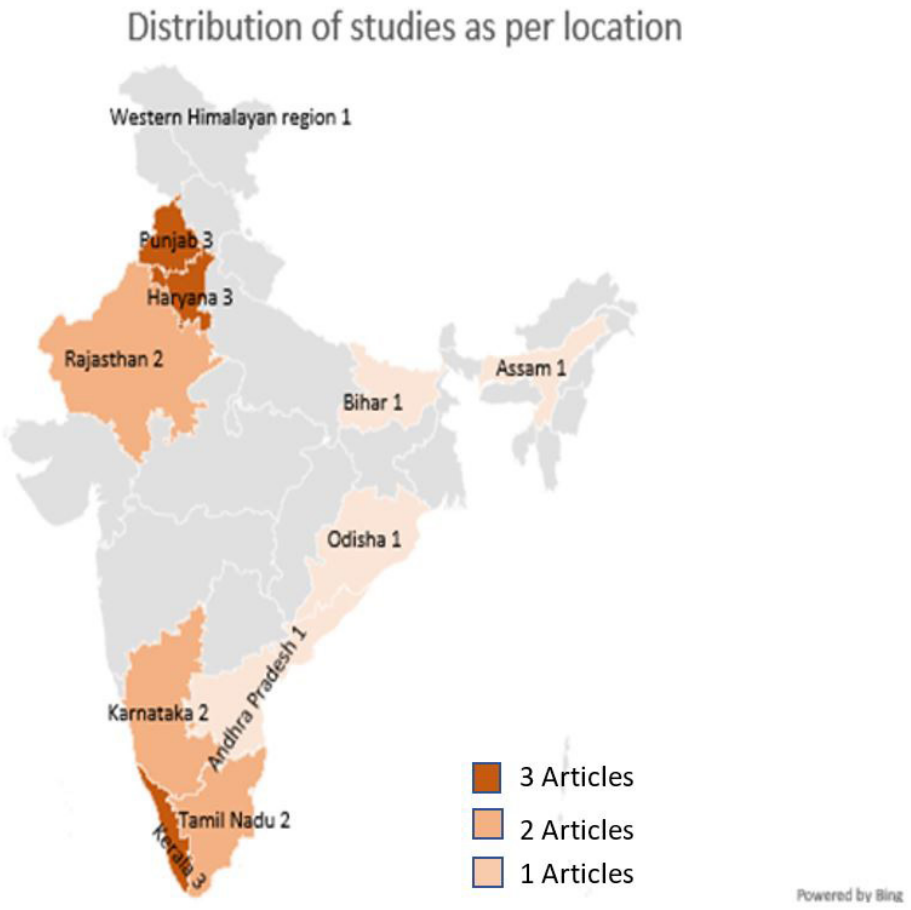


**Figure 1.** Distribution of studies as per location


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in only 6, that is, Rajasthan (Jaipal et al., 2021; Sharma et al., 2019), Andhra Pradesh (Kalla et al., 2015), Punjab(Gaurav et al., 2014; Moudgil, Jasbir S Bedi, et al., 2019; Moudgil, Jasbir S. Bedi, et al. 2019), Haryana (Chauhan, 2019; Jadhav, Lata Chauhan, and Garg, 2019b, 2019a), Bihar(Nirala et al., 2017), and Tamil Nadu (Moharana et al., 2015; Raosaheb et al., 2020).

### 3.2.2. Year of Publication

Among the studies selected, predominantly the studies were published in the year 2019 (Chauhan, 2019; Jadhav et al., 2019b, 2019a; M Kurjogi et al., 2019; Moudgil, Jasbir S Bedi, et al., 2019; Moudgil, Jasbir S. Bedi, et al., 2019; Sardar et al., 2019; Sharma et al., 2019) and the no studies were found in the year 2011 and 2012 (Fig.2).

### 3.3. Type of Antibiotic found

In this review, the detected antibiotics were grouped into 6 classes of antibiotics, namely, Tetracyclines, Beta-Lactam antibiotics, Quinolones, Sulphonamides, Aminoglycosides, and Macrolides (Fig. 3). Out of the 19 selected studies, the Tetracycline group of antibiotics was the most commonly detected residue found across the studies, that is, it was mentioned in 33% of studies Macrolides (Hebbal et al. 2020; Gaurav et al., 2014; Jaipal et al., 2021; Kalla et al., 2015; Kumar, Panda, and Sharma, 2021; Kumarswamy et al., 2018; M Kurjogi et al., 2019; Lejaniya et al., 2017; Moudgil, Jasbir S Bedi, et al., 2019; Moudgil, Jasbir S. Bedi, et al., 2019; Sharma et al., 2019).

Within the Tetracycline group, the identified antibiotics were Tetracycline (18%) and Oxytetracycline (16%). Quinolones were the second most commonly detected antibiotics, that is, in 28% of the studies, followed by Beta-lactams (25%), Sulphonamides (8%), and Aminoglycosides (5%). The least encountered antibiotic in the studies was the Macrolides group of antibiotics (Azithromycin), which was found in only one study (3%). Among Quinolones, Enrofloxacin (18%) was frequently encountered, followed by ciprofloxacin (3%) and Norfloxacin (3%). Table 1 shows the list of antibiotics found in the studies under the six classes of antibiotics.

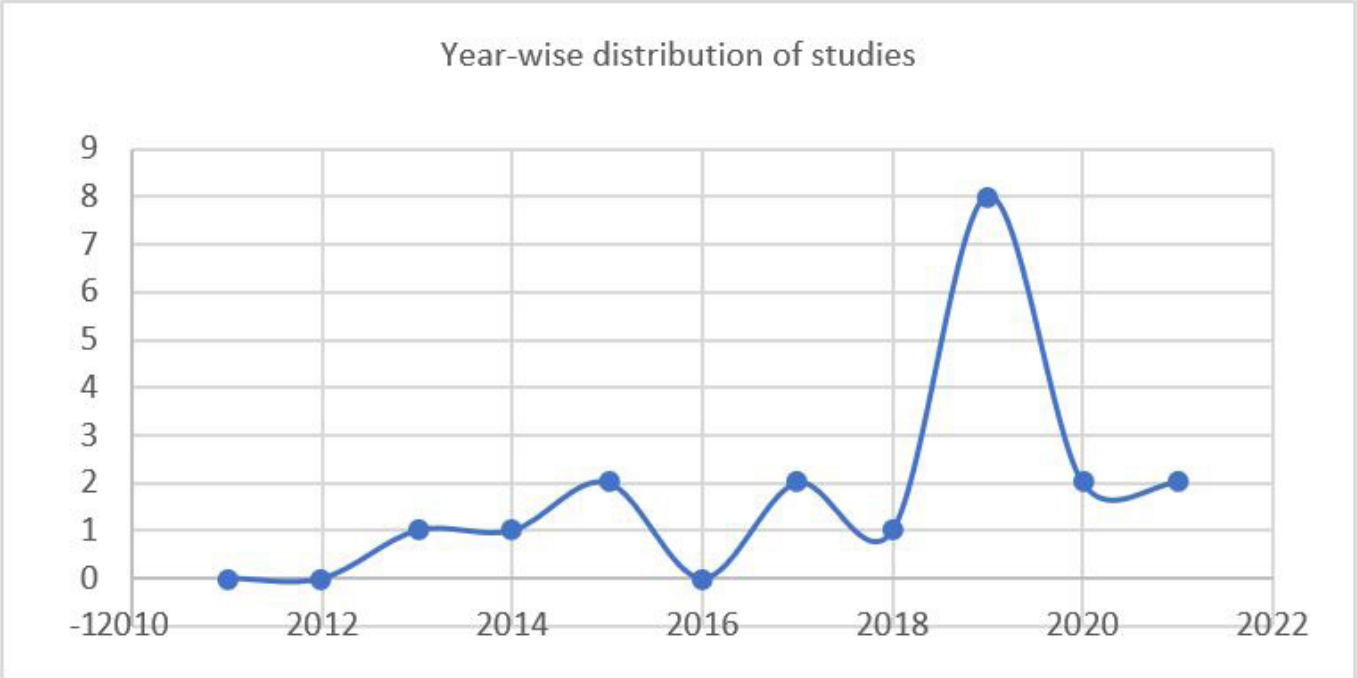


**Figure 2.** Distribution of studies as per year of publication

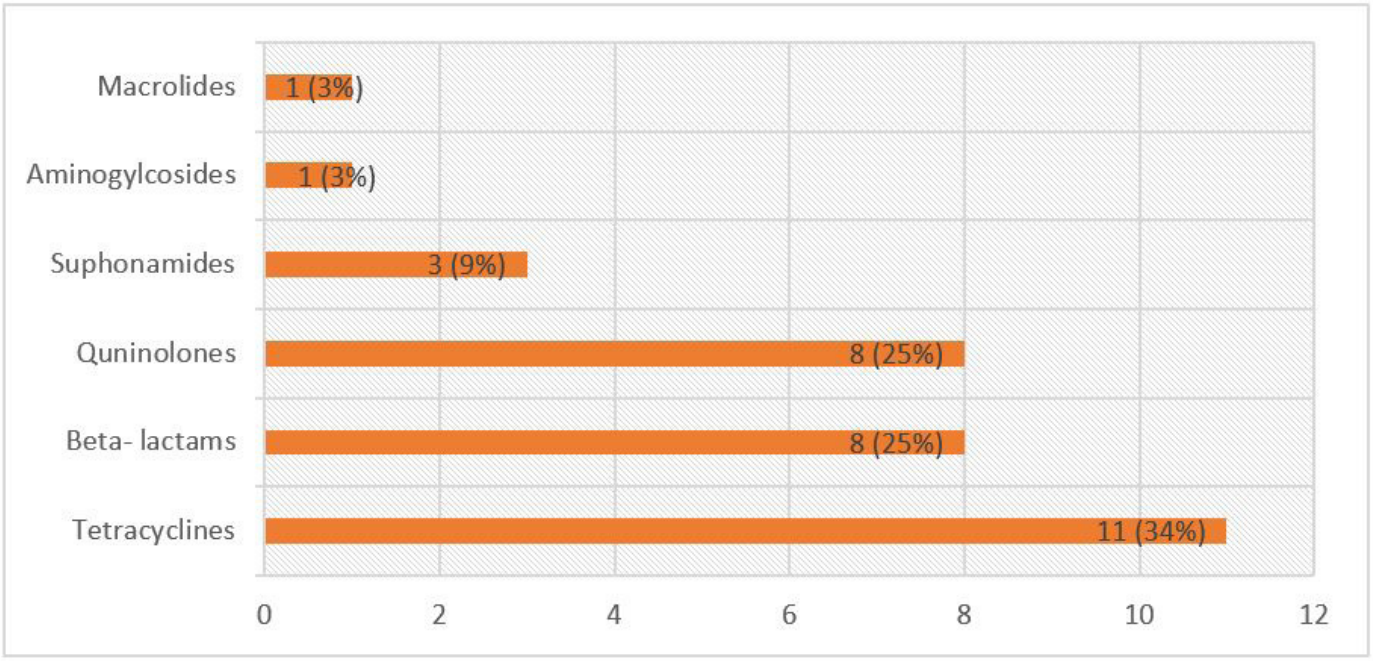


**Figure 3.** Distribution of studies as per the type of antibiotics

**Table 1.** The list of antibiotics found in the studies under the different classes of antibiotics

| Tetracycline group | Beta-lactam Antibiotics | Quinolones | Sulphonamides | Aminoglycosides | Macrolides |
|---|---|---|---|---|---|
| Oxytetracycline | Penicillin G | Enrofloxacin | Sulphamethoxazole | Kanamycin | Azithromycin |
| Tetracycline | Amoxicillin | Norfloxacin | Sulpha containing drugs | Gentamycin | |
| | Cloxacillin | Ciprofloxacin | | | |
| | Ceftriaxone | Quinolones (broadly found) | | | |
| | Beta-lactam (broadly found) | | | | |

### 3.4. Type of Risk Assessment tool used

To understand the risk that may be posed due to the occurrence of ARs in milk samples, the identified studies have used two types of risk assessment measures, Maximum Residual limits (MRL), which are the permissible limits established for safe human consumption, and the Hazard Quotient (HQ), which is a ratio that assesses the adverse health effects based on the exposure to the substance. Out of the 19 studies, Fig. 4 shows that 58% of studies used Maximum re-


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sidual limits (MRL) (Hebbal et al., 2020; Gaurav et al., 2014; Jadhav et al., 2019a, 2019b; Nirala et al.,, 2017; Kumarswamy et al., 2018; Mahantesh Kurjogi et al., 2019; Moharana et al., 2015; Moudgil, Jasbir S Bedi, et al., 2019; Sardar et al., 2019; Sharma et al., 2019), 16% of studies used both MRL and Hazard Quotient (HQ) (Chauhan, 2019; Kumar et al., 2021; Moudgil, Jasbir S. Bedi, et al., 2019), and 26% of studies did not evaluate any form of risk assessment measure to assess the potential risk that can occur due to the detection of ARs of milk (Dinki and Balcha, 2013; Jaipal et al., 2021; Kalla et al., 2015; Lejaniya et al., 2017; Raosaheb et al., 2020).

### 3.5. Identified Hazardous Health Effects

The studies mentioned the potential health hazards of consuming milk containing ARs. The risks identified were primarily long-term effects. All the hazardous effects mentioned across the studies were grouped based on five domains for this review, namely (Fig. 5):

1. Gastrointestinal Effects
2. Effects on Vital Organs
3. Sensitive Reactions,
4. Drug Resistance, and
5. Other Pathophysiological Effects

Of the Six domains, 33% of studies mentioned other pathophysiological effects comprising carcinogenicity, teratogenicity, phototoxicity, etc. This was followed by drug resistance (25%) and sensitive reactions (16%). The effect under each domain is summarised in Table 2.

#### 3.5.1. Gastrointestinal Effects

The gastrointestinal effects indicated in the chosen articles were Gastrointestinal (GI) disturbances and interferences in intestinal microflora. Gastrointestinal disturbances were mentioned in 5% of the studies, whereas Interferences in microflora were indicated in 8% of the articles. Drugs like penicillin may cause gastrointestinal effects (Moudgil, Jasbir S. Bedi, et al., 2019).

#### 3.5.2 Effects on vital organs

The most affected vital organs due to the consumption of milk containing antibiotics, as mentioned in the articles, were the kidney, liver, and neural organs. A total of 13% of articles mentioned effects on vital organs, of which 8% of studies mentioned damage to renal organs in the form of either nephrotoxicity or nephropathy. Whereas 5% of studies mentioned

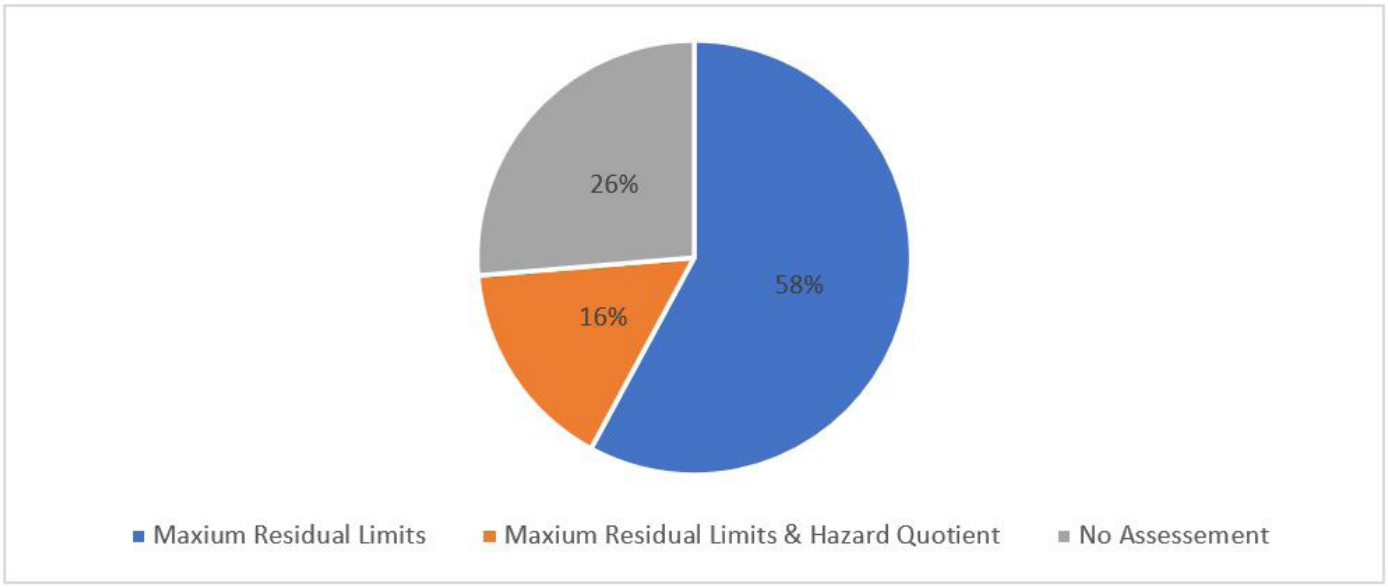


**Figure 4.** Distribution of studies as per the type of risk assessment tool used

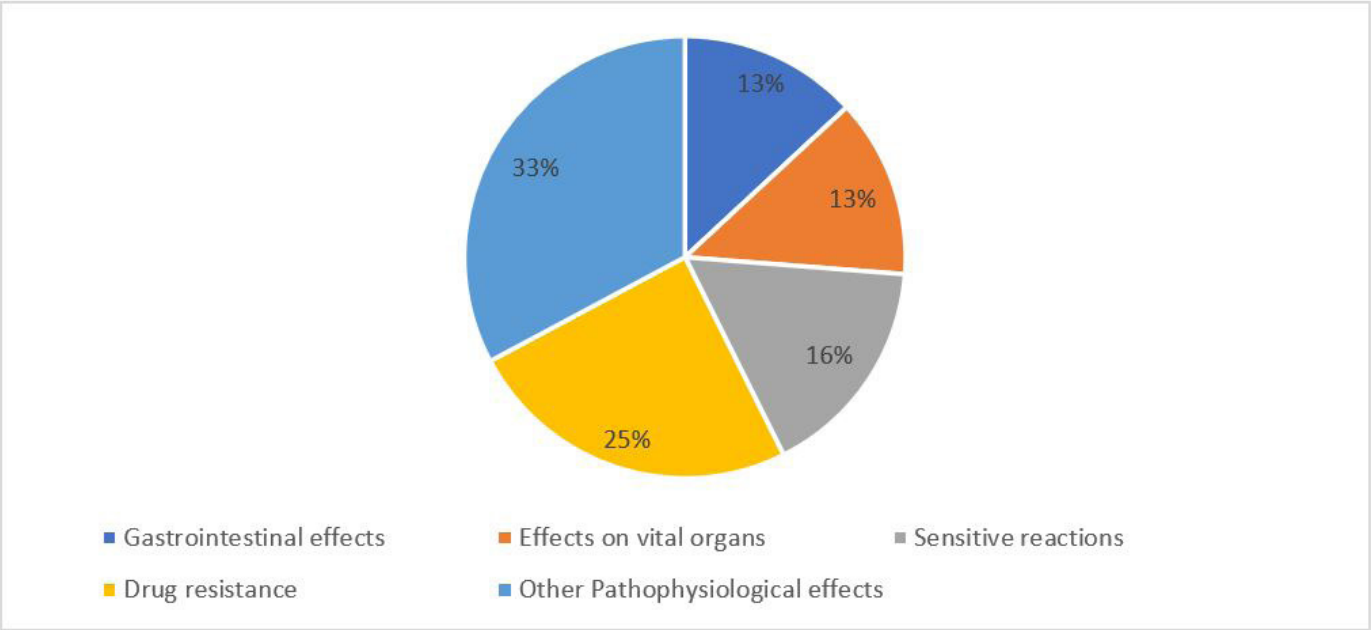


**Figure 5.** Distribution of studies as per the type of health hazard identified

**Table 2.** The list of hazardous health effects found in the articles under the domains

| GIT Effects | Effects on Vital Organs | Sensitive Reactions | Drug Resistance | Pathophysiological Effects |
|---|---|---|---|---|
| GI disturbances (n=3) | Nephrotoxicity/ Nephropathy (n=4) | Allergic Reactions (n=9) | AMR (n=15) | Carcinogenicity (n=5) |
| Interferences of intestinal micro-flora (n=5) | Neurotoxicity (n=1) | Anaphylactic Reactions (n=1) | | Teratogenicity (n=1) |
| | Hepatotoxicity (n=3) | | | Bone marrow toxicity (n=4) |
| | | | | Mutagenicity (n=3) |
| | | | | Toxicological and microbiological (n=1) |
| | | | | Immunopathological risks (n=2) |
| | | | | Photosensitivity/ Phototoxicity (n=2) |
| | | | | Discolouration of teeth/ nails (n=2) |


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hepatotoxicity, and 2% mentioned neurotoxicity may arise. Some of the drugs that may lead to effects on vital organs were aminoglycosides, gentamycin, enrofloxacin, oxytetracycline, and Azithromycin (Hebbal et al., 2020; M Kurjogi et al., 2019; Moudgil, Jasbir S Bedi, et al., 2019).

### 3.5.3 Sensitive Reactions

Two kinds of sensitive reactions were highlighted by the studies. First, allergic reactions were mentioned in 15% of the articles. Second, anaphylactic reactions were highlighted in 2% of studies. Of all the findings, after AMR, the most reported health hazard was allergic reactions to antibiotics by consuming milk contaminated with antibiotics after prolonged exposure. Sensitivity reactions may be caused by antibiotics such as penicillin, sulphamethoxazole, and enrofloxacin (Jadhav et al., 2019a; Moudgil, Jasbir S. Bedi, et al., 2019).

### 3.5.4 Drug Resistance

AMR was the most highlighted health hazard, which was mentioned in 15 articles, that is, 25% of all the studies. Prolonged exposure to antibiotics may develop drug resistance bacteria, which can further enter the food cycle of humans through milk, was the most common understanding of all the authors that mentioned AMR as a hazard in their study.

### 3.5.5 Other Pathophysiological Effects

Cumulatively, 33% of studies mentioned pathophysiological effects, of which, Carcinogenicity was mentioned in 5% of papers. Followed by bone marrow toxicity (7%), mutagenicity (5%), immunopathological effects (3%), and discoloration of teeth (3%). The least commonly identified pathophysiological effects were teratogenicity (2%) and toxicological or microbiological effects (2%). Antibiotics like Oxytetracycline, Tetracycline, Sulphonamides, Chloramphenicol, and Azithromycin may induce pathophysiological effects in humans (Hebbal et al., 2020; Chauhan, 2019; Gaurav et al., 2014; M Kurjogi et al., 2019; Moudgil, Jasbir S. Bedi, et al., 2019).

## 3.6. Government Initiatives in Dairy Industry

A total of 11 documents were found on the government initiatives in the dairy industry. The initiatives were divided based on three domains:

1. Initiatives on Quality and Control of Antibiotic Use
2. Initiatives Related to Dairy Animal Health
3. Initiative to Control AMR Arising Due to Food-Animals

Of the three domains, most of the documents were found for the first domain (55%), that is, Initiatives on Quality and Control of Antibiotic Use, with a total of six documents (27%). Three documents (18%) were found for Initiatives related to Dairy Animal Health, and two pieces of literature were found for the third domain (Appendix 4).

### 3.6.1 Initiatives for Quality and Control of Antibiotic Use

The Government of India, the Food Safety and Standards Authority of India (FSSAI), the Department of Animal Husbandry, Dairying & Fisheries, and the Central Drug Standards Control Organisation are some of the governing bodies identified that have been recognised and have worked towards controlling and maintaining standards of milk in India. FSSAI is a safety regulatory body for food that has been established under the Food safety and standard Act, 2006 in India (FSSAI). The Ministry of Health and Family Welfare is the administrative unit responsible for the functioning of FSSAI (FSSAI). It formulates various regulations to ensure the quality and safety of food products in India. Among these is the Food safety standards (Contaminants, Toxins & Residues) Regulations, 2011. Under this regulation, acceptable standards for various toxins and residues are mentioned for various animal-derived food products. This regulation first came in the year 2011 and in the 1st version of the document, antibiotic residual limits were only set for seafood. Even though in 2011, the Jaipur Declaration on antimicrobial resistance (AMR) stated that AMR is caused not only because of irrational use of antimicrobials among humans but also because of its injudicious use in animals (World Health Organization, 2011). It was only in 2018 that residual limits for antibiotics in milk and meat were introduced by FSSAI. These regulations are formulated to limit the quantity of antibiotic residue consumed by humans,


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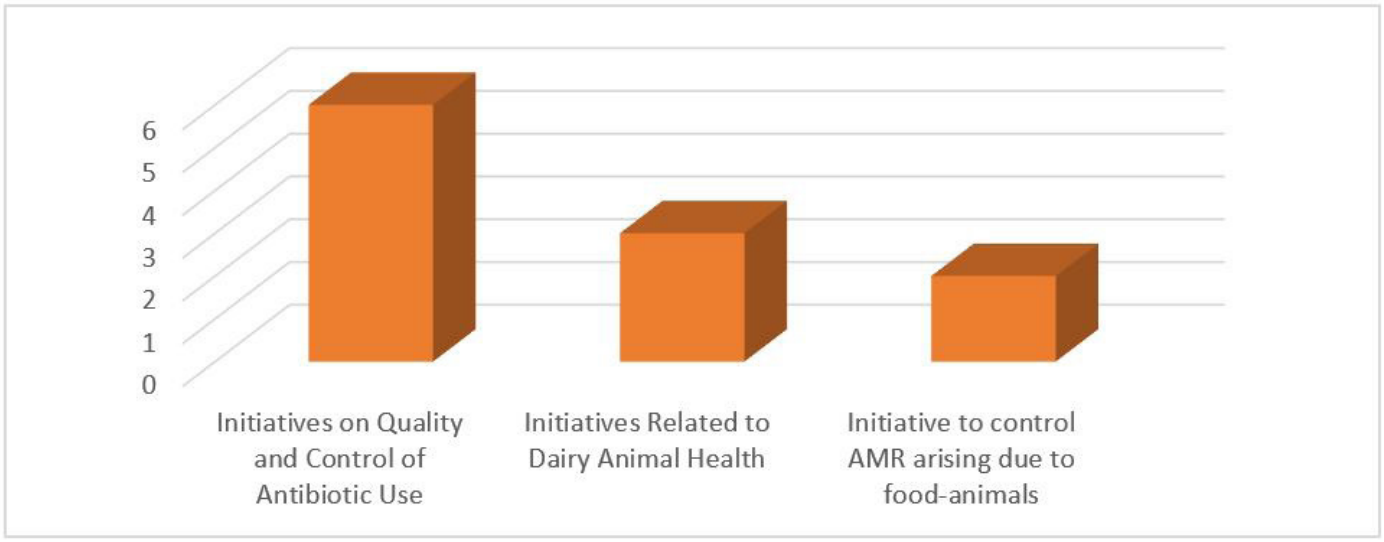


**Figure 6.** Distribution of reports based on the domains

hence preventing the spread of antibiotic resistance.

In 2013, the National Animal Policy was announced, and one of the major goals was to ensure that quality livestock products met international food safety requirements. The policy highlights the growing concern of excessive residues of antibiotics, fertilizers, pesticides, and other toxins in milk. Thereby, encouraging the states to carefully use antibiotics in livestock (D. & F. Department of Animal Husbandry, 2013). Another organization that identified the public health problem and issued advisories to regulate the use of antibiotics in farm animals is the Central Drugs Standard Control Organization, which also recommends labelling medicine containers with appropriate withdrawal periods to ensure judicious and monitored utilization of antibiotics in food animals (Department of Animal Husbandry, 2013). In 2013, Central Drugs Standard Control Organisation added another category of Schedule H1 drugs under the Drugs and Cosmetics Rule, 1945, along with the existing Schedule H category. The Schedule H1 classification of drugs was predominantly done to control the indiscriminate use of antibiotics both in the human and animal sector (Central Drugs Standard Control Organization, 2017).

Two initiatives concerning the maintenance of the standard quality of milk for consumers were found. First, is the National Programme for Dairy Development, which was implemented by the department of animal husbandry and dairying in 2014. The programme focuses on developing robust dairy infrastructure, quality, and manpower training (Department of Animal Husbandry and Dairying). The second initiative on quality is the Export of Milk and Milk Products (Quality Control, Inspection, and Monitoring) Rules, 2020, which came as a successor to the Export of Milk and Milk Products (Quality Control, Inspection, and Monitoring) Rules, 2000 (Priya, 2020). As per the rule, the standard quality of milk should be maintained and checked before exporting. The rule also mentions that the milk should be free of residues like antibiotics, pesticides, and other harmful substances (Priya, 2020). The milk is to be assessed for residual limits as per national or international standards, and any batch of milk contaminated with such residues is to be discarded as per the rule (Priya, 2020).

### 3.6.2 Initiatives Related to Dairy Animal Health

To maintain quality milk standards and ensure complete profitability, it is important to maintain proper animal health. To maintain livestock health in India the two regulatory bodies were the Department of Animal Husbandry & Dairying and the National Dairy Development Board were the two regulatory bodies identified to maintain livestock health in India. These institutions have certain initiatives that control and monitor livestock health. In 2011, the National Animal Disease Reporting System (NADRS) was es-


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tablished to collect data on animal health to monitor disease control efforts. (NADRS). Recently, a new version, NADRS 2.0, was released in the year 2021.

Unlike Norway, Australia, and the Netherlands, India does not have a national mastitis control programme (Brightling et al., 2009; Jansen et al., 2010; Østerås, 2013). In one of the states in India a pilot programme called Mastitis Control Popularisation Programme (MCPP) was launched in 2014 to rationalize antibiotic drug usage in infected cattle (National Dairy Development Board). Many other state-run initiatives are there in states like Kerala, Assam, etc. To counter diseases such as Brucellosis and Foot & Mouth Disease, the Department of Animal Husbandry and Dairying launched the National Animal Disease Control Program in 2019, with the goal of eradicating the diseases by vaccinating all livestock animals by 2030 (Department of Animal Husbandry & Dairying, 2019).

### 3.6.3 Initiative to Control AMR Arising Due to Food-Animals

To control AMR arising due to food animals, the Indian Council of Agriculture Research (ICAR) and the National Centre for Disease Control (NCDC), were the two organizations found. The previous National policy on AMR containment (2012-2017) did not recognize the spread of AMR through the animal sector as well; it was only limited to irrational use by humans. Although in 2017, the National action plan on AMR (NAP-AMR) (2017-2021) was introduced with one of its objectives to optimize antibiotic usage in the human, animal, and food sectors (Sharma and Anuj 2017). The action plan was formulated keeping the Global Action Plan on AMR (GAP-AMR) into consideration (Sharma and Anuj, 2017). A national surveillance network of veterinary laboratories exists in India, which was established in collaboration with the Food and Agriculture Organisation (FAO) and ICAR in 2017 (FAO, 2017). The network is known as the Indian Network for Fishery and Animal Antimicrobial Resistance (INFAAR). The surveillance system was created to capture all AMR-related data from the veterinary industry.

## 4. Discussions

This scoping review was undertaken to collate all the evidence available on the occurrence of ARs in milk. The findings of the analysed publications demonstrated high evidence of ARs in milk across India's several states. The antibiotics in milk were a considerable cause of concern as most of the antibiotics found in this scoping review belong to the WHO's critically Important Antibiotics List. It is a list that categorizes antibacterial drugs based on their importance in human medicine (WHO, 2011). Of the antibiotics found, Tetracycline and sulphonamides belong to the "Highly Important" group. In the critically important category, Aminoglycosides and Penicillin belong to "High Priority", whereas Cephalosporins (3rd generation), Macrolides, and Quinolones belong to the "Highest Priority" groups (WHO, 2011). The European Medicines Agency (EMA) puts these Critically Important Antibiotics under their "Restricted" category, which recommends limited use of these antibiotics in animals to safeguard public health (European Medicines Agency, 2020). Also, with measures such as banning the preventative use of antibiotics and using certain models, the Netherlands successfully reduced the use of antibiotics in dairy animals by 2015 (Lam, Jansen, and Wessels, 2017; Scherpenzeel et al., 2016). Therefore, urgent attention and similar stringent measures are required to control the use of antibiotics in India as well.

Also, 79% of articles selected for the study indicated AMR as a significant long-term public health hazard, which may arise from consuming milk containing AR. Bhattacharya et al found Vancomycin-resistant strains of bacteria in the milk samples and stated that the irrational use of antibiotics may have contributed to the development of such strains (Bhattacharyya et al., 2016). Some of the enabling factors responsible for the development of AMR through the food-animal industry are lack of antibiotic awareness and information, use of fake and substandard drugs, poor 'one health' integration in developing countries, and lack of alternatives to antibiotics for animal use (Grace, 2015).

In India in terms of government activities, there are some restrictions in place to control the use of antibiotics in the dairy business, but there is a need for more stringent controls. Requirement for appropriate regulations, robust surveillance system, strengthened quality control system, and training programs for

farmers were highlighted by several authors included in the review (Hebbal et al., 2020; Gaurav et al., 2014; Kalla et al., 2015; Nirala et al., 2017; Moudgil, Jasbir S Bedi, et al., 2019; Moudgil, Jasbir S. Bedi, et al., 2019; Raosaheb et al., 2020). According to a report, some guidelines to limit the use of antibiotics exist in India, but they have never been implemented into laws or regulations (CDDEP, 2016). Another report on antibiotic usage in food animals stated that India does not have a stringent regulatory framework to limit the utilization of antimicrobials in livestock and food animals (Sivaraman and Yann, 2018).

## 5. Conclusion

The present scoping review portrayed India's situation in terms of ARs in milk with the help of studies conducted in India. Although there is growing evidence on the subject year by year, there is a paucity of literature concerning public health effects that may arise from consuming milk contaminated with antibiotics. Antibiotics in milk are primarily an issue of prime importance in India as it has the most extensive consumer base for milk. The majority of antibiotics detected in this study were on the WHO's Critically Important Antibiotics list, which is a cause of great concern for humans. AMR was also identified as the most common public health risk associated with antibiotic-contaminated milk. Given that India is one of the world's hotspots for AMR, a more proactive approach involving stricter laws and monitoring systems is the need of the hour to prevent the spread of AMR that can result from the overuse of antibiotics in dairy animals and other food-producing animals in India.

## 6. Recommendations

The recommendations for this scoping study were based on the information presented in the literature reviewed.

• Regulating WHO recommended critically important antibiotics used in food animals.

• Awareness and training for dairy farmers and veterinarians about the use of antibiotics (public health implications, appropriate withdrawal periods, and residual limits) and about AMR that can arise due to their irrational use in animals.

## Conflict of interest

The authors declare no conflict of interest.

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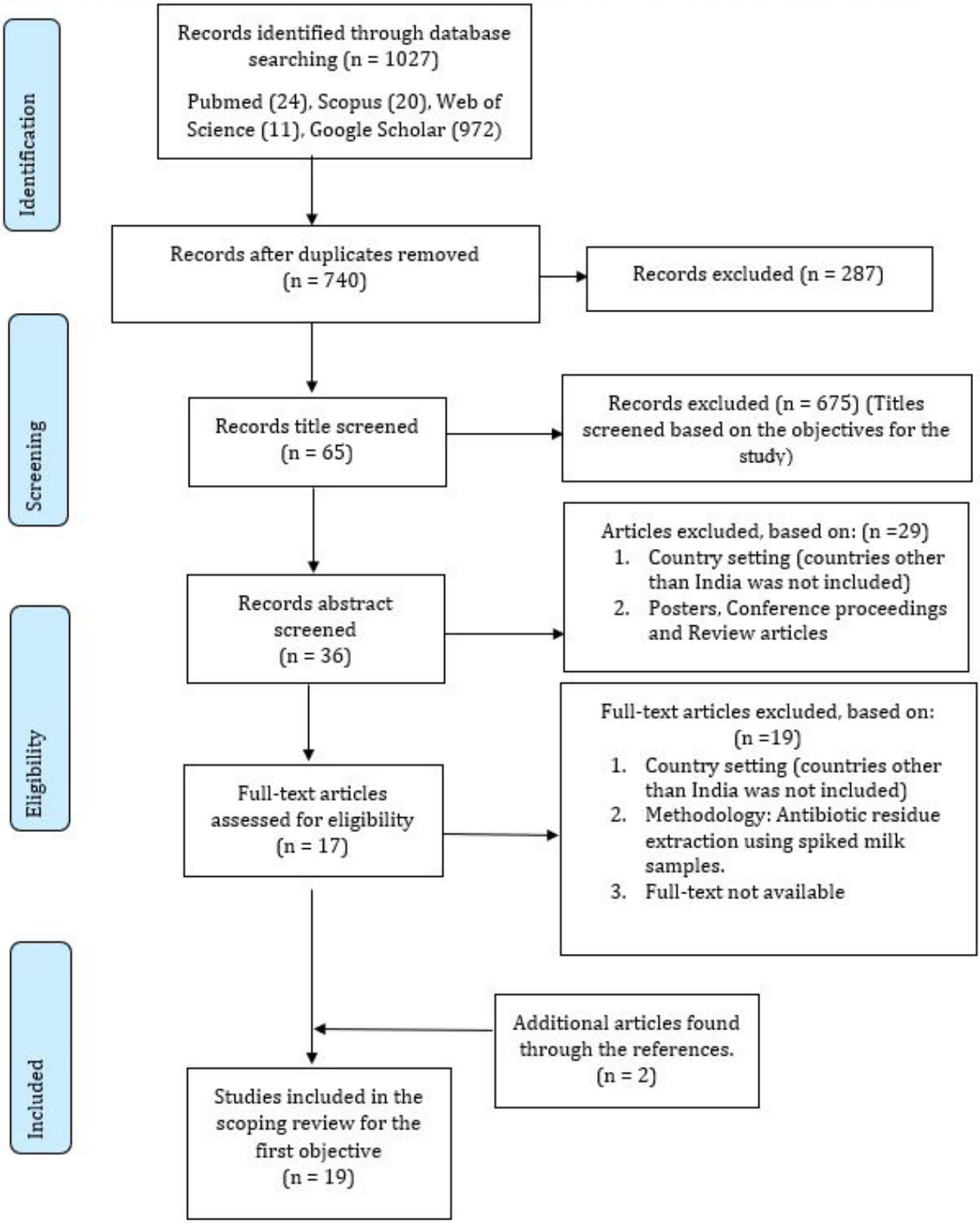
**Appendix 1: PRISMA Flow diagram demonstrating the study selection process.**



**Appendix 2:** Search Strategy

| Database | Keywords | Number of Articles retrieved |
|---|---|---|
| **PubMed** | (Antibiotics) OR (Oxytetracycline)) OR (tetracycline)) OR (Fluoroquinolones)) OR (Sulfonamides))<br><br>AND<br><br>((Residues) OR (traces))<br><br>AND<br><br>(((Milk) OR (Milk samples)) OR (Dairy milk)) OR (cow milk)) OR (buffalo milk)) OR (cattle milk))) AND (India)<br><br>Filter: English, 2011-2021 | 24 articles |
| **Scopus** | TITLE-ABS-KEY ( antibiotics OR oxytetracycline OR tetracycline OR penicillin OR sulfonamides OR quinolones )<br><br>AND<br><br>TITLE-ABS-KEY ( residues OR traces )<br><br>AND<br><br>TITLE-ABS-KEY ( "milk" OR "milk samples" OR "dairy milk" OR "cow milk" OR "buffalo milk" )<br><br>AND<br><br>TITLE-ABS-KEY ( india )<br><br>AND<br><br>( LIMIT-TO ( PUBYEAR , 2021 ) OR LIMIT-TO ( PUBYEAR , 2020 ) OR LIMIT-TO ( PUBYEAR , 2019 ) OR LIMIT-TO ( PUBYEAR , 2018 ) OR LIMIT-TO ( PUBYEAR , 2017 ) OR LIMIT-TO ( PUBYEAR , 2016 ) OR LIMIT-TO ( PUBYEAR , 2015 ) OR LIMIT-TO ( PUBYEAR , 2014 ) OR LIMIT-TO ( PUBYEAR , 2012 ) ) | 20 articles |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**Continue Appendix 2:** Search Strategy

| Database | Keywords | Number of Articles retrieved |
|---|---|---|
| **Web of science** | #4 AND #3 AND #2 AND #1<br><br>TS=(antibiotics OR oxytetracycline OR tetracycline OR penicillin OR sulfonamides OR quinolones)<br><br>AND<br>TS=("milk" OR "milk samples" OR "dairy milk" OR "cow milk" OR "buffalo milk" OR "cattle milk")<br><br>AND<br><br>TS=(residues OR traces )<br><br>AND<br><br>TS=(india)<br><br>Refined by: [excluding] PUBLICATION YEARS: (2007 OR 2001 OR 2005 OR 1995) | 11 articles |
| **Google Scholar** | Antibiotic AND Residue AND Milk AND India AND Oxytetracycline OR tetracycline OR quinolones OR Sulfonamides OR Residues OR traces OR "Milk samples" OR "Dairy milk" OR "cattle milk" -Meat -Pesticides -Toxins -honey -Fish -antibody -poultry | 972 articles |


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**Appendix 3:** Data Extraction Sheet

| S.No. | Author name, Year of publication | Study Location | Study Purpose | Methodology | Key findings | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | No. of samples contaminated with antibiotic residues | Antibiotic(s) found | Type of public health risk assessment used | Public health risks identified in the paper |
| 1. | Dinki & Balcha, 2013 | Guwahati | Antibiotic residues were detected and the microbial content of samples taken from six different consumers' collection points were assessed. | 120 milk samples were obtained aseptically from milk cans chosen at random. | 28 samples | Not mentioned | No Assessment used | -allergic reactions<br>-Drug resistance |
| 2 | Gaurav et al, 2014 | Punjab | In five areas of Punjab, the study investigated and monitored the presence of Tetracycline in cattle milk. | Tetracycline residual contamination was examined and evaluated in 133 cattle milk specimens collected from dairies in five Punjab districts. | 18 samples | Tetracycline | Maximum Residual Limits (MRL) assessed | -Tetracycline can cause Phototoxicity |
| 3. | Kalla et al, 2015 | Coastal districts of Andhra Pradesh | Pathogen isolation and identification of antibiotics, aflatoxins, pesticide, and industrial contaminants residues in the milk supply chain. | 600 samples were obtained and sent to the laboratory for testing.<br>Antibiotic residues were detected using Delvotest, and fast test kits. | 62 samples | Penicillin-G, Tetracycline, and Oxytetracycline | Not assessment used | -allergic reactions<br>-Drug resistance |
| 4. | Moharana et al, 2015 | Chennai, Tamil Nadu | The study identified Enrofloxacin residues in samples of cow's milk | 125 milk specimens were obtained and analyzed for residues. | 21 samples<br>(16.8%) | Enrofloxacin | Maximum Residual Limits (MRL) assessed | -Drug resistance |
| 5. | Nirala et al, 2017 | Bihar | The study conducted a sample survey in both organized and unorganized dairy industries to assess the antibiotic presence in milk samples. | The study assessed 250 milk samples from the organized the unorganized sectors in the districts of Bihar, India. High-performance liquid chromatography (HPLC) was used to analyze samples. | 8 samples | Enrofloxacin and Ciprofloxacin | Maximum Residual Limits (MRL) assessed | -Allergic reactions and interferences of intestinal micro-flora |

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**Continue Appendix 3:** Data Extraction Sheet

| S.No. | Author name, Year of publication | Study Location | Study Purpose | Methodology | Key findings | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | **No. of samples contaminated with antibiotic residues** | **Antibiotic(s) found** | **Type of public health risk assessment used** | **Public health risks identified in the paper** |
| 6. | Lejaniya et al, 2017 | Thrissur, Kerala | The study calculated the amount of antibiotic residue in pooled milk samples. | Pooled morning milk samples were collected from milk cooperatives and organized farms in and around Thrissur district of Kerala. A total number of 50 samples were collected in sterile | 7 samples | Beta-lactam and Tetracycline | No assessment | Implicitly stated. |
| 7. | Kumarswamy et al, 2018 | Thrissur, Kerala | The goal of this study was to assess the residues of antibiotics in milk. | Morning milk samples were obtained in the Thrissur district of Kerala from milk unions and organized farms. Antibiotic Test Kit (BEIJING YF-MARISGLOBAL CO., LTD, CHINA) was used to check for beta-lactam and tetracycline antibiotic residue in the samples. | 14 samples | Tetracycline, ß-lactams, and enrofloxacin | Maximum Residual limits (MRL) assessed | -Carcinogenicity, teratogenicity, allergic reactions, bone marrow toxicity, mutagenicity, and Gastrointestinal disorders.<br>-Antimicrobial Resistance (AMR). |
| 8. | Moudgil et al, 2019 | Punjab | The purpose of this study was to examine the prevalence of antibiotic residues in raw and marketed milk, to establish its suitability for human consumption. | Using high-performance liquid chromatography (HPLC), the study examined 524 milk samples, for antibiotic residues, from Punjab, India. | Dairy farms- 78 (16%) samples.<br>Commercial milk samples- 4 (12.5%) samples. | enrofloxacin, oxytetracycline, tetracycline, and Sulphamethoxazole | -Maximum Residual limits (MRL) assessed.<br>-Hazard Quotient (HQ) estimation | Allergic reactions, disorders of intestinal flora, carcinogenicity, and neurotoxicological effects.<br>- Antimicrobial resistance (AMR) |

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**Continue Appendix 3:** Data Extraction Sheet

| S.No. | Author name, Year of publication | Study Location | Study Purpose | Methodology | Key findings | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | No. of samples contaminated with antibiotic residues | Antibiotic(s) found | Type of public health risk assessment used | Public health risks identified in the paper |
| 9. | Moudgil et al, 2019 | Punjab | The study was conducted to detect antibiotics and mycotoxins in raw milk samples obtained from dairy farms in Punjab, India. | The study collected 168 raw milk samples from nine districts of Punjab and analyzed them using competitive Enzyme-linked immunosorbent test kits that are commercially available. | 19 samples | enrofloxacin,<br><br>oxytetracycline, penicillin G, sulphamethoxazole<br><br>and chloramphenicol | Maximum Residual limits (MRL) assessed. | allergic reactions (Enrofloxacin, Penicillin G), disruption of intestinal flora, carcinogenicity, mutagenicity, nephropathy, hepatotoxicity (Oxytetracycline), and bone marrow toxicity (Oxytetracycline).<br><br>- Drug resistance |

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**Continue Appendix 3:** Data Extraction Sheet

| S.No. | Author name, Year of publication | Study Location | Study Purpose | Methodology | Key findings | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | No. of samples contaminated with antibiotic residues | Antibiotic(s) found | Type of public health risk assessment used | Public health risks identified in the paper |
| 11. | Chauhan et al, 2019 | Hisar, Haryana | The purpose of this study was to detect aminoglycoside residues in milk from the local market and to quantify the risk of dietary exposure. | this study assessed the presence of aminoglycoside residues in milk accessible on the local market and quantified the risk of dietary exposure to aminoglycoside residues. | 15 samples | Aminoglycosides (Kanamycin and Gentamycin) | -Maximum Residual limits (MRL) assessed<br>-Hazard Quotient (HQ) assessed | -Chronic exposure to aminoglycosides can cause nephrotoxicity leading to renal failure, neuromuscular blockage, and irreversible ototoxicity.<br>-Drug resistance |
| 12. | Kurjogi et al, 2019 | Dharwad, Karnataka | In this study, a microbiological technique was used to identify antibiotic residues in cow milk specimens. | the study obtained 13 raw cow milk samples from the dairy farms in the Dharwad area of Karnataka, India. | 2 samples | Azithromycin and Tetracycline | Maximum Residual limits (MRL) assessed. | -Allergic reaction, intestinal alterations, photosensitivity reaction with nail discoloration, and discoloration of teeth in children<br>-Drug resistance |
| 13. | Priyanka et al, 2019 | Hisar, Haryana | The study detected and quantified residues of penicillin in milk. | For the study, 100 milk samples were obtained from Hisar's local market, Samples of raw milk and pasteurized milk of various brands were randomly collected. | 14 samples | Penicillin (Amoxicillin and Cloxacillin) | Maximum Residual limits (MRL) assessed | -Penicillin residues can cause health risks such as allergic reactions<br>-Drug resistance |
| 14. | Jadhav et al, 2019 | Hisar, Haryana | The goal of this study was to standardize the use of high-performance liquid chromatography (HPLC) for the identification and quantification of quinolone residues. | The study randomly obtained 100 milk samples from Hisar's local market, Both pasteurized and raw milk samples were collected. | 8 samples | Quinolones (Norfloxacin and Enrofloxacin) | Maximum Residual limits (MRL) assessed | -bone marrow aplasia and can alter the normal gastrointestinal microflora resulting in GI disturbances<br>- Drug resistance |

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**Continue Appendix 3:** Data Extraction Sheet

| S.No. | Author name, Year of publication | Study Location | Study Purpose | Methodology | Key findings | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | No. of samples contaminated with antibiotic residues | Antibiotic(s) found | Type of public health risk assessment used | Public health risks identified in the paper |
| 15. | Hebbal et al, 2020 | Palakkad, Kerala | The study was conducted to detect antibiotic residues in samples of milk. | Raw milk specimens were taken from three of Kerala's Palakkad districts. For screening, 215 samples were collected and screened using. Microbial inhibition assay (MIA). The analyzed positive samples were further processed by enzyme-linked immunosorbent assay (ELISA) to assess oxytetracycline residues and validated using high-performance liquid chromatography (HPLC). | 22 samples<br><br>(5 for oxytetracycline) | Oxytetracycline and other antibiotics (not mentioned) | Maximum Residual limits (MRL) assessed | -These residues can cause toxic effects such as anaphylactic reactions, carcinogenicity (oxytetracycline, sulfamethazine, and furazolidone), nephropathy (gentamicin), mutagenicity, reproductive disorders, bone marrow toxicity (chloramphenicol), hepatotoxicity, and immunopathological effects in humans.<br><br>-AMR |
| 16. | Das et al, 2019 | Bhubaneswar, Odisha | The goal of this investigation was to detect antibiotic residues in mastitic cow milk after therapy. | The samples of milk were obtained from cured mastitic cows that underwent antibiotic therapy. The cows were divided in six groups and different doses of ceftriaxone and enrofloxacin were administered to each group. | Not specific | Ceftriaxone and Enrofloxacin | Maximum Residual limits assessed | -Intestinal flora interferences<br><br>-Drug resistance |

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**Continue Appendix 3:** Data Extraction Sheet

| S.No. | Author name, Year of publication | Study Location | Study Purpose | Methodology | Key findings | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | No. of samples contaminated with antibiotic residues | Antibiotic(s) found | Type of public health risk assessment used | Public health risks identified in the paper |
| 17. | Raoshaeb,2020 | Tamil Nadu and Karnataka | Across chosen districts of Tamil Nadu and Karnataka, the study was conducted to estimate antimicrobial utilization for multiple clinical diseases within organized and unorganized dairy sectors. | The information was gathered through surveys from 104 working veterinarians and treatment records on farms were obtained. To quantitatively analyze antibiotic residues in milk, 100 samples were collected from those farms. | 13 samples | Not mentioned | No assessment | -AMR |
| 18. | Kumar et al, 2021 | The North-western Himalayan state of India. | The study determined the health risks posed by the presence of antibiotics in milk. | For analysis of residues, 173 raw and pasteurized samples were collected in India's northern. Himalayan state. | 16 samples | Oxytetracycline and amoxicillin | -Maximum Residual limits (MRL) assessed.<br>- Hazard Quotient | -Allergic reactions, GIT disruption, carcinogenicity, nephropathy, hepatotoxicity.<br>-Drug resistance |
| 19. | Jaipal et al, 2021 | Bikaner, Rajasthan | The study describes relationships between the prevalence of antibiotic residues in cattle milk and various farm management strategies used by farmers. | For the study, 200 milk samples were gathered from dairy farms. Samples were retrieved from both indigenous and cross-breed cattle. | 28 samples | Beta-lactam antibiotics, Tetracycline, Quinolones, and sulpha drugs | No assessment | Implicitly stated. |

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## Appendix 4: List of all government issues under the three domains

| S. No. | Government initiative domains | Government Initiative | Year | Brief |
|---|---|---|---|---|
| 1. | **Initiatives Quality and Control Antibiotic Use** | • Version 1: FSSAI Food safety standards (Contaminants, toxins & Residues) Regulations, 2011 (Anon) introduced | 2011 | Antibiotic residues permissible limits only for seafood |
| | | • Version 2 | | |
| | | • Version 3 | | changes were made and antibiotic residues permissible limits only for seafood and honey. |
| | | • Version 4 (Anon) | 2017 | changes were made and antibiotic residues permissible limits for animal food products such as meat, milk etc. were added. |
| | | • Version 5 | 2018 | updated version |
| | | | 2019 | updated version. |
| | | | 2020 | |
| | | National livestock Policy (Anon) | 2013 | encouraged states for careful use of antibiotics. Also highlighted the growing concern of excessive residues of antibiotics, fertilizers, pesticides, and other toxins in milk and milk products. |
| | | Schedule H1 Drugs (Anon) | 2013 | to control the indiscriminate use of antibiotics both in human and animal |
| | | National programme for dairy development (2013-2017) (2018-2022) (Department of Animal Husbandry and Dairying) | 2013 | To build dairy infrastructure, ensure milk quality, and manpower training. |
| | | Central Drugs Standard Control Organisation (Rational use of antibiotics for limiting antimicrobial Resistance) (Anon) | 2017 | Recommends putting labels on antibiotics with appropriate withdrawal periods meant for animal use. |
| | | Export of Milk and Milk Products (Quality Control, Inspection, and Monitoring) Rules, 2020 (Anon) | 2020 | standard quality of milk should be maintained and checked before exporting and ilk should be free of any residues like antibiotics, pesticides, and other harmful substances. |

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**Continue Appendix 4: List of all government issues under the three domains**

| S. No. | Government initiative domains | Government Initiative | Year | Brief |
|---|---|---|---|---|
| **2.** | **Initiatives Related to Dairy Animal Health** | National Animal Disease Reporting System (NADRS) (Anon)<br><br>NADRS 2.0 | 2011<br><br>2021 | Primary objectives of the Animal Disease Reporting System is the collection and collation of animal health information.<br><br>Updated version |
| | | Animal disease control project by National dairy development board (Anon) | 2014 | Mastitis Control Popularisation Programme (MCPP) was launched in Gujarat |
| | | National animal disease control programme (Anon) | 2019 | Disease control programmes for foot and mouth disease and Brucellosis. |
| **3.** | **Initiative to control AMR arising due to food-animals** | National Action Plan on AMR (NAP-AMR) (Sharma and Anuj, 2017) | 2017 | optimise the use of antibiotics in humans, animals and food sector |
| | | Indian Network for Fishery and Animals Antimicrobial Resistance (INFAAR) (Anon) | 2017 | National network of veterinary laboratory for antimicrobial resistance. |

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# Prevalence of some enteric pathogens in table eggs with special reference to *E. coli* O157: H7

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*E. coli*; *E. coli* O157: H7; Shiga like toxins; *Salmonella typhimurium*; *Yersinia enterocolitica*

The present investigation aims to study the incidence of some enteric pathogens in table eggs with special references to *E. coli* O157: H7. A total of 250 table egg samples (75 Baladi hen's, 75 white farm hen's, 75 brown farm hen's, and 25 duck eggs) were collected randomly from poultry farms, groceries, supermarkets, and street vendors in El Fayoum city, Egypt. Each Baladi hen's egg sample is represented by five eggs, while each farm hen's and duck egg are represented by three eggs. The samples were analysed for the presence of coliforms, faecal coliforms, *E. coli*, *E. coli* O157: H7, Shiga like toxin genes 1&2, *Salmonella typhimurium*, and *Yersinia enterocolitica*. The isolates were identified by biochemical, serological & molecular (PCR) methods. The obtained results in the present study showed that the examined samples of shells and contents of Baladi hens', poultry farms' (white and brown) and ducks' eggs were contaminated with coliforms with incidences of 25.33, 5.33, 1.33, 4.00, 5.33, 0.00, 0.00 and 2.66%, respectively, while faecal coliforms were in 8.0, 2.7, 0.0, 20.0, 0.0, 0.0, 0.0 and 0.0 %, respectively, *E. coli* was present in 2.7, 1.3, 0.0, 8.0, 0.0, 0.0, 0.0 and 0.0 %, respectively. Despite Shiga like toxin genes 1 &2 being found in the shells of Baladi hens' eggs and ducks' eggs, respectively, *E. coli* O157: H7 failed to be detected. Moreover, *Salmonella typhimurium* was isolated only in 4% of ducks' eggshells, while *Yersinia enterocolitica* failed to be isolated in this study. The highest rates of contamination were observed in ducks' and Baladi hens' eggs, while poultry farms' (white and brown) eggs were the best types and advised to be consumed. The potential health hazards and the proposed control measures for the isolated strains were discussed.

## 1. Introduction

Table eggs are consumed worldwide and are a reasonable choice as part of a healthy, balanced diet. Eggs are one of the most balanced and economical sources of protein. In addition, eggs contain all the vitamins and minerals needed for human beings except vitamin C. Fully mixed eggs contain 65% water, 12% protein and 11% fat (Jay et al., 2005).

Eggs own a natural defence system against contaminating microorganisms, such as cuticles, calcium hard shells, and shell membranes (Jerzy & Dagmara, 2009). The albumen proteins have antimicrobial properties, especially the lysozyme. Another proteinase is ovomucoid, which prevents bacteria from using the protein in albumen. Also, the pH in albumen is about

9–10, and the viscosity of the egg white is not suitable for microbial growth (Froning, 1998).

The egg can be contaminated with a variety of pathogens both on the eggshell and on the contents, such as Escherichia coli, *Yersinia enterocolitica*, and *Salmonella* (Ricke et al., 2001). Food poisoning related to egg-borne pathogens may cause severe morbidity or mortality with diarrhoea, vomiting, nausea, and abdominal cramps.

Coliforms are faecal bacteria that indicate some kind of faecal contamination of the food and give an index of poor sanitation. *E. coli* is one of the coliform bacteria that naturally inhabit the gastrointestinal tract of all warm-blooded animals. *E. coli* is commonly used as an indicator of faecal contamination of food, although most of its strains are not considered pathogenic (Willey et al., 2009). *E. coli* strains are enterohaemorrahagic, enteropathogenic, enterotoxigenic, enteroaggregative, shiga toxin-secreting, and haemolytic- diarrhea. Some strains can cause food poisoning due to their pathogenicity. Pathogenic strains such as O157:H7 are highly virulent and may have an infective dose as low as ten organisms (Foodborne Pathogenic Microorganisms & Natural Toxins Handbook 2009).

Shiga toxin-producing *E. coli* (STEC) is a bacterium that can cause serious foodborne diseases. In most cases, the illness is self-limiting but can progress to a life-threatening condition, including haemolytic uraemic syndrome (HUS), particularly in young children and the elderly. HUS is characterised by acute renal failure, haemolytic anaemia, and thrombocytopenia (low platelet count). STEC produces toxins known as Shiga-toxins (Stx1 & Stx2) because of their similarity to the toxins produced by Shigella dysenteriae. These are potent bacterial toxins that cause severe damage to the lining of the intestine and are also known as Vero toxins, or previously as Shiga-like toxins ( Melton-Celsa, 2014).

STEC is destroyed by cooking the food thoroughly until the temperature of all parts reaches 70 °C or higher. Although *E. coli* O157: H7 is the most important STEC serotype in terms of public health, other serotypes have been implicated in sporadic outbreaks and cases.

Salmonellosis is a zoonotic infection transmitted to humans by contact with the bird itself or its eggs (Willey et al., 2009). Eggs can become contaminated by exposure to contaminants such as dust or droppings that are found in the nest or on the littered floor. Salmonellosis can cause gastrointestinal illness in humans (Perry, 2004).

Y. enterocolitica is a zoonotic enterobacterium that causes enterocolitis and other clinical manifestations in humans, including immunological signs (Bottone, 1997). Yersiniosis is often characterised by symptoms such as diarrhoea and gastroenteritis with vomiting. However, the hallmark symptoms are fever and abdominal pain. Yersinia infection mimics appendicitis and mesenteric lymphadenitis, but the bacteria can also cause infections in other sites such as joints, wounds, and the urinary tract. (Foodborne Pathogenic Microorganisms and Natural Toxins Handbook, 2009).

Eggshell quality is of primary importance to the egg industry worldwide. Eggshells need to be firmly intact throughout the chain from when the egg is laid until it is used by the consumer (Roberts, 2010). Cracks in the eggshells affect the quality, as eggs with cracks spoil faster than intact eggs (Gietema, 2005). Duck eggs are more highly contaminated than hen's eggs as they are laid near damp places and due to the rapid deterioration of the antibacterial activity of albumen by the unfavourable surroundings. Bahout (2001) studied the public health implications resulting from the consumption of duck's and hen's eggs.

Due to the risk of spreading diseases, hygiene is not only important for health and production performance but also for food safety (Vucemilo et al., 2010). The target of the present investigation was to study the incidence of some enteric pathogens in table eggs, with particular reference to *E. coli* O157: H7, which were collected from poultry farms, street vendors, groceries, and supermarkets located in Fayoum city, Egypt.

## 2. Materials and Methods

### 2.1. Collection of samples

A total of 250 table egg samples (75 Baladi hen 's, 75 white farm hen 's, 75 brown farm hen 's, and 25 duck

eggs) were collected randomly from poultry farms, groceries, supermarkets, and street vendors in El Fayoum city, Egypt. Each Baladi hen's egg sample is represented by five eggs, while each farm hen's and duck eggs are represented by three eggs. Each sample was put in a sterile plastic bag and immediately taken to the laboratory, where they were prepared and examined microbiologically.

**2.2. Preparation of samples: as described by Wehr, H. M., & Frank, J. F. (2012)**

Eggshell: The eggshell was washed by a surface rinse method.
Egg content: The eggs were prepared to evacuate their contents.

**2.3. Microbiological examination**

**2.3.1. Enumeration of total coliform count: (Most Probable Number)**

This was done using lauryl sulphate tryptose broth (LST) with inverted Durham 's tubes according to (Wehr, H. M., & Frank, J. F. 2012).

**2.3.2. Enumeration of faecal coliform count (Most Probable Number)**

A loopful from each LST-positive broth was inoculated into sterile tubes of *E. coli* broth (EC broth). The inoculated and control tubes were incubated in a thermostatically controlled water bath at 44.5 °C for 48 h. Positive tubes showing gas production were recorded according to Wehr, H. M., & Frank, J. F. 2012.

**2.3.3. Enumeration, isolation, biochemical identification and serology of Escherichia coli of true faecal type**

A loopful from each EC-positive broth tube was streaked onto Eosin Methylene Blue (EMB) (Oxoid, Ltd, Basingstoke, UK). The inoculated and control plates were incubated at 35+1 °C for 24 hrs. The plates were examined for the presence of typical nuclear colonies with a dark centre and a green metallic sheen. Positive EMB plates for *E. coli* were recorded. The numbers of Escherichia coli/ml. or gm. were calculated after the IMViC pattern from the most probable number (MPN) tables for the three-tube method. Agar slants were prepared from EMB for further biochemical identification (Wehr, H. M., & Frank, J. F. , 2012).

The serological characterization of *E. coli* isolates by the slide agglutination method was performed using polyvalent and monovalent antisera. The isolates were tested first with OK polyvalent antisera. Substantially, two separate glass slides were used. A saline solution was added to the slide glass, followed by some of the colonies from the suspicious culture, mixed to form a smooth, dense suspension. To the first glass slide (control), only a drop of saline was added and mixed. To the second, an undiluted antiserum was added and then tilted forward and backward for one minute. Agglutination was noticed using indirect lighting over a dark background. When a colony agglutinated strongly positive with one of the polyvalent serum pools, a further part was inoculated onto a nutrient agar slant (Oxoid, Ltd, Basingstoke, Hampshire, UK) and incubated at 37°C for 24 hours to grow as a culture before testing with O monovalent antisera for serogroups O26, O44, O86, O111, O114, O126, O142, O157 and O158. The strains were members of the same serogroups and isolated from the same samples were reported only once. Positive control strains gained from the Animal Health research institute, Dokki, Giza, Egypt, were involved in each experimental run.

**2.3.4. Isolation of Escherichia coli O157:H7**

Twenty-five ml. /gm. of each sample was separately homogenised with 225 ml of modified tryptone soy broth supplemented by Novobiocin (20 mg / l) for 2 minutes using a sterile homogenizer (universal Laboratory Aid, Poland). The inoculated broth was incubated at 37 °C for 24 hours. A loopful from the incubated broth was streaked onto a Tellurite Cefixime Sorbitol MacConkey agar plate and incubated at 37 °C for 24 hours. Sorbitol-negative colonies (colourless) were picked up and purified, and then examined biochemically and serologically (De-Boer & Heuvelink, 2000).

**2.3.5. Molecular identification of Shiga-Like Toxins (Stx1&Stx2)**

**2.3.5.1. Extraction of DNA**

DNA was extracted using QIAamp DNA Mini K (Qia-


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gen, Hilden, Germany). Briefly, 1.5 ml of an overnight broth culture of *E. coli* grown in MacConke broth at 37°C was centrifuged at 8000 rpm for 5 min and the supernatant was discarded. The cell pellet was resuspended in phosphate-buffered saline (PBS) to a final volume of 200 ml. Twenty ml of QIAGEN protease were put into the bottom of a 1.5 ml microcentrifuge tube, then 200 ml of the sample followed by 200 ml of buffer A were added and mixed by pulse vortexing for 15 seconds. The mixture was then incubated at 56˚Cfor 10 min and centrifugated to remove droplets from inside the lid. Then 200 ml of ethanol (96%) was added and mixed again for 1 second. After that, centrifugation was done to discard droplets from inside the lid. The mixture was gently applied to the QIAamp Mini spin column (in a 2 ml collecting tube) for DNA extraction. The DNA concentration was weighted using a spectrophotometer (DU530, Beckman, CA). An average of 10 mg of DNA was gained.

#### 2.3.5.2. Cycling conditions of the primers during PCR

The Stx1 and Stx2 genes for *E. coli* were amplified by duplex PCR as described by Dipineto et al. (2006). 6 ml of template DNA was tested in a reaction mixture containing 25 µL of Emerald Amp GT PCR master mix (2x premix), 15 µL of PCR grade water, and 1 ml of both forwarding and reverse primer (20 pmol) according to Emerald Amp GT PCR master mix (Takara), code number RR310AKit. The primary denaturation for Stx1 and Stx2 was for 5 min at 94°C followed by 35 cycles at 94°C for the 30 s, 58°C for 40 s, then 72°C for 45 s, and a final extension at 72°C for 10 min. Twenty ml of the reaction product were subjected to running gel electrophoresis in a 1.5% agarose gel (AppliChem, Ottoweg 4, Darmstadt, Germany) at 1–5 volts/cm of the tank length for 30 min, and the gel was sent to a UV cabinet and photographed using a gel documentation system. The data were analysed using the computer software Automatic Image Capture Software, Protein Simple formerly Cell Biosciences, the USA at the reference lab for veterinary quality control on poultry production, Animal health research institute, Dokki, Giza, Egypt (Sambrook et al., 1989).

#### 2.3.6. Isolation, identification, and serology of *Salmonella typhimurium*

Twenty-five ml./gm. of prepared samples, both of eggs rinsing solution and homogenous eggs contents, were added septically to 225 ml of sterile buffered peptone water and incubated at 37 °C for 24 ±2 hours, one ml of the incubated pre-enriched broth was inoculated into 10 ml Rappaport Vassiliadis broth tube, after that the tube was incubated at 41.5 ± 0.5 °C for 24 hours. Loops from the inoculated tubes were streaked separately onto Xylose lysine deoxycholate agar (XLD) agar medium and incubated at 37 °C for 24 hrs. Suspected colonies were red with or without black centres. The suspected colonies were sub-cultured onto a nutrient agar plate and incubated at 37 °C for 24 hours. The purified isolates were identified morphologically, biochemically (IMViC, Urea hydrolysis and Triple sugar iron agar) and serologically by using polyvalent group and specific antisera for the determination of somatic (O) and flagellar (H) antigens at the serology unit, Animal Health Research Institute, Ministry of Agriculture; Dokki, Giza, Egypt (FDA, 2010).

#### 2.3.7. Isolation of *Yersinia enterocolitica*

Twenty-five ml/gm of samples was transferred aseptically to 225 ml tryptic soy broth (TSB, Biolife, 1996), mixed thoroughly and incubated at 22 °C for 24 hours (Scheimann & Wauters, 1992). Then loops from the incubated broth were streaked directly onto Cefsulodin- Irgasan-Novobiocin (CIN) agar media plates (Difco, 1997) and were incubated at 25 °C for 48 hours. The colonies of *Yersinia enterocolitica* having a characteristic appearance (bull-eye like, dark red centre surrounded by a translucent zone) were picked and streaked onto Trypticase Soy agar (TSA) slants and incubated at 25 °C for 24 hours for further identification (morphologically, motility test and biochemically as Urease test and sugar fermentation reaction) (Walker & Glimour,1986).

## 3. Results

### 3.1. Statistical analytical results and frequency distribution of coliform counts.

Table 1 & Figure 1 show that coliforms were found in 19 (25.33%) of Baladi hens' eggshells samples in the counts ranging from 4 CFU / ml to 4.3×10 CFU / ml with a mean count of 1.34×10 CFU / ml and the highest frequency distribution, 10 (52.6%), lies within

the range of 3- < 10 CFU/ ml., while coliforms were detected in 4 (5.33%) of Baladi hens 'egg contents in counts ranging from 4 CFU/gm to 9 CFU/gm with a mean count of 6 CFU/gm with a frequency distribution of 100% that lies within the range of 3- < 10 CFU/ gm. Four(5.33%) of the examined white poultry farms 'eggshells were contaminated with coliforms in counts ranging from 4 CFU/ml to 2.1×10 CFU/ml with a mean count of 1.03×10 CFU/ml and the highest frequency distribution of 3(75%) that lies in the range of 3 -< 10 CFU/ml, while one (1.33%) of the examined brown poultry farms 'eggshells were contaminated with coliforms with a mean count of 7 CFU/ml and the highest frequency distribution 1(100%) of positive samples lies between 3 -< 10 CFU/ml, while coliforms couldn't be detected from all samples of poultry farms' egg contents. Concerning duck egg samples, coliforms were found in 11 (44%) of ducks 'eggshells samples with the counts ranging from 9 CFU/ml to $1.5\times10^{2}$CFU/ml with a mean count of 5.03×10 CFU/ml with the highest frequency distribution of positive samples was 8 (72.7%) that lies between 10- < 10 2 CFU/ml, while coliforms were detected in 2 (8 %) of ducks 'egg contents in counts ranging from 7 CFU/gm to 9 CFU/gm with a mean count of 8 CFU/gm with the highest frequency distribution (100%) that lies between 3 -< 10 CFU/gm.

### 3.2. Statistical analytical results and frequency distribution of faecal coliform count.

Table 2 & Figure 2 illustrate that faecal coliforms were found in 6 (8%) of Baladi hens ' eggshells samples in counts ranging from 4 CFU/ml to 9 CFU/ml with a mean count of 6.7 CFU/ml and 100% of the positive samples contained faecal coliforms within the range of 3 -< 10 CFU/ml. Faecal coliforms were found in 2 (2.7%) of white poultry farms ' eggshells samples in counts ranging from 4 CFU/ml to 7 CFU/ml with a mean count of 5.5 CFU/ml. 100% of the positive

**Table1:** Statistical analytical results of coliform count in the examined samples of shells and contents of Baladi, poultry farm (white and brown), and duck eggs:

| Examined samples | No. of examined samples | Positive samples | | Minimum | Maximum | Mean | ± SEM |
|---|---|---|---|---|---|---|---|
| | | No. | % | | | | |
| Baladi hens ' eggshells | 75 | 19 | 25.33 | 4 | 4.3×10 | 1.34×10 | 2.23 |
| White poultry farms ' eggshells | 75 | 4 | 5.33 | 4 | 2.1×10 | 1.03×10 | 3.73 |
| Brown poultry farms ' eggshells | 75 | 1 | 1.33 | 7 | 7 | 7 | 0 |
| Ducks ' eggshells | 25 | 11 | 44 | 9 | $1.5\times10^{2}$ | 5.03×10 | 1.65×10 |
| Baladi hens ' egg contents | 75 | 4 | 5.33 | 4 | 9 | 6 | 1.23 |
| White poultry farms ' egg contents | 75 | 0 | 0 | 0 | 0 | 0 | 0 |
| Brown poultry farms ' egg contents | 75 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ducks ' egg contents | 25 | 2 | 8 | 7 | 9 | 8 | 1 |


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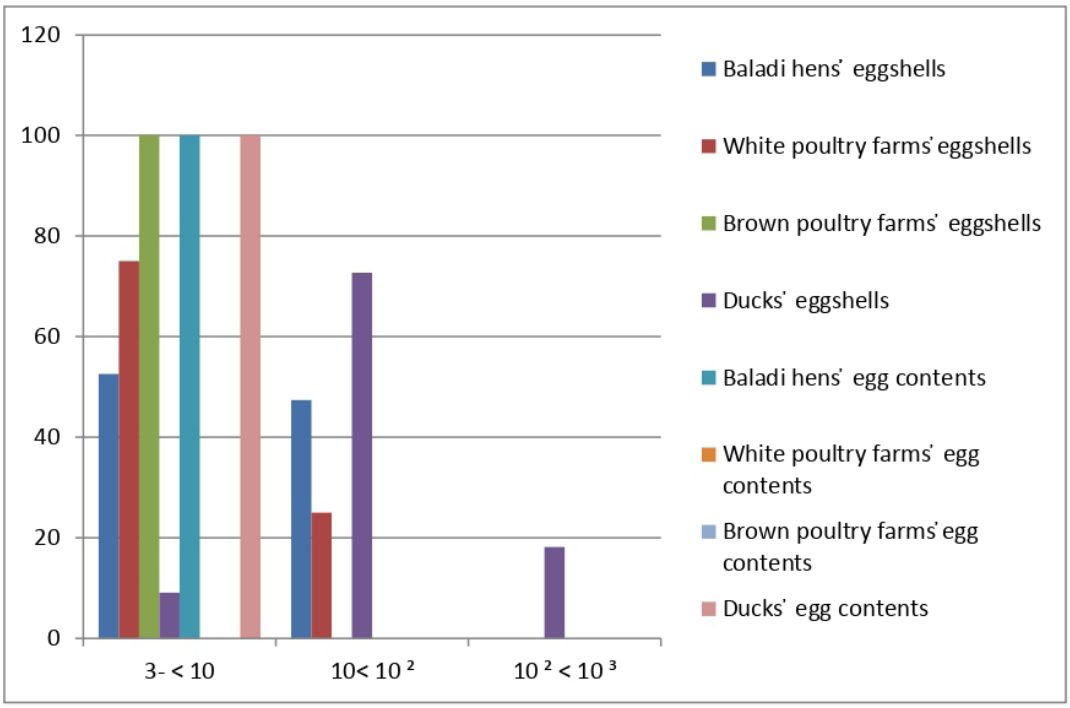


**Figure 1.** Frequency distribution of coliform count in the examined samples of shells and contents of Baladi, farm (white and brown), and duck eggs.

samples were contaminated with faecal coliforms within the range of 3 -< 10 CFU/ml. However, no faecal coliforms were detected in brown poultry farms ' eggshells, whereas 5(20%) of the examined ducks ' eggshells samples were contaminated with faecal coliforms in the counts ranging from 4 CFU/ml to 23CFU /ml with a mean count of 12.6 CFU/ml. From the positive samples, 3(60%) were found in the range of 3 -< 10 CFU/ml., and 2 (40%) within 10- < 10 2 CFU/ml. On the other hand, faecal coliforms couldn't be detected from all examined content samples of Baladi hens', poultry farms', and ducks 'eggs.

### 3.3. Statistical analytical results and frequency distribution of *E. coli* count.

Table 3 & Figure 3 show that *E. coli* was found in Baladi hens' eggshells at an incidence of two (2.7%) in counts ranging from 7 CFU/ml to 9 CFU/ml with a mean count of 8 CFU/ml. All the positive samples, two (100%), were found in the range of 3 -< 10 CFU/ml, but only one (1.3%) *E. coli* isolate was found in white Poultry farm 's eggshell sample, which lies within the range of 3 -< 10 CFU/ml with a mean count of 7 CFU/ml, while *E. coli* was detected in two (8%) of examined ducks ' eggshells samples in counts ranging from 2×10 CFU/ml to 2.3×10 CFU/ml with a mean count of 2.15×10 CFU/ml and the highest frequency distribution two (100%) lies between the range 10- < 10 2 CFU/ml.

On the other hand, *E. coli* couldn't be found in brown poultry farms ' eggshells and all examined content samples of Baladi hens', poultry farms', and duck eggs.

### 3.4. Molecular identification of Shiga-like toxins (Stx1 & Stx2) from the recovered *E. coli* strains of the examined egg samples.

It is apparent in Figure 4, PCR results for Shiga like toxins 1 & 2 genes. Stx1 (614 bp) was found in one Baladi hen's eggshell sample, and Stx2 (779 bp) was detected in one duck 's eggshell.

### 3.5. Incidence of *Salmonella typhimurium* and *Yersinia enterocolitica* in the examined samples.

Table (4) shows that only one (4%) isolate was identified as S. typhimurium, which was isolated from a duck 's eggshell sample, while S. typhimurium couldn't be detected in Baladi hens' and poultry farms' eggshells and contents samples, no S. typhimurium was found in ducks' egg contents samples. *Yersinia enterocolitica* was not found in all the samples tested.

**Table 2.** Statistical analytical results of faecal coliform count in the examined samples of shells and contents of Baladi, poultry farm (white and brown), and duck eggs:

| Examined samples | No. of examined samples | Positive samples | | Minimum | Maximum | Mean | ± SEM |
|---|---|---|---|---|---|---|---|
| | | No. | % | | | | |
| Baladi hens' eggshells | 75 | 6 | 8 | 4 | 9 | 6.7 | 0.92 |
| White poultry farms' eggshells | 75 | 2 | 2.7 | 4 | 7 | 5.5 | 1.5 |
| Brown poultry farms' eggshells | 75 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ducks' eggshells | 25 | 5 | 20 | 4 | 23 | 12.6 | 3.8 |
| Baladi hens' egg contents | 75 | 0 | 0 | 0 | 0 | 0 | 0 |
| White poultry farms' egg contents | 75 | 0 | 0 | 0 | 0 | 0 | 0 |
| Brown poultry farms' egg contents | 75 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ducks' egg contents | 25 | 0 | 0 | 0 | 0 | 0 | 0 |

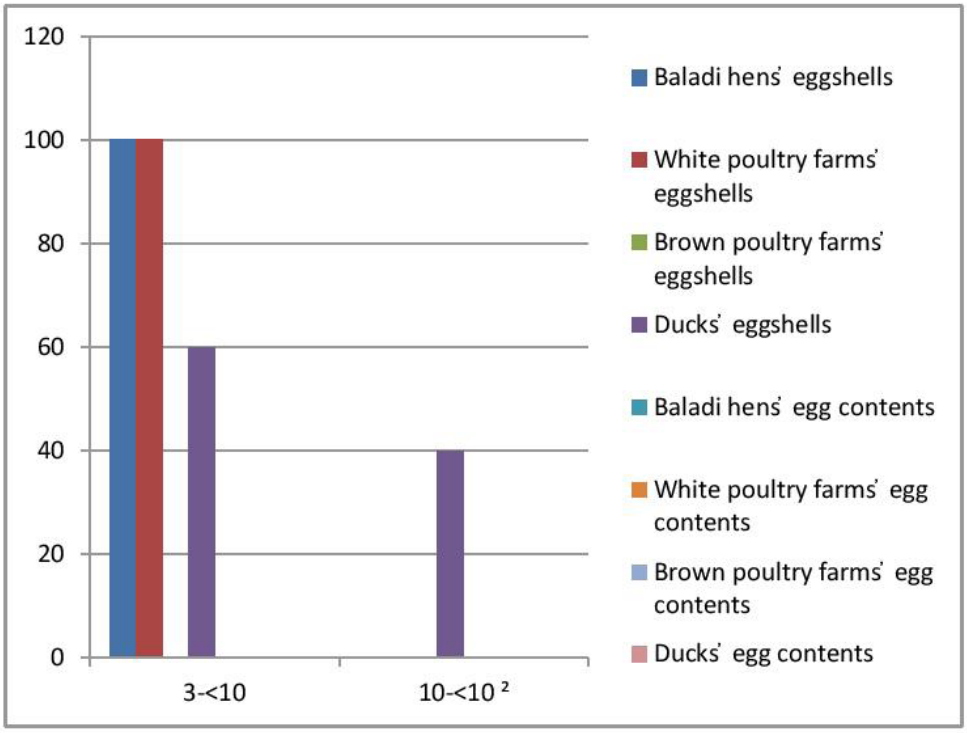


**Figure 2.** Frequency distribution of faecal coliform count in the examined samples of shells and contents of Baladi, poultry farm (white and brown), and duck eggs samples.


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**Table 3.** Statistical analytical results of *E. coli* count in the examined samples of shells and contents of Baladi, poultry farm (white and brown), and duck eggs

| Examined samples | No. of examined samples | Positive Samples | | Minimum | Maximum | Mean | ± SEM |
|---|---|---|---|---|---|---|---|
| | | No. | % | | | | |
| Baladi hens' eggshells | 75 | 2 | 2.7 | 7 | 9 | 8 | 1 |
| White poultry farms' eggshells | 75 | 1 | 1.3 | 7 | 7 | 7 | 0 |
| Brown poultry farms' eggshells | 75 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ducks' eggshells | 25 | 2 | 8 | 2×10 | 2.3×10 | 2.15×10 | 1.5 |
| Baladi hens' egg contents | 75 | 0 | 0 | 0 | 0 | 0 | 0 |
| White poultry farms' egg contents | 75 | 0 | 0 | 0 | 0 | 0 | 0 |
| Brown poultry farms' egg contents | 75 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ducks' egg contents | 25 | 0 | 0 | 0 | 0 | 0 | 0 |

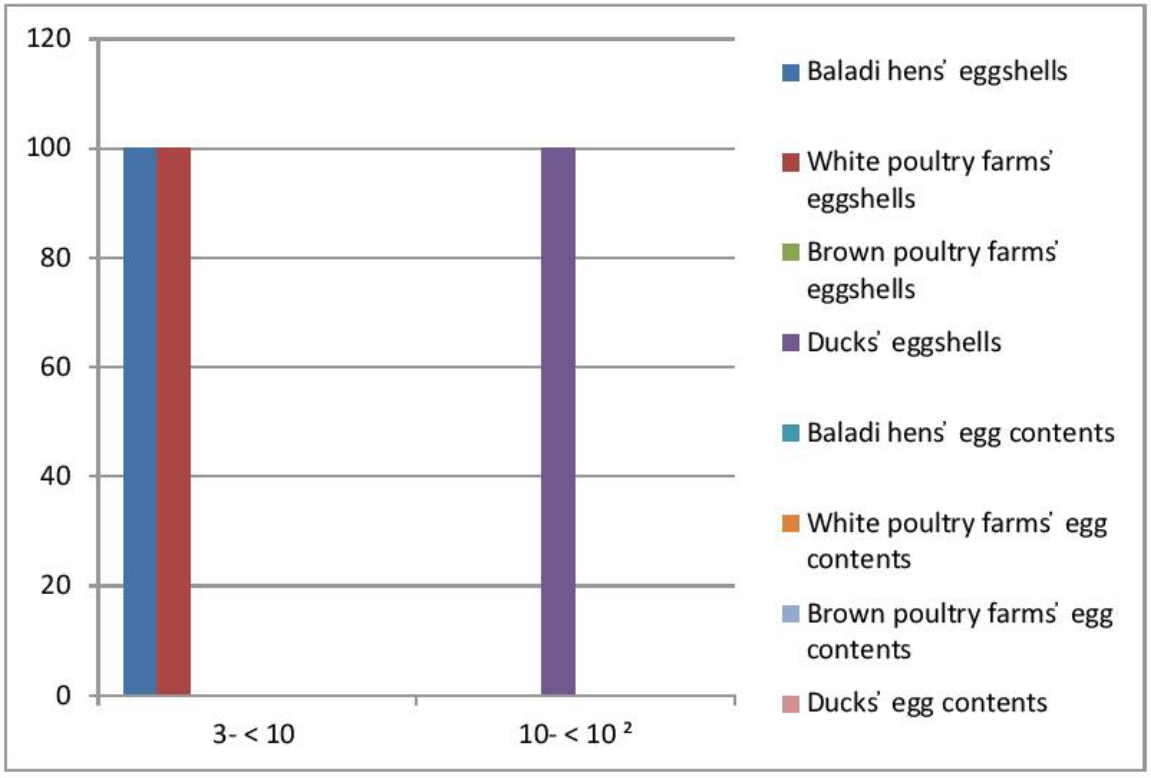


**Figure 3.** Frequency distribution of *E. coli* count in the examined samples of shells and contents of Baladi, farm (white and brown), and duck's eggs.

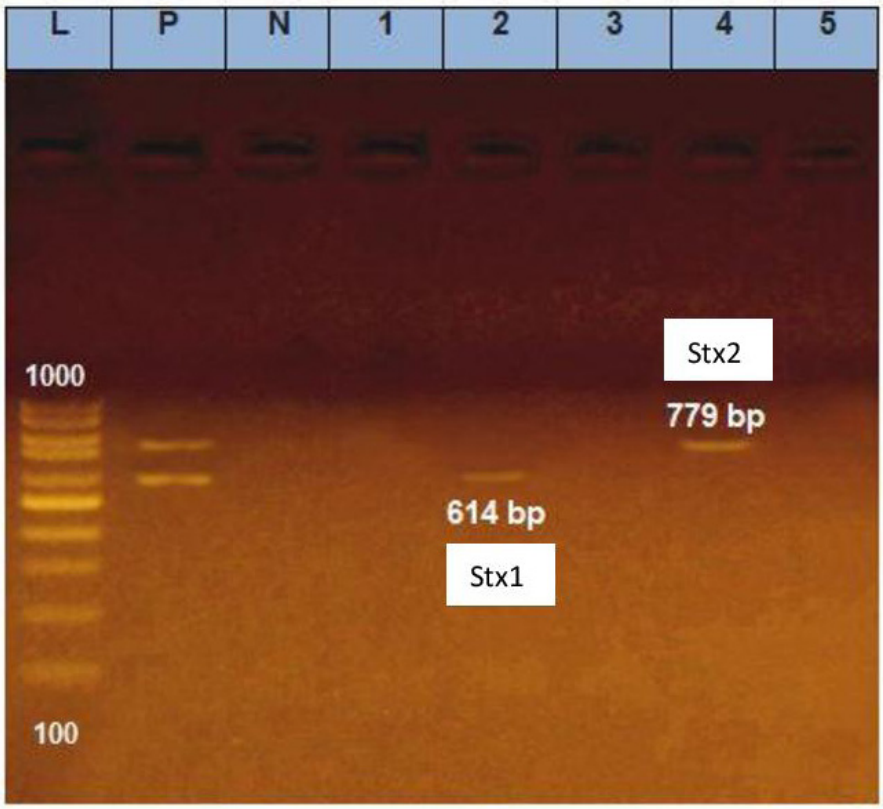


**Figure 4.** PCR results for Shiga-like toxins 1 & 2 genes, Stx1 (614 bp) and Stx2 (779 bp) from *E. coli* strains. Lane L: DNA ladder, Lane Pos.; control +ve, Lane Neg.; control –ve, Lane 2 (+ve Stx1) and Lane 4 (+ve Stx2).

**Table 4.** Incidence of *Salmonella typhimurium* & *Yersinia enterocolitica* in the examined samples of shells and contents of Baladi, poultry farm (white and brown), and duck eggs:

| Examined samples | No. of examined samples | *Salmonella typhimurium* | | *Yersinia enterocolitica* | |
|---|---|---|---|---|---|
| | | No. of positive samples | % | No. of positive samples | % |
| Baladi hens' eggshells | 75 | 0 | 0 | 0 | 0 |
| White poultry farms' eggshells | 75 | 0 | 0 | 0 | 0 |
| Brown poultry farms' eggshells | 75 | 0 | 0 | 0 | 0 |
| Ducks' eggshells | 25 | 1 | 4 | 0 | 0 |
| Baladi hens' egg contents | 75 | 0 | 0 | 0 | 0 |
| White poultry farms' egg contents | 75 | 0 | 0 | 0 | 0 |
| Brown poultry farms' egg contents | 75 | 0 | 0 | 0 | 0 |
| Ducks' egg contents | 25 | 0 | 0 | 0 | 0 |
| Total | 500 | 1 | 0.2 | 0 | 0 |


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## 4. Discussion

### 4.1. Coliform counts

#### 4.1.1. Baladi hens' eggs

Results recorded in Table 1& Figure 1, showed that coliforms were found in 19 (25.33%) Baladi hens' eggshells samples with a mean count of 1.34×10 CFU/ml and the highest frequency distribution, 10 (52.6%), lies within the range of 3- < 10 CFU/ ml.

Higher results were recorded by El-Leboudy & El-Mossalami (2006), Refaat (2009), El-Kholy (2014), Sadek et al. (2016), and El-Kholy et al. (2020), and lower results were obtained by Bahobail et al. (2012). On the other hand, coliforms could be detected in 4(5.33%) of Baladi hens 'egg contents with a mean count of 6 CFU / gm and a frequency distribution of 100% that lies in the range of 3- < 10 CFU/gm. Higher incidences were reported by El-Leboudy & El-Mossalami (2006), El-Kholy (2014), and Sadek et al. (2016). Moreover, our result is nearly similar to that obtained by El-Kholy et al. (2020), while Refaat (2009) couldn't find coliforms in Baladi hens' egg contents.

#### 4.1.2. Poultry farms' eggs

According to the findings in Table 1 & Figure 1, 4(5.33%) of the examined white poultry farms' eggshells were contaminated with coliforms with a mean count of 1.03×10 CFU/ml with the highest frequency distribution of 3(75%) that lies in the range of 3 -< 10 CFU/ml, while 1(1.33%) of the examined brown poultry farms' eggshells were contaminated with coliforms with a mean count of 7 CFU / ml. The highest frequency distribution 1(100%) of positive samples lies between 3 -< 10 CFU/ml.

Higher results of 33.3, 37.1, 30, 47.06, and 10% were estimated by El-Leboudy & El-Mossalami (2006), Refaat (2009), El-Leboudy et al. (2011), El-Kholy et al. (2014), and Sadek et al. (2016), respectively. A slightly lower result (4%) was found by El-Kholy et al. (2020). On the other hand, coliforms couldn't be detected from all samples of poultry farm's egg contents. Similar results were obtained by Refaat (2009), Sadek et al. (2016), and El-Kholy et al. (2020), while higher results were detected by El-Leboudy & El-Mossalami (2006), and El-Kholy et al. (2014).

#### 4.1.3. Ducks' eggs:

From the data presented in Table 1 & Figure 1, coliforms were detected in 11(44%) of ducks ' eggshell samples with a mean count of 5.03×10 CFU / ml. The highest frequency distribution of positive samples was 8 (72.7%) which lies between 10- < 10 2 CFU/ml. A lower result was obtained by Refaat (2009).

On the other hand, coliforms were detected in 2 (8 %) of ducks ' egg contents with a mean count of 8 CFU / gm, and the highest frequency distribution (100%) lies between 3 -< 10 CFU/gm. Higher results were found by El-Leboudy & El-Mossalami (2006) and Awny et al. (2018), while Refaat (2009) couldn't find coliform organisms in ducks egg contents.

Coliforms are an intestinal and non-intestinal inhabitant, so coliform count is a traditional indicator of faecal contamination, microbial quality, and reflects food hygiene standards (Musgrove et al., 2008). The existence of coliforms in Baladi hens 'eggs and ducks eggs is an indicator of poor hygiene. Therefore, eggs that contain a high percentage of coliforms are of economic and public health importance (Sabreen, 2001), while the lower rate of contamination of poultry farm eggs is due to the egg cleaning process before marketing and its hygienic handling. Also, eggshell surface disinfection has an important role in preventing egg spoilage and egg-related diseases (De Reu et al., 2006).

### 4.2. Faecal coliform counts

#### 4.2.1. Baladi hens' eggs

As recorded in Table 2 & Figure 2, faecal coliforms were found on 6(8%) of Baladi hens' eggshells samples, and 100% of positive samples contained faecal coliforms with a mean count of 6.7 CFU / ml within the range of 3 -< 10 CFU/ml. Our results disagreed with the results estimated by Refaat (2009), El-Kholy (2014), and Sadek et al. (2016), who detected faecal coliforms with higher as 22.9, 57.1, and 73.3%, respectively.

On the other hand, faecal coliforms couldn't be detected from all samples of Baladi hens' egg contents.

This result was in harmony with those obtained by Refaat (2009), who mentioned that faecal coliforms couldn't be detected from all samples of Baladi hens 'egg contents, while higher incidences were estimated by El-Kholy (2014) and Sadek et al. (2016).

**4.2.2. Poultry farms' eggs**

According to the data reported in Table 2& Figure 2, faecal coliforms were found in 2 (2.7%) of the white poultry farms' eggshells samples with a mean count of 5.5 CFU/ml. 100% of the positive samples contained faecal coliforms within the range of 3 -< 10 CFU/ml, while no faecal coliforms were detected in brown poultry farms' eggshells.

Higher incidences of 11.4, 20.59, and 6.7 % were estimated by Refaat (2009), El-Kholy et al. (2014), and Sadek et al. (2016), respectively.

On the other hand, faecal coliforms couldn't be detected in the poultry farms ' egg contents in this study. Our results agreed with those reported by Refaat (2009), and Sadek et al. (2016), while faecal coliforms were detected in 20.59% of poultry farms 'egg contents by El-Kholy et al. (2014).

**4.2.3. Ducks' eggs**

The summarised results in Table 2 & Figure 2 showed that 5 (20%) of the examined ducks' eggshell samples were contaminated with faecal coliforms with a mean count of 12.6 CFU/ml. From the positive samples, 3 (60%) were found in the range of 3 -< 10 CFU/ml., and 2 (40%) within 10- < 10 2 CFU/ml.

A lower result of 11.4% was recorded by Refaat (2009). Regarding the examined ducks ' egg contents samples, no faecal coliforms were detected, and this result agreed with that estimated by Refaat (2009).

**4.3. *E. coli* counts**

**4.3.1. Baladi hens' eggs**

The results presented in Table 3 & Figure 3 revealed that *E. coli* was found in Baladi hens' eggshells in an incidence of 2 (2.7%) with a mean count of 8 CFU/ml. All the positive samples, 2 (100%), were found in the range of 3 -< 10 CFU/ml. Higher results of 32, 42.8, 44, and 53.3 were recorded by Al-Ashmawy (2013), El-Kholy (2014), Ibrahim et al. (2014) and Sadek et al. (2016), respectively, while *E. coli* couldn't be detected by Refaat (2009).

On the other hand, in our study, *E. coli* couldn't be found in Baladi hens' egg contents samples. A similar result was recorded by Refaat (2009). Higher incidences of 23, 19, and 6.7% were found by Al-Ashmawy (2013), Ibrahim et al. (2014), and Sadek et al. (2016), respectively.

**4.3.2. Poultry farms' eggs**

Table 3 & Figure 3 show that only 1(1.3%) *E. coli* isolate was found in a white poultry farm's eggshell sample with a mean count of 7 CFU/ml, which lies within the range of 3 -< 10 CFU/ml, whereas *E. coli* couldn't be isolated from brown farms ' eggshells samples and all samples of farms ' egg contents.

Concerning poultry farms' eggshells, higher incidences of 5.7, 14.71, 27.5, and 6.7 % were reported by Refaat (2009), El-Kholy et al. (2014), Ibrahim et al. (2014), and Sadek et al. (2016), respectively.

Regarding the farms' egg content samples, many authors failed to isolate *E. coli* as Refaat (2009), Al-Ashmawy (2013), El-Malt (2015), and Sadek et al. (2016), while El-Kholy et al. (2014) detected *E. coli* in 11.76%.

**4.3.3. Ducks' eggs**

*E. coli* was detected in 2 (8%) of examined ducks ' eggshells samples with a mean count of 2.15×10 CFU / ml as reported in Table 3, and the highest frequency distribution, as shown in Figure 3, 2 (100%), lies between the range 10- < 10 2 CFU/ml. A lower incidence of 5.7% was recorded by Refaat (2009).

Concerning ducks ' egg contents, *E. coli* couldn't be isolated from all examined samples. A higher result was obtained by El-Leboudy & El-Mossalami (2006). According to Tables (2&3), no faecal coliforms and *E. coli* were found in all examined samples of contents of Baladi, poultry farm hens, and ducks 'eggs. This may be due to the internal antimicrobial defence mechanisms of eggs and the use of antibiotics in farms.


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Serological identification of the suspected *E. coli* isolates was done. Moreover, molecular identification of Shiga-like toxins (Stx1 & Stx2) from the recovered *E. coli* strains of the examined egg samples was done. It is apparent in Figure 4, PCR results for Shiga-like toxins 1 & 2 genes, Stx1 (614 bp) was found in one Baladi hen's eggshell sample, and Stx2 (779 bp) was detected in one duck's eggshell.

Shiga toxin-producing *E. coli* (STEC) can cause intense foodborne illnesses and haemolytic uraemic syndrome (HUS), which is characterised by acute renal failure, haemolytic anaemia, and thrombocytopenia (low platelet count). Although *E. coli* O157:H7 is the most critical STEC serotype in terms of public health, other serotypes have been implicated in sporadic cases and outbreaks.

In the current study, *E. coli* O157:H7 failed to be detected.

Generally, the presence of *E. coli* in eggs is an excellent indicator of faecal pollution and the presence of some enteric pathogens, which may lead to foodborne infection and intoxication. It constitutes a public health hazard to humans and a significant economic menace to the poultry industry (Quiroga et al., 2000).

### 4.4. Isolation of *Salmonella typhimurium*

#### 4.4.1. Baladi hens' eggs

As shown in Table (4), S. typhimurium couldn't be detected in Baladi hen's eggshells and contents samples. Higher results were reported by Refaat (2009) and El-Kholy (2014) for eggshells and contents.

#### 4.4.2. Poultry farms' eggs

It is evident from the results recorded in Table (4), that S. typhimurium couldn't be detected in all examined poultry farms' eggshells and contents samples. Several investigators failed to isolate *Salmonella* Spp. from table eggs, such as El-Kholy et al. (2014), Awny et al. (2018), and Mahdavi et al. (2012). This result in poultry farms' eggs may return to the strict hygienic measures applied in egg production and prophylactic treatment against pathogens. Also, the use of probiotics in the ration of layer poultry farms to establish beneficial gut microflora may reduce colonisation by pathogenic organisms like *Salmonella* by competitive exclusion. It represents a potential risk to consumers because all *Salmonella* are potentially pathogenic (Kabir, 2009).

#### 4.4.3. Ducks' egg

In the present study, 1 (4%) isolate was identified as S. typhimurium as shown in Table (4), which was isolated from ducks' eggshell samples. The presence of *Salmonella* on eggshells indicates contamination with duck faeces. Our result is nearly similar to the incidence of 4.29% that was estimated by Harsha et al. (2011), while higher values were demonstrated by Korashy et al. (2008) and Suksangawong (2008). However, Adzitey et al. (2012), and Sedeek & Aioub (2014) couldn't detect *Salmonella* in all of the examined ducks' eggshell samples.

On the other hand, it couldn't be detected in ducks' egg contents in our study. Also, Sedeek & Aioub (2014) failed to detect *Salmonella* in ducks' egg contents. Ducks' eggs were associated with S. typhimurium outbreaks in Germany between 1974 and 1996 (Rabsch et al., 2002).

### 4.5. Isolation of *Yersinia enterocolitica*

#### 4.5.1. Baladi hens' eggs

As recorded in Table (4), Y. enterocolitica failed to be detected in our study from both the shells and contents of Baladi hen's eggs. A higher result was obtained by Abdel-Haleem & Ali (2005), who isolated the pathogen in incidences of 6.7% and 20% from eggshells and contents, respectively.

#### 4.5.2. Poultry farms' eggs

In the present study, as reported in Table (4), we failed to isolate Y. enterocolitica from the examined samples of both shells and contents of poultry farm hens' eggs (white and brown). Also, other investigators couldn't isolate Y. enterocolitica from poultry farm hens' eggs (shells and contents), such as Abdel-Haleem & Ali (2005), while Favier et al. (2005) found Y. enterocolitica at a percentage of 2.27% on eggshells.

### 4.5.3. Ducks' eggs

From Table (4), it is clear that no Y. enterocolitica was found in all examined samples of both shells and contents of ducks' eggs, while the investigation carried out by Korashy et al. (2008) pointed out that Y. enterocolitica could be detected in 10% and 6.7% of eggshells and contents, respectively.

## 5. Conclusion

This study revealed that Baladi hens' and ducks' eggs have a higher microbial load than poultry farm hens' eggs. There are higher incidences of coliforms, faecal coliforms, and *E. coli* organisms in Baladi hens' and ducks' eggs than those from poultry farm hens' eggs, and the presence of S. typhimurium on ducks' eggshells.

Duck eggs contain a relatively high contamination percentage as they lay their eggs nearer to damp places (ponds) with high moisture and pick up flies and other infective materials. On the other hand, the antibacterial activity of their egg albumen (Con albumen) deteriorates rapidly on storage, and the eggshell is thinner than that of a hen's egg (Burley & Vadehra, 1989).

Therefore, hygienic measures should be applied to home-produced hens and ducks to lower the bacterial load in their eggshells and subsequently in their egg contents. In addition, strict sanitary measures should be implemented in farms to safeguard egg consumers from infection and to save eggs from deteriorating. Also, egg preservation, handling, and distribution should be done with care. Thorough cooking and preparation of eggs and egg-containing foods should be applied to safeguard human beings from being infected with pathogenic organisms.

Future recommendations include the following: Routine microbiological screening, control programs, and prompt vaccination for Baladi, poultry, and duck farms should be adopted to reduce herd infections. Make sure the egg-laying areas are clean and perform frequent egg collection to minimise egg contamination. Prevention of egg washing and application of eggshell sanitation or fumigation programs properly. From laying to consumption, eggs should be stored at a temperature of less than 4 °C and at a relative humidity of 70% to 80%. Prevention of eating raw and half-cooked eggs to avoid the risk of food poisoning.

## Conflict of interest

The authors declare no conflict of interest.

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# The effect of *Lactobacillus acidophilus* on the changes in the acidity of probiotic milk during storage

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**Keywords**

*Lactobacillus acidophilus*; Probiotic products; *Bifidobacteria*; Fermentation process; Health promotion; Dairy products.

Probiotic products are functional products for human health. To evaluate the acidity changes of milk containing probiotic bacterium *Lactobacillus acidophilus*, three containers containing 3 litre of sterilized low-fat milk with 1.5% fat prepared by Mihan Company were considered in three groups. The results showed changes in milk containing *Lactobacillus acidophilus* bacteria because the acidity index was recorded for two hours to reach the acidity of 42 °C in milk in the incubator at 38 °C and during storage in the refrigerator. An increase in the acidity of probiotic milk compared to the control sample was observed on the first day after production. The acidification rate increased during the fermentation under the influence of L. *acidophilus* bacterium also the final acidity after the end of the fermentation period in the probiotic milk was higher than in the control sample.

## 1. Introduction

Functional food is a food that contains at least one specific and proven health property in addition to the basic nutritional properties which is functionally recommended by the manufacturer or by nutritionists and consumed by the consumer (Farahmand et al., 2021). One of the important things related to the selection and production of functional foods is their safety. Nowadays, considering the practical proof of the unfavourable results due to imbalance in human societies, the tendency to produce and consume a variety of functional foods has increased significantly (Bull et al., 2013). Nowadays, food consumers pay special attention to the items such as pleasant flavour, low calories and fat, and the beneficial effect of food on health (Widyastuti et al., 2021). Therefore, the food industry tries to manufacture products with better flavour and properties, among which a dairy product processed with probiotics is of great importance in promoting health (Zhang et al., 2019).

Probiotics are one of the most emerging and popular functional products which are of particular importance in this regard (Ye et al., 2017). Probiotic products with valuable nutritional and therapeutic properties are among the most controversial topics in the field of industry, nutrition, and medicine (Raygan et al., 2018). According to FAO/WHO definition, probiotics are "living microorganisms that have beneficial effects on maintaining the health of their host if consumed in sufficient quantities" (Depree & Savage, 2001). Probiotics are used not only as dietary and pharmaceutical supplements but also in dairy products, juices, chocolates, and even meat products (Parker et al., 2018). Two major groups of probiotic microorganisms are *Lactobacillus* pp and Bifidobacterium (Toropov et al., 2020). Probiotics proliferate in the gastrointestinal

tract and compete with the production of antagonistic metabolites with saprophytic micro flora. This ability can be observed in lactic acid bacteria such as Lactobacilli and *Bifidobacteria* (Motey et al., 2021). Epidemiological studies showed that regular use of probiotics may reduce the risk of cancer, improve heart health, improve the immune system, improve gastrointestinal health, anti-inflammatory, antimicrobial and antiviral effects, lower blood pressure, prevent weight gain, prevention of urinary tract infections, and treatment of allergic diseases such as eczema (Parker et al., 2018; Barros et al., 2020). Continuity and amount of consumption of probiotics are effective in playing their beneficial therapeutic roles. A daily intake of 106 to 108 CFU/g live bacteria was considered an acceptable minimum. Therefore, daily consumption of 100 g of probiotic product with 1×106 to 5×108 CFU of live bacteria per gram of product can provide the optimal limit (Cruz et al., 2009).

Probiotic bacteria (*Bifidobacteria* and *Lactobacillus acidophilus*) are substantially decreased during refrigerated storage, particularly in acidic conditions, according to extensive studies. As a result, it is critical to keep the quantity of these microorganisms constant throughout storage (Nyanzi et al., 2021). Research has looked at the probiotic properties of this group of bacteria, and strains with specified probiotic characteristics have been introduced (Saarela, 2019).

Besides, consumer-perceived sensory characteristics are important from a commercial and market point of view (Wilkinson, 2018). Fermented dairy products always carry a significant population of bacteria of the starter cultures that are common in terms of the type or even species of well-known and commercially known probiotics and for which many health properties have been mentioned (Sarkar, 2018). Milk is one of the most often eaten items in families' food baskets, and it provides important products to the customer in addition to providing a favourable environment for the development and survival of probiotics due to its unique nutritional characteristics (Vinderola et al., 2019). Probiotic milk is produced in a variety of ways, such as by adding bacteria to fresh milk without fermentation (such as sweet milk) or fermenting milk by probiotic bacteria (such as Yakult) (Patro et al., 2016). Adding probiotics to fermented dairy products leads to milk protection by producing lactic acid and antimicrobial compounds and increasing the nutritional value of food by producing compounds such as free amino acids and vitamins (Turkmen et al., 2019). Of its technological and health characteristics, milk is one of the most popular products in the country. The inclusion of probiotic bacteria in such a high-consumption product can help promote community health (Sotoudegan et al., 2019). Among the most common probiotic dairy products in the global market, we can mention yogurt, buttermilk, kefir, probiotic cheese, and other types of fermented milk (Sah et al., 2016). The increasing prevalence of probiotic products and their widespread advertising has led to the fact that in addition to the common control of these products by regulatory agencies, a part of the scientific study is limited to the quality of biomarkers of probiotic microorganisms in these products (Rezazadeh et al., 2019). This research tried to study the possibility of survival and growth of *Lactobacillus acidophilus* in milk.

## 2. Materials and Methods

The current research used a fully randomized design. Three containers holding 3 litre of sterilized low-fat milk with 1.5% fat produced by Mihan Company were considered three groups to create milk containing the probiotic bacteria *Lactobacillus acidophilus*-5. The probiotic bacterium of *Lactobacillus acidophilus* (LA-5®) (Chr. Hansen, Hørsholm, Denmark) was in freeze-dried (Direct Vat Set) form at the time of purchase. According to the manufacturer's recommendation, it was kept in freezing conditions (-18°C) until consumption.

### 2.1. Microorganisms

Single-strain probiotic culture of *Lactobacillus acidophilus* (La-5) was purchased in freeze-dried form and DVS type from the Danish company of Kristin Hansen. The reason to choose *Lactobacillus acidophilus* La-5 as a probiotic strain was between two common probiotic bacteria species (*Lactobacillus acidophilus* and Bifidobacterium) in probiotic dairy products, Bifidobacterium Lactis Bb-12 does not survive well at the pH value of about 5.5. *Lactobacillus acidophilus* survives well at this pH (Taheri et al., 2008). Incompatibility of Bb-12 at 45 °C of aerobic medium (42-45°C) and acidic conditions and at the same time, the optimal growth of *Lactobacillus acidophilus* La-5 in these conditions was shown (Taheri et al., 2008).

According to the incubation conditions and the final pH of the product, *Lactobacillus acidophilus* La-5 was selected as the probiotic bacterium used in this study.

Tested starter culture and preparation of culture rennet (1% Tween 80) was added to the one-way probiotic culture of *Lactobacillus acidophilus* La-5 by surface cultured in an MRS agar medium. This culture was then sterilized for 15 minutes at 121°C. The 1 g of probiotic strain was then transferred to 100ml of Lactose Broth and incubated for 48 hours at 42°C. Next, at 40°C, 5ml of the produced medium was inoculated into 1 litre of 1.5% fat sterilized milk, homogenized, and incubated at 37°C until about 80°D acidities. The cells were centrifuged at 2360g for 8min and washed twice in NaCl solution (0.85%). The bacterial mass was dissolved in a normal saline solution to contain approximately 109-1010 CFU/ml of viable bacteria (Pedroso et al., 2012; Florence et al., 2012). Finally, the acidity test was performed; also the last sample of fermented milk was used as a culture starter in various experiments.

### 2.2. Determination of the optimum growth temperature

To determine the optimum growth temperature, first, one litre of low-fat sterile milk was placed in an Erlenmeyer for 20 minutes at the 80 °C temperature of incubator and then the temperature was reduced to about 40 °C using cold water, then 5 ml of the initial culture medium was inoculated and homogenized under sterile conditions. The resulting milk was evenly distributed in the presence of flame in four 250 ml Erlenmeyer and incubated at 38 and 42 °C, respectively, and the acidity was evaluated at 0, 2, 4, 6, and 8 hours.

### 2.3. Sampling

To evaluate the survival of *Lactobacillus acidophilus* probiotic bacteria during storage and its effect on the characteristics of probiotic milk and comparison with the control sample, sampling was performed on the control and probiotic samples at 38 and 42°C.

### 2.4. Evaluation of titratable acidity

In the presence of phenolphthalein reagent, 0.1 N soda, and according to the technique described in Iranian National Standard No. 2852, the titration method was employed. In the presence of phenolphthalein reagent, 10 ml of the sample was mixed with 10 ml of distilled water and titrated with 0.1 N soda. The value of this index was calculated based on the Dornic degree (Mortazavian et al., 2010). The acidity of the milk was controlled and recorded every 2 hours, and the total acidity was calculated from Equation (1). One millilitre of 0.1 N soda is equivalent to 0.009008 g of lactic acid.

Equation (1):

$$\%titratable\ acidity = \frac{ml \times N \times 90 \times 100}{V \times 1000}$$

Where:
ml= ml 0.1 NaOH used
N= Normality of 0.1N NaOH
V= ml sample solution used

### 2.5. Statistical analysis

In this study, the tests were performed in three periods, and each period with three replications. The comparison among the results was performed by ANOVA statistical tests with a 95% confidence level by SPSS 11.5 software. Excel software was used to draw graphs and figures.

## 3. Results and Discussion

Acidity rate during storage: The changes in milk containing *Lactobacillus acidophilus* bacteria in terms of acidity index in two hours to achieve acidity of 42 degrees Dornic (milk) in the incubator at 38 °C and during storage in the refrigerator were recorded. Acidity values in terms of degree Dornic were almost constant in the early hours, which were assessed because the activities of the probiotics are not started. Figures (1 to 4) compare the acidification process of probiotic milk with the control sample. The first point in this diagram was the higher acidity of probiotic milk than the control sample on the initial day after production.

In the first milk sample at 38 ° C, the process of increasing the acidity was slow so that in the first 6 hours the acidity was from 19 to 30 Dornic degrees, and then in the next 2 hours with faster growth reached 41 Dornic degrees (Figure1). Also, in the second sam-


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ple at 42 ° C, the process of increasing the acidity is done more rapidly so that in 4 hours, the acidity has reached 25 to 41 Dornic degrees (Figure2). Then, the third sample was examined with twice the amount of the L. *acidophilus* and a temperature of 42 ° C, and the result was somewhat similar to the second sample, so that in four hours, the acidity increased by 19 degrees, reaching 43 Dornic degrees (Figure3). Finally, for a more accurate comparison, the results of all three samples were placed on a graph (Figure4). Therefore, it can be claimed that the presence of L. *acidophilus* increased the acidification rate during fermentation, and the final acidity after the end of the fermentation period in probiotic milk was higher than in the control sample. It was found that the acidification of the probiotic product was more than the control, during production and incubation. Furthermore, to the rate and speed of acidification during production, which is technologically important, acidification occurs during product storage, which is the high rate and speed of acidification at this stage of the maintenance problems of these products (Parker et al., 2018). In addition to post-acidification, the two-phase phenomenon is also one of the problems in maintaining this type of fermented milk. Research on similar products indicated that sedimentation occurs up to day 14 and then stops. This problem was more common in the samples with higher acidification during storage (Oliveira et al., 2002).

Considering the topic of probiotic products is one of the new scientific subjects in Iran and knowing the production and maintenance capacities of such products will open more windows to its industrialization and improve the health level of society. The present study was designed and conducted following recent years' research to evaluate a type of dairy health product according to Iranian taste. This study's results showed that the probiotic microorganism *Lactobacillus acidophilus* La-5 could be used in milk production. In addition to growth, the acidification of the starter bacteria is important during production and maintenance (Farahmand et al., 2021).

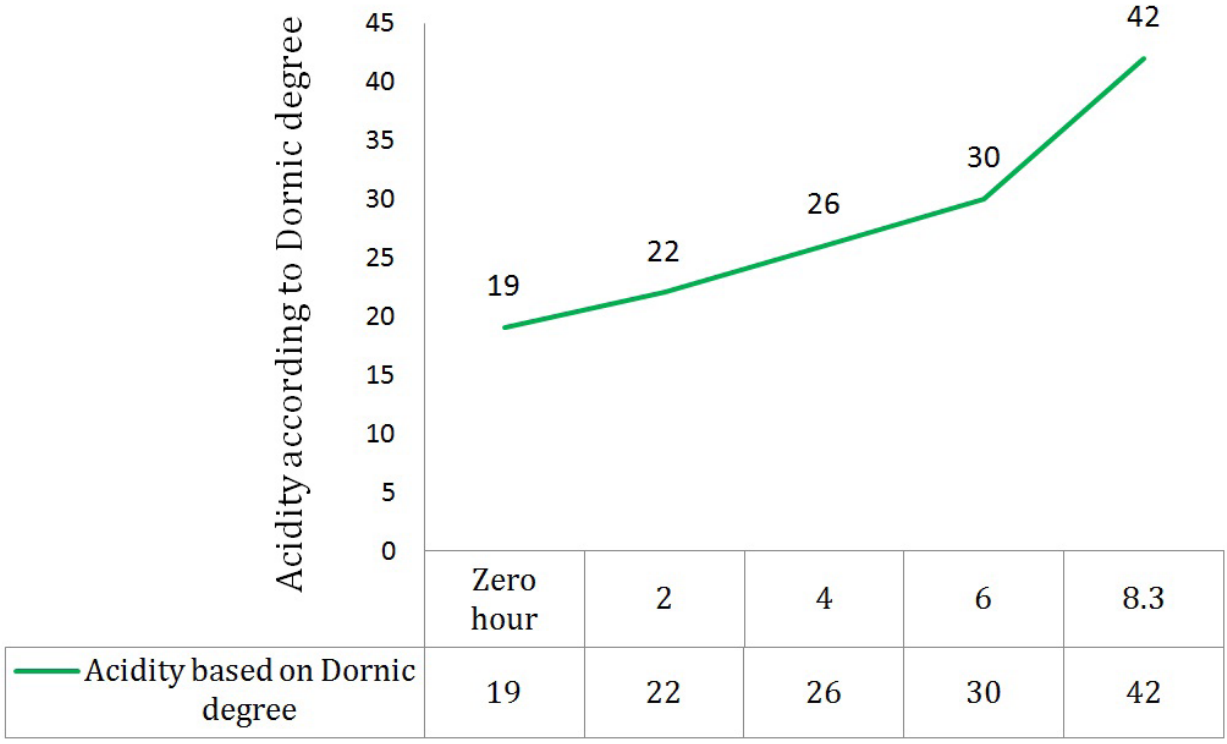


**Figure1.** The investigation of acidity in the production of *acidophilus* milk at low temperatures (at 38 °C)

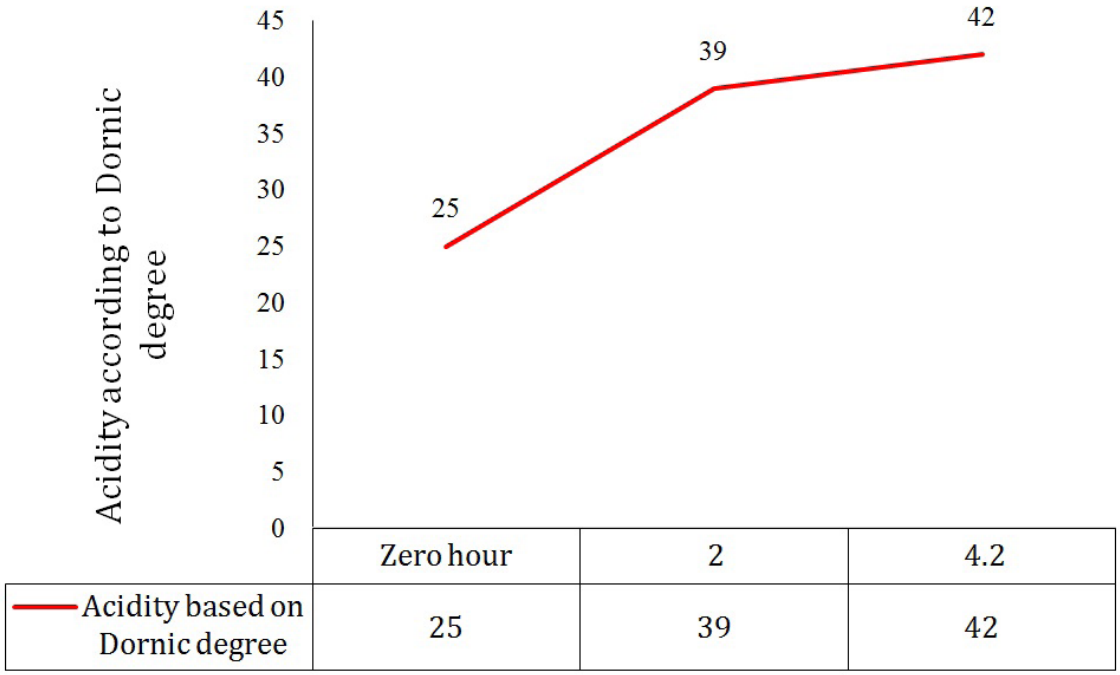


**Figure2.** The investigation of acidity in the production of *acidophilus* milk (at 42°C)

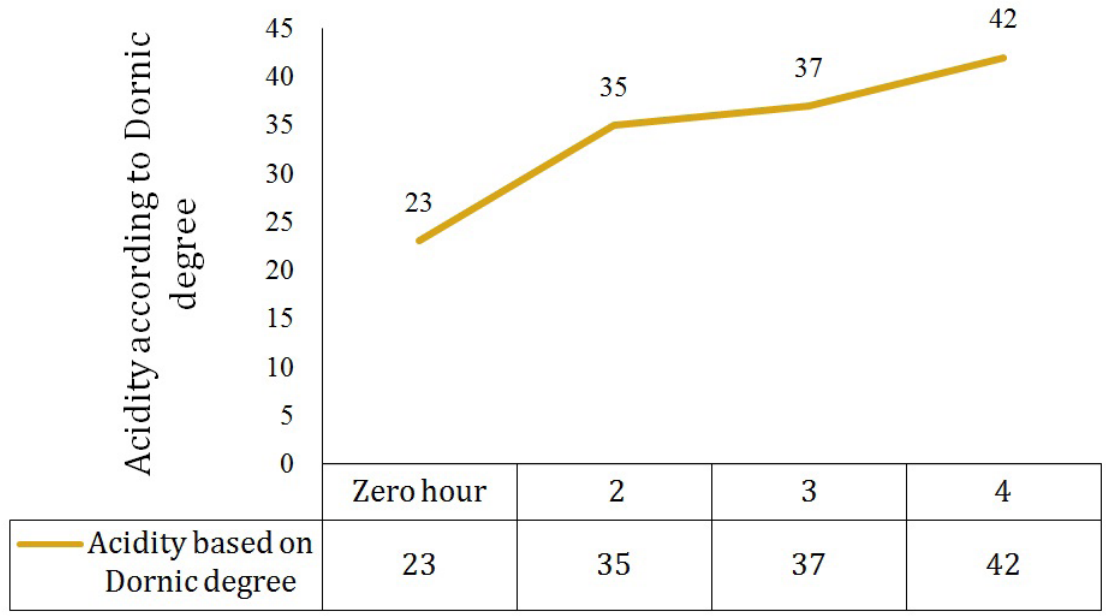


**Figure3.** The investigation of acidity in the production of the third high-dose *acidophilus* milk (Twice the dose and temperature of 42°C)


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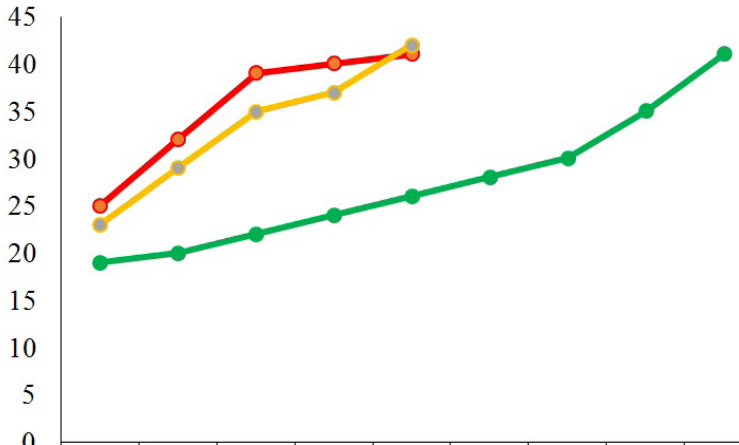


| | Zero hour | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| The first milk (temp 38°C) | 19 | 20 | 22 | 24 | 26 | 28 | 30 | 35 | 41 |
| The second milk (temp 42°C) | 25 | 32 | 39 | 40 | 41 | | | | |
| The third milk (double dose & temp of 42°C) | 23 | 29 | 35 | 37 | 42 | | | | |

**Figure4.** Comparative study of acidity development in the production of all three probiotic milk according to Dornic degree

## 4. Conclusions

The basis of probiotic products is their medicinal properties (bioavailability). The sensory properties of these products also play a significant role. In other words, the advantage of consuming probiotics via food and not in the form of medicine is their sensory properties. Among probiotic products, fermented products are universally accepted due to their unique sensory properties. Generally, this study's results showed that the acidity rate in probiotic milk during the shelf life is higher compared to the control sample.

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## Conflict of interest

The authors declare no conflict of interest. Besides, the funders had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Research Paper

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# Physico- chemical characterization of Indonesian mangroves fruits species

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*Avicennia* sp.; *Bruguiera* sp.; mangrove flour; *Rhizophora* sp.; *Sonneratia* sp.

Carbohydrates in flour are essential ingredients for the food industry, often used as thickening, gelling, bulking, and water retention agents. In Indonesia, mangrove fruits have traditionally been used as a carbohydrate source. However, studies related to the physicochemical properties of the fruit, flour, and starch of mangroves as a food source are still minimal. This work reported the physico-chemical characteristic of four species of Indonesian mangrove, namely *Avicennia* sp., *Bruguiera* sp., *Rhizophora* sp., and *Sonneratia* sp. All mangrove fruits are not safe to be consumed because they contain cyanide more than a safe level (> 50 ppm). However, proper food processing can reduce cyanide to safe levels, depending on the characteristics of those fruits. Our results suggest that mangrove fruit flour can be utilized as a food source. *Bruguiera*'s can provide thickness in a short cooking time based on the pasting properties. *Rhizophora*'s is not suitable for use as a thickening agent in cold and semi-solid food products. *Avicennia* sp. and *Sonneratia* sp. require a long cooking time to produce a good consistency, but this consistency can withstand well at cold temperatures.

## 1. Introduction

Indonesia has the largest mangrove forest area globally, followed by Australia and Brazil, which are amount ± 3 million Ha (KLHK, 2019; Rahadian et al., 2019). This amount is about 23% or almost a quarter of all the world's mangrove ecosystems, from a total area of ± 16 million Ha (ITTO, 2017; KLHK, 2019), divided into the proximal, intermediate, and distal areas. They are distributed throughout the Indonesian archipelago, especially along the east coast of Sumatra, the north coast of Java, the west and east coasts of Kalimantan, the protected landscape in Sulawesi, Maluku, and the southern coast of Papua (Rahadian et al., 2019).

The proximal site is the area closest to the sea which is dominated by *Rhizophora* apiculata, *Rhizophora mucronata*, and *Sonneratia alba*. The intermediate zone, which is the area between the sea and land, is dominated by *Rhizophora* sp., *Avicennia* sp., *Bruguiera* sp., *Sonneratia* sp., and *Ceriops sp*. (Rahim & Baderan, 2017). Mangrove species that are often found in Indonesia are api-api (*Avicennia* sp.), pedada (*Sonneratia* sp.), lindur (*Bruguiera* sp.), and bakau (*Rhizophora* sp.) (Bengen, 2001; Putri et al., 2015). *Avicennia marina* and *Rhizophora* are the dominant mangrove species in the area near the sea in the mangrove zon-

ing of Pantai Indah Kapuk, North Jakarta. The density of mangrove forests in Pantai Indah Kapuk is in the category of sparse (<10 ind/100 $m^2$) to dense (≥15 ind/100 $m^2$) (Putri et al., 2015). Several studies reported that fishery potential obtained from mangrove litter reached 3.45 g/$m^2$/days (Aida et al., 2014), 548780 kg/ha/year (Aida et al., 2014; Mahmudi et al., 2012), and 1405.25 kg/ha/years (Aida et al., 2014).

Although the availability of mangrove fruits is abundant in Indonesia, information about the nutritional properties of mangroves from Indonesia is still limited. It makes this mangrove resource unable to become a valuable commodity, both economically and functionally. Some basic research related to the physicochemical qualities of mangrove fruit is still rarely carried out, even though in some areas in Indonesia, mangrove fruits have been consumed as a food source, mainly for traditional food products.

Mangrove fruit flour is rich in dietary fiber and bioactive compounds suitable for developing functional food products (Handayani et al., 2015; Jariyah et al., 2015, 2018; Widjanarko et al., 2014). Various kinds of existing mangrove fruits can be used as flours to become the essential ingredients of multiple foods, including crispy sticks, crackers, cakes, and others (Subandriyo et al., 2015). Mangrove fruits such as *Bruguiera gymnorrhiza* and *Avicennia marina* have high carbohydrate contents (Amalia et al., 2016; Sumartini et al., 2021). Carbohydrates are the primary source of calories for humans. About 60-80% of calories are obtained from carbohydrates (Hwanhlem et al., 2014).

Mangrove fruit also can reduce blood glucose levels after being processed into flour containing 7.50% soluble dietary fiber and 38.60% insoluble dietary fiber. Thus, mangrove fruit flour is a potent candidate for functional food, especially antidiabetic (Hardoko et al., 2015). Besides the beneficial properties, mangrove fruits also contain several antinutritional factors such as tannins, saponins, and hydrogen cyanide (Dewi et al., 2017). Hence, this study is of importance to demonstrate the potential of mangrove fruits as food sources, concomitantly exploring their benefits and reducing the antinutritional compounds.

## 2. Materials and Methods

Mangrove fruits, species *Avicennia marina*, *Bruguiera gymnorrhiza*, *Sonneratia* caseolaris, and *Rhizophora mucronata*, were obtained from Pantai Indah Kapuk (PIK), North Jakarta, Indonesia. The PIK was in the northern part of the Jakarta city (S: 06°07′28″ and E: 106°45′15″), Jakarta, Indonesia (Figure 1).

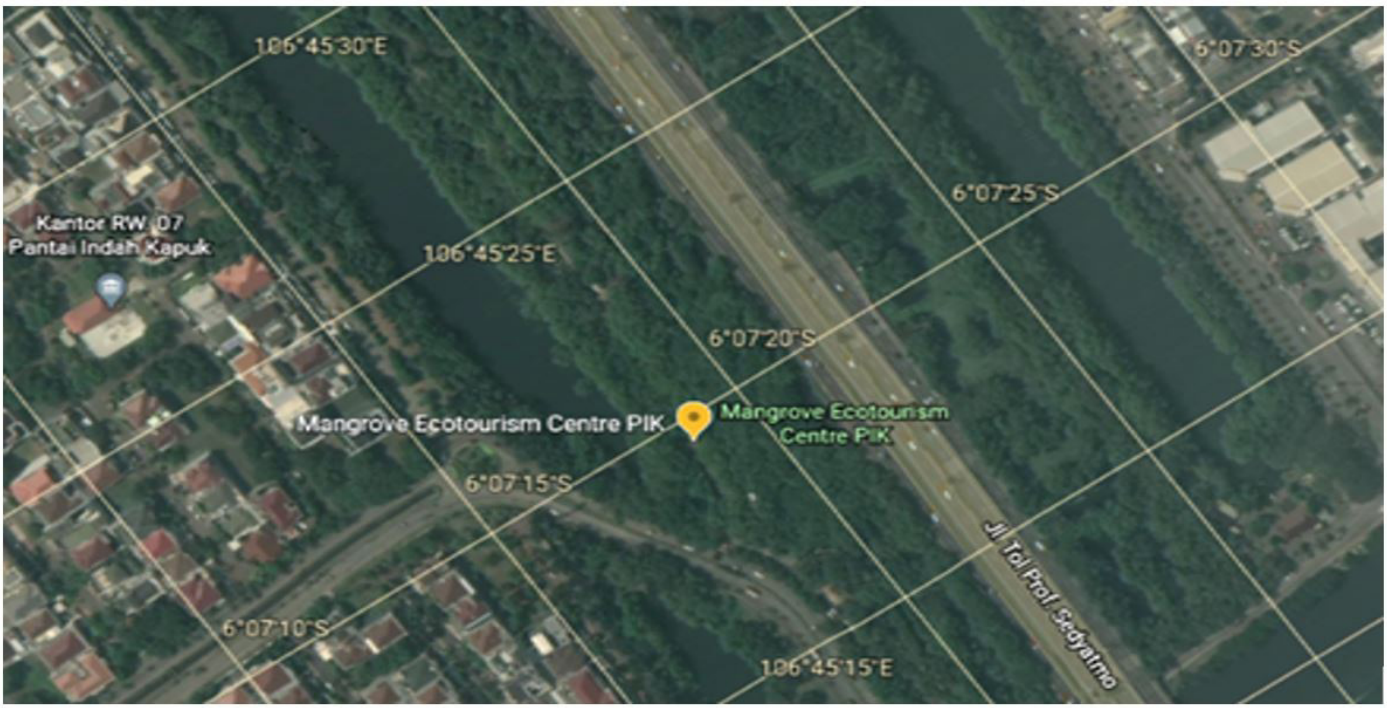


**Figure 1.** Location of sampling sites of mangrove fruits (Mangrove Ecotourism Centre, PIK, North Jakarta Sites, Indonesia).

### 2.1. Sample preparations of mangrove fruits

Mangrove fruits that had excellent physical conditions and were not damaged were collected. The fruits were labelled for each species, kept in a box containing dry ice, and brought back to the Food Engineering Laboratory located at IPB University. Only clean fruits which were free from damage were selected and immediately packed in sealed plastic and then stored in the freezer at -20 ºC before further processing.

### 2.2. Flour preparation

Samples of mangrove fruits were prepared by peeling, soaking, blanching, slicing/reducing the size, drying, and shading. The drying process was carried out using a rack-type cabinet dryer (ND4-60 SP tray dryer, Teraba Seisakusho, Japan) at a temperature of 60-70 °C for 4-6 hours. Drying medicinal plants by oven at 70 °C for 5 hours warrants further research based on the level of phytochemicals that remain in the treated samples and the relatively low cost involved (Mahanom et al., 1999), and drying at 70 oC or below can provide reasonable drying time (Djaeni & Sari, 2015). Cabinet Dryer was suitable for food ingredients in the form of fruit pieces. The potential for food degradation due to high temperatures can be minimized (A'yuni et al., 2022). The dried fruits were comminuted using a Y2112M-2 laboratory grinding machine (Bartex Electric Motor, Japan). The flour was sieved using a 100 Tyler Mesh and stored prior to analyses.

### 2.2. Starch preparation

Starch was prepared by following Nurindra (2015) with modification. Mangrove flour was added with 0.25 % (w/v) sodium metabisulfite and water (1:4) (w/v). Mangrove flour is filtered using gauze until the dregs and the filtrate are separated. Milk starch obtained was deposited for 6 hours at room temperature. The water was discarded, and the starch was dried at 50 °C for 12 hours. The dried starch was mashed and sieved (150 mesh) to obtain a starch powder.

### 2.3. Proximate analysis of mangrove fruit and flour

Moisture content was determined using an ED series 53 hot air-drying oven (Binder, USA ) at 100 °C for 18 hours (AACC, 2013 with modification). Determination of flour ash content by ignition of flour for 2 hours at a temperature of 600 °C (AACC, 2001 with modification). This was followed by the determination of crude fiber and fat (solvent extraction)(AOAC, 2012 with modification). The Kjeldahl method determined crude protein content with digestion and sample distillation. The distillate was titrated with 0.1 N hydrochloric acid solution until the solution changed from bluish-green to pink (AACC 46-13.01 with modifications) (AACC, 2010). The calculation of carbohydrates followed the "by-difference" method (FAO, 2003).

### 2.4. Physicochemical characterization of mangrove flour

#### 2.4.1 Cyanide acid analysis

The study of cyanide acid from the flour followed the Lian and Hamir method (Marlina, 2000), which accelerated the release of cyanide glucoside compounds, using 3 N hydrochloric acids with incubation at room temperature for 3 hours. Measurement of the absorbance of each eluate was done at a wavelength of 490 nm using a UVmini-1240 Spectrophotometer (Shimadzu, Japan).

#### 2.4.2. Starch analysis

A 2.5 g sample was transferred to a 10-mL graduated flask and added with 75 mL hydrochloric acid stepwise. The hydrolysis was performed in the autoclave with a heating pressure of 103.42 kN/m² for 10 min. The hydrolysed starch was cooled immediately down to a temperature of 20 oC. Carrez solution I (5 mL) and II (5 mL) were added, and subsequently, the mixture was diluted with 100 mL of distilled water. The solution was transferred to a 200 mm polarimeter tube and the optical rotation was measured by means of a polarimeter or saccharimeter (Sarmin NF et al., 2018).

#### 2.4.3. Amylose and amylopectin analysis

A 40 mg mangrove flour was put in a tube, and then 1.0 mL 95 % ethanol and 9 mL 1 N NaOH were added. The next step was to heat the solution in a water bath at 100 oC for 10 min and cool it down for 1 h. The solution was diluted with distilled water to 100 mL. About 5 ml of the solution was placed into a 100 mL volumetric flask containing 60 mL of distilled water,


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then added with 1.0 mL of 1 N acetic acid and 2.0 mL of 2 % iodine solution, respectively. The final volume was tared to 100 mL using distilled water. The solution was shaken and allowed to stand for 20 min, and the absorbance was monitored using a spectrophotometer at a wavelength of 625 nm (Apriyantono et al., 1989). Amylopectin content was obtained as follows: Amylopectin content = Starch content - Amylose content.

### 2.4.4. Colour measurement

The colour measurements were performed using CR 300 Chroma Meter (Konica Minolta, Japan) according to CIE 1976-Lab Color Space. A standard white plate was used to standardize the instrument. The colour of flour in the CIE-Lab parameters was L (white/black), a (red/green), and b (yellow/blue). Results were presented as the mean value of five measurements ± standard deviation (SD). The whiteness index (WI) was calculated based on the following equation $WI = 100 - [(100 - L)^2 + a^2 + b^2]^{½}$ (Lin et al., 2009).

### 2.4.5. Analysis of Birefringence, size, and shape of starch granule

The starch was suspended in distilled water at a concentration of 1 % (w/v). The suspension was dropped onto the slide using a pipette. The specimen was observed under a C-35AD-4 polarizing microscope (Olympus, Japan) with the help of a camera (ToupTek, China) connected to a computer (Chen et al., 2015). The observation of the birefringence structure, size, and shape of the starch granules was done at a magnification of 400X. Measurement diameters were analyzed using a histogram to determine granule size distribution (Sahin & Sumnu, 2006).

### 2.4.6. Pasting properties of mangrove flour

The pasting properties of the samples (flour and starch) were determined using a Tec-Master instrument, Rapid Visco Analyzer (RVA) (Newport Scientific, Australia) according to AACC 76.21.01 (1999).

### 2.4.7 Statistical analysis

One-way analysis of variance (ANOVA) and multiple comparisons (Duncan's post hoc test) were used to evaluate the significant difference in the data at $p < 0.05$. Pearson's correlation coefficient (r) was used to analyze the linear correlation between specific parameters. A two-way t-test (2-tailed) was used to test the statistical significance of the correlation coefficient ($p<0.05$). All the statistical analyses were done using SPSS, Version 22 (IBM, USA) (Allen et al., 2014).

## 3. Results

Mangrove fruits had different sizes and shapes (Figure 2). Table 1 shows that *Avicennia* sp. had the smallest size of the others, with a length of 1.50-3.80 cm, a diameter of 0.80-1.60 cm, and a weight of 0.30-2.37 gram. The *Sonneratia* sp. had a length of 1.00-5.40


**Figure 2.** Mangrove fruit of (a) *Avicennia* sp. (b) *Bruguiera* sp. (c) *Rhizophora* sp. (d) *Sonneratia* sp.

**Table 1**. Size of mangrove fruits *)

| Dimensional | *Avicennia* sp. | *Bruguiera* sp. | *Rhizophora* sp. | *Sonneratia* sp. |
|---|---|---|---|---|
| **Length (cm)** | 1.50- | 16.50-24.40±3.85[b] | 36.70-58.80±6.14[c] | 1.00-5.40±1.20[a] |
| **Diameter (cm)** | 0.80- | 1.00-2.00±0.20[a] | 1.10-1.90±0.20[ab] | 1.60-6.00±1.32[c] |

cm, a diameter of 1.60-6.00 cm, and a weight of 15.00-50.00 grams. The *Bruguiera* sp. had a length of 16.50-24.40 cm, a diameter of 1.00-2.00 cm, and a weight of 16.00-50.00 grams. The biggest size observed was *Rhizophora* sp. with a length of 36.70-58.80 cm, a diameter of 1.10-1.90 cm, and a weight of 22.00-70.00 grams.

The proximate compositions of wet basis (wb) of Indonesian mangroves fruits were presented in Table 2. The moisture contents of the fruits varied significantly from 50.77 to 77.73 g/100 g (wb). The level of moisture contents found in this research is in the range previously reported by other researchers, especially for *Avicennia* sp. was 52.94 % (67.50 % by (Chrissanty 2012)), *Bruguiera* sp. was 57.48 % (62.92 % by (Jacoeb et al. 2013)), *Rhizophora* sp. was 50.77 % (52.38 % by (Mile et al. 2021)), and *Sonneratia* sp. was 77.73 % (77.10 % by (Jariyah et al. 2014)). The ash contents were in the range of 1.05 to 2.05 g/100 g (wb). This value is slightly higher than previously reported, where *Avicennia* sp. was 2.05 % (1.22 % by (Chrissanty, 2012)), *Bruguiera* sp. was 1.18 % (1.15 % by (Sudirman et al., 2014)), *Rhizophora* sp. was 1.05 % (0.98 % by (Podungge et al., 2015)), and *Sonneratia* sp. was 1.63 %. The crude protein contents of mangrove fruits were in the range of 1.51 to 5.02 g/100 g (wb). This value is closer than previously reported, where *Avicennia* sp. was 5.02 % (4.83 % by (Chrissanty, 2012)), *Bruguiera* sp. was 2.09 % (2.11 % by (Sudirman et al., 2014)), *Rhizophora* sp. was 1.51 % (1.75 % by (Podungge et al., 2015)), and *Sonneratia* sp. was 2.12 % (2.24 % by (Jariyah et al., 2014)). The crude lipid contents were between 0.21 to 1.18 g/100 g (wb). This value is slightly higher than previously reported, where *Avicennia* sp. was 0.34 % (0.24 % by (Chrissanty, 2012)), *Bruguiera* sp. was 0.32 % (0.79 % by (Jacoeb et al., 2013), *Rhizophora* sp. was 0.21 % (1.69 % by (Podungge et al., 2015)), and *Sonneratia* sp. was 1.18 % (0.86 % by (Jariyah et al., 2014)). The crude fiber content was between 14.76 to 20.58 g/100 g (wb), which is *Bruguiera* sp. was 14.76 % and higher than ported by Sarungallo et al. (2010) of 11.48 %. The carbohydrate contents were in the range of 17.34 to 46.46 g/100 g (wb). Our results on carbohydrate content were not much different from those reported by others, they were *Avicennia* sp. was 39.65 % (25.25 % by (Chrissanty, 2012)), *Bruguiera* sp. was 38.93 % (32.91 % by (Priyono et al., 2010)), *Rhizophora* sp. by 46.46 % (34.68 % by (Podungge et al., 2015)), and *Sonneratia* sp. was 17.34 % (15.95 % by (Jariyah et al., 2014)). The proximate compositions amongst the species, on a wet basis, were significantly different ($p<0.01$).

The proximate compositions of dry basis (db) of Indonesian mangroves fruits were presented in Table 2. The moisture contents of the fruits varied significantly from 105.13 to 349.43 g/100 g (db). The ash contents were in the range of 2.13 to 7.32 g/100 g (db).

Our result on ash content of *Sonneratia* sp. was 7.32 %, not much different from reported by (Manalu et al., 2013) (8.40 %). The crude protein content was in the range of 3.37 to 10.67 g/100 g (db). Our result on the crude protein content of *Sonneratia* sp. was 9.56 %, not much different from reported by (Manalu et al., 2013) (9.21 %). The crude lipid content was in the range of 0.43 to 5.29 g/100 g (db). Our result on the crude lipid content of *Sonneratia* sp. was 9.56 %, not much different from reported by (Manalu et al., 2013) (9.21 %). The crude fiber content was in the range of 34.39 to 72.35 g/100 g (db). The value of fiber content of *Sonneratia* sp. was 72.35 g/100 g. The total dietary fiber in mangrove fruits found in this research is in the range previously reported by other researchers, especially for *Sonneratia* sp. was about 63.70 %,


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**Table 2**. Proximate compositions of Indonesian mangroves fruits.

| Proximat (g/100 g) | *Avicennia* sp. | | *Bruguiera* sp. | | *Rhizophora* sp. | | *Sonneratia* sp. | |
|---|---|---|---|---|---|---|---|---|
| | wb | db | wb | db | wb | db | wb | db |
| Moisture | 52.94±0.07$^{ab}$ | 112.54±0.33$^{ab}$ | 57.48±0.33$^{b}$ | 135.51±1.78$^{b}$ | 50.77±0.73$^{a}$ | 105.13±2.51$^{a}$ | 77.73 ±0.44$^{c}$ | 349.43±8.79$^{c}$ |
| Ash | 2.05 ±0.01$^{c}$ | 4.36±0.03$^{b}$ | 1.18±0.39$^{a}$ | 2.78±1.30$^{a}$ | 1.05±0.06$^{a}$ | 2.13±0.16$^{a}$ | 1.63±0.03$^{b}$ | 7.32±0.19$^{c}$ |
| Protein | 5.02±0.01$^{c}$ | 10.67±0.01$^{c}$ | 2.09±0.02$^{b}$ | 4.92±0.04$^{b}$ | 1.51±0.05$^{b}$ | 3.37±0.10$^{a}$ | 2.12±0.02$^{a}$ | 9.56±0.09$^{C}$ |
| Lipid | 0.34±0.22$^{a}$ | 0.72±0.00$^{a}$ | 0.32±0.08$^{c}$ | 0.76±0.19$^{c}$ | 0.21±0.04$^{a}$ | 0.43±0.09$^{a}$ | 1.18±0.05$^{b}$ | 5.29±0.18$^{b}$ |
| Fiber | 20.58±0.12$^{b}$ | 43.73±0.02$^{b}$ | 14.76±0.34$^{a}$ | 34.71 ±0.81$^{a}$ | 16.93±0.26$^{a}$ | 34.39±0.53$^{a}$ | 16.11±1.30$^{c}$ | 72.35±0.65$^{c}$ |
| Carbohydrate | 39.65±0.08$^{bc}$ | 84.25±0.08$^{bc}$ | 38.93±0.05$^{bc}$ | 91.54±0.05$^{c}$ | 46.46±0.80$^{c}$ | 94.07±0.80$^{c}$ | 17.34±0.11$^{a}$ | 77.83±0.11$^{a}$ |

Note: Different letters in the row indicate significant differences in the proximate composition amongst mangrove fruits

which is distributed amongst soluble (9.8%) and insoluble components (53.9%) (Jariyah et al., 2014). In comparison, seaweeds have comparable fiber content (74.11%) (Ahmad et al., 2012). It is well recognised that dietary fiber is important in human wellness because, for example, it binds and/or encapsulates bile salts to reduce cholesterol (Brown et al., 1999). The carbohydrate content was between 77.83 to 94.07 g/100 g (db). Our result on ash content of *Sonneratia* sp. was 77.83 %, not much different from reported by (Manalu et al., 2013) (77.57 %). The proximate compositions amongst the species, on a dry basis, were significantly different ($p<0.01$).

The proximate analyses of flours were presented in Table 3. For wet basis (wb), the moisture contents of the flours varied significantly from 4.94 to 9.86 g/100 g (wb). The level of moisture contents found in this research is in the range previously reported by other researchers, especially for *Avicennia* sp. was 8.08 % (9.36 % by (Chrissanty, 2012)), *Bruguiera* sp. was 4.94 % (6.68% by (Patil & Chavan, 2013)), *Rhizophora* sp. was 7.69 % (8.34 % by (Chrissanty, 2012)), and *Sonneratia* sp. was 9.86 % (9.63 % by (Ardiansyah et al., 2020)). The ash contents were to between 2.27 to 6.65 g/100 g (wb). This value is slightly higher than previously reported, where *Avicennia* sp. was 3.89 % (2.36 % by (Chrissanty, 2012)), *Bruguiera* sp. was 3.24% (2.70 % by (Priyono et al., 2010)), *Rhizophora* sp. was 2.27 % (1.27 % by (Hardoko et al., 2015)), and *Sonneratia* sp. was 6.65 % (wb) (5.39 % by (Ardiansyah et al., 2020)). The crude protein contents were between 3.43 to 9.83 g/100 g (wb). This value is closer than previously reported, where *Avicennia* sp. was 9.83 % (12.25 % by (Permadi et al., 2012)), *Bruguiera* sp. was 6.03 % (5.59 % by (Sulistyawati & Kumalaningsih, 2012)), *Rhizophora* sp. was 3.43 % (3.50 % by (Hardoko et al., 2015)), and *Sonneratia* sp. was 7.33 % (wb) (8.34 % by (Ardiansyah et al., 2020)). The crude lipid contents were approximately 1.14 to 3.14 g/100 g (wb). This value is closer than previously reported, where *Avicennia* sp. flour was 1.14 % (0.81 % by (Permadi et al., 2012)), *Bruguiera* sp. was 1.93 % (1.79 % by (Sulistyawati & Kumalaningsih, 2012)), *Rhizophora* sp. was 1.40 % (0.86 % by (Purwaningsih et al., 2013)), and *Sonneratia* sp. was 3.14 % (4.70 % by (Jariyah et al., 2014)). The crude fiber contents were between 5.65 to 14.28 g/100 g (wb). The level of crude fiber contents found in this research is in the range previously reported by other researchers, especially for *Avicennia* sp. was 5.65 % (4.85 % by (Chrissanty, 2012)), *Bruguiera* sp. was 10.21 % (10.09 % by (Patil & Chavan, 2013)), *Rhizophora* sp. was 8.25 % (9.01% by (Yamamoto et al., 1983)), and *Sonneratia* sp. was 14.28 % (9.80 % soluble fiber by (Jariyah et al., 2014)). The carbohydrate contents were in the range of 73.02 to 85.21 g/100 g (wb). This value is closer than previously reported, where *Avicennia* sp. flour was 77.06 % (78.13 % by (Permadi et al., 2012)), *Bruguiera* sp. was 83.36 % (82.09 % by (Sulistyawati & Kumalaningsih, 2012)), *Rhizophora* sp. was 85.21 % (87.68 % by (Chrissanty, 2012)), and *Sonneratia* sp. was 73.02 % (74.12 % by (Ardiansyah et al., 2020). The proximate compositions amongst the species, on a wet basis, were significantly different ($p<0.01$).

The proximate compositions of dry basis (db) of In-

**Table 3.** Proximate compositions of Indonesian mangroves flours

| Proximate (g/100 g) | *Avicennia* sp. | | *Bruguiera* sp. | | *Rhizophora* sp. | | *Sonneratia* sp. | |
|---|---|---|---|---|---|---|---|---|
| | wb | db | wb | db | wb | db | wb | db |
| **Moisture** | 8.08±0.07$^{b}$ | 8.75±0.40$^{b}$ | 4.94±0.07$^{a}$ | 5.21±0.18$^{a}$ | 7.69± 0.09$^{b}$ | 8.33±0.13$^{b}$ | 9.86± 0.06$^{c}$ | 10.94±0.07$^{c}$ |
| **Ash** | 3.89±0.06$^{c}$ | 4.23±0.41$^{c}$ | 3.24±0.06$^{b}$ | 3.42±0.22$^{b}$ | 2.27±0.06$^{a}$ | 2.46±0.30$^{a}$ | 6.65±0.05$^{d}$ | 7.38±0.13$^{d}$ |
| **Protein** | 9.83±0.01$^{d}$ | 10.69±0.02$^{d}$ | 6.03±0.01$^{b}$ | 6.43±0.04$^{b}$ | 3.43±0.05$^{a}$ | 3.72±0.10$^{a}$ | 7.33±0.03$^{c}$ | 8.13±0.15$^{c}$ |
| **Lipid** | 1.14±0.88$^{a}$ | 1.24±0.94$^{a}$ | 1.93±0.54$^{c}$ | 2.03±0.56$^{c}$ | 1.40±0.09$^{a}$ | 1.52±0.44$^{a}$ | 3.14±0.74$^{b}$ | 15.84±0.83$^{b}$ |
| **Fiber** | 5.65±0.12$^{a}$ | 6.15±0.20$^{a}$ | 10.21±0.17$^{b}$ | 10.72±0.40$^{b}$ | 8.25±0.26$^{b}$ | 8.94±1.64$^{b}$ | 14.28±0.15$^{c}$ | 15.84±0.52$^{c}$ |
| **Carbohydrate** | 77.06±0.08$^{a}$ | 83.83±0.08$^{a}$ | 83.86±0.05$^{c}$ | 88.88±0.05$^{c}$ | 85.21±0.80$^{d}$ | 92.31±0.80$^{d}$ | 73.02±0.11$^{b}$ | 81.01±0.11$^{a}$ |

Note: Different letters in the row indicate significant differences in the proximate composition amongst mangrove flours

donesian mangroves flours were presented in Table 3. The moisture contents of the flours varied significantly from 5.21 to 10.94 g/100 g (db). The ash contents were 2.46 to 7.38 g/100 g (db). The crude protein contents were in the range of 3.43 to 10.69 g/100 g (db). The crude lipid contents were in the range of 1.24 to 3.48 g/100 g (db). The crude fiber contents were in the range of 6.15 to 15.84 g/100 g (db). The carbohydrate contents were in the range of 81.01 to 92.31 g/100 g (db). The proximate compositions amongst the species, on a dry basis, were significantly different ($p < 0.01$).

Table 4 shows the cyanide acid contents of the mangrove fruits and their respective flours. The cyanide acid content of mangrove fruit of *Avicennia* sp. was 130 ppm, *Rhizophora* sp. was 120 ppm, *Bruguiera* sp. was 60 ppm, and the highest content is *Sonneratia* sp. was 140 ppm. The safe limit for cyanide acid in food is 50 ppm (Baskin & Brewer, 2006). Generally, mangrove fruits contain HCN of more than 50 ppm, so it is not safe for direct consumption. The cyanide acid content was reduced after flour preparation, ranging from less than 0.25 ppm to 79.65 ppm. The cyanide contents for *Avicennia* sp. and *Sonneratia* sp., flours were detected (limit of detection (LoD) was 0.25 ppm), while cyanide contents for *Bruguiera* sp. and *Rhizophora* sp. were 79.65 ppm and 21.19 ppm, respectively.

Figure 4 shows the results of the colour measurements of mangrove flour as recorded in terms of the L*, a*, b*, and whiteness index (WI). Positive values obtained for coordinates a* and b* were significantly different ($p < 0.05$) among samples. As seen in Table 5, a* values ranged from 2.59 ± 0.01 for *Avicennia* sp. to 14.81 ± 0.01 for *Sonneratia* sp. The b* values ranged from 12.07 ± 0.01 for *Avicennia* sp. to 26.30 ± 0.01 for *Bruguiera* sp. As seen in Table 5, L* values ranged from 43.27 to 64.79. The a* values ranged from 2.59 to 14.81, and b* values ranged from 12.07 to 26.30. L*, a*, and b* of *Avicennia* sp. are the lowest. The L* and b* values of *Bruguiera* sp are the highest of than others. The whiteness index (WI) values ranged from 40.13 ± 0.01 for *Rhizophora* sp. to 55.74 ± 0.00 for *Bruguiera* sp. The L*, a*, b*, and WI values were significantly different ($p<0.01$).

Table 6 shows the values of amylose, amylopectin, and starch compound from four mangrove starch. *Avicennia* sp. flour contains 19.84 % starch, with an amylose content of 2.46 % and amylopectin of 17.27 % of starch. *Bruguiera* sp. flour contains 27.59 % starch, 3.98 % amylose, and 25.19 % amylopectin from starch. *Rhizophora* sp. flour contains 25.48 % starch, 0.61 % amylose and 21.72 % amylopectin from starch. *Sonneratia* sp. flour contains 22.17 % starch, 0.61% amylose and 21.71% amylopectin from starch.

Among the mangrove fruit flours shown in Table 6, the highest starch, amylose, and amylopectin content were recorded from *Bruguiera* sp. (27.59 % for starch; 3.98 % amylose and 25.19 % amylopectin of the starch fraction). The lowest starch and amylopectin content came from *Sonneratia* sp. (0.61 % of the starch fraction). The highest starch content was found in *Bruguiera* sp. (27.59 %) and the least starch con-


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**Table 4.** The values of cyanide acid in flour

| Mangrove | Cyanide acid from mangrove fruits (db)(g/100 g) | Cyanide acid from mangrove flour (db) (g/100 g) |
|---|---|---|
| *Avicennia* **sp.** | 130 ±0.10[a] | < 0.25 [a] |
| *Bruguiera* **sp.** | 120 ±0.10[c] | 79.65 ±0.14[c] |
| *Rhizophora* **sp.** | 60 ±0.10[d] | 21.19 ±0.02[b] |
| *Sonneratia* **sp.** | 140 ±0.10[b] | < 0.25 [a] |


**Figure 3.** Mangrove flour of (a) *Avicennia* sp. (b) *Bruguiera* sp. (c) *Rhizophora* sp. (d) *Sonneratia* sp.

**Table 5.** The whiteness index (WI) and parameters L*, a*, b* for the colour of mangrove fruit flour.

| Flour mangrove | L* | a* | b* | WI |
|---|---|---|---|---|
| *Avicennia* **sp.** | 43.27 ±0.06[a] | 2.59± 0.01[a] | 12.07 ±0.01[a] | 41.94 ±0.06[b] |
| *Bruguiera* **sp.** | 64.79 ±0.06[d] | 5.25 ±0.01[b] | 26.30 ±0.01[d] | 55.74 ±0.00[d] |
| *Rhizophora* **sp.** | 46.76 ±0.01[b] | 6.62 ±0.01[d] | 23.02 ±0.02[c] | 40.13 ±0.01[a] |
| *Sonneratia* **sp.** | 49.53 ±0.01[c] | 14.81 ±0.01[c] | 20.97 ±0.01[b] | 44.94 ±0.01[c] |

Note: Different letters in the column indicate significant differences in colour attribute composition amongst mangrove fruits

**Table 6**. The values of starch compound of flours from mangrove fruits

| Mangrove Species | Amylose (g/100 g basis flour) | Amylopectin (g/100 g basis flour) | Starch (%) |
|---|---|---|---|
| *Avicennia* **sp.** | 2.46 ±0.33[b] | 17.27 ±0.08[a] | 19.84±0.14[a] |
| *Bruguiera* **sp.** | 3.98 ±0.11[c] | 25.19 ±0.29[d] | 27.59±2.24[c] |
| *Rhizophora* **sp.** | 2.89 ±0.77[b] | 22.57 ±0.19[c] | 25.48±0.02[bc] |
| *Sonneratia* **sp.** | 0.61 ±0.03[a] | 21.72 ±0.01[b] | 22.17±0.23[ab] |

*Note: Different letters in the column indicate significant differences in colour attribute composition amongst mangrove fruits*

tent was found in *Avicennia* sp. (19.84 %). The highest amylopectin content was recorded from *Bruguiera* sp. (of the starch fraction). Higher amylose content lowers the gelatinization profile of starch. The higher the amylose content, the more difficult it will be to form a gel. Because the amorphous structure formed will increase the gelatinization temperature so that gelatinization will run slowly.

Birefringence structure, size, and shape of granule analysis of starch of flour of *Avicennia* sp. could be seen in Figure 4, *Bruguiera* sp. could be seen in Figure 5, *Rhizophora* sp. could be seen in Figure 6, and *Sonneratia* sp. could be seen in Figure 7.

The values of pasting temperature analysis of flour from mangrove fruits could be seen in Table 7. The time for *Bruguiera* sp. flour to fully gelatinize was the fastest compared to others. It is indicated by the peak time; it was 7.5 minutes for flour, and 8.3 minutes for starch. Meanwhile, to complete gelatinization, *Rhizophora* sp. took 11.6 minutes. *Avicennia* sp. and *Sonneratia* sp. flour took the same time to gelatinize fully, which was 13 minutes for flour. For starch, *Sonneratia* sp. took 8.5 minutes, *Avicennia* sp. and *Rhizophora* sp. took the same time, which was 10 minutes. flour gelatinization of *Avicennia* sp., *Sonneratia* sp., *Rhizophora* sp., and *Bruguiera* sp. was 72 °C, 78 °C, 80 °C, and 82.5 °C. Meanwhile, the starch gelatinization of *Rhizophora* sp., *Avicennia* sp., *Bruguiera* sp., and *Sonneratia* sp. was 50 °C, 50.2 °C, 51.25 °C, and 56.25 °C, respectively.

## 4. Discussion

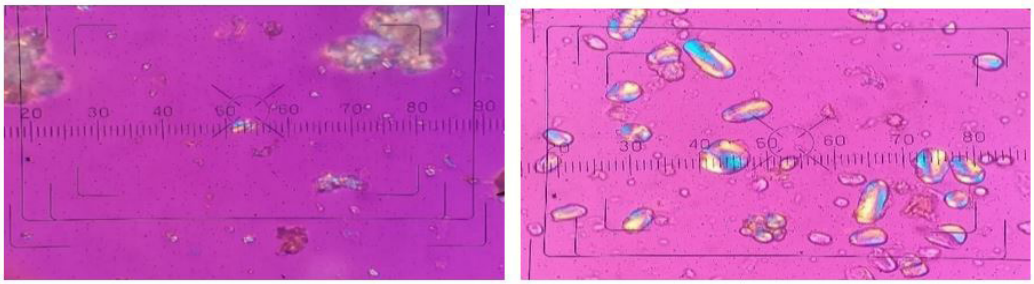


**Figure 4.** Birefringence structure, size, and shape of granule analysis of *Avicennia* sp's flour (a) and starch (b)

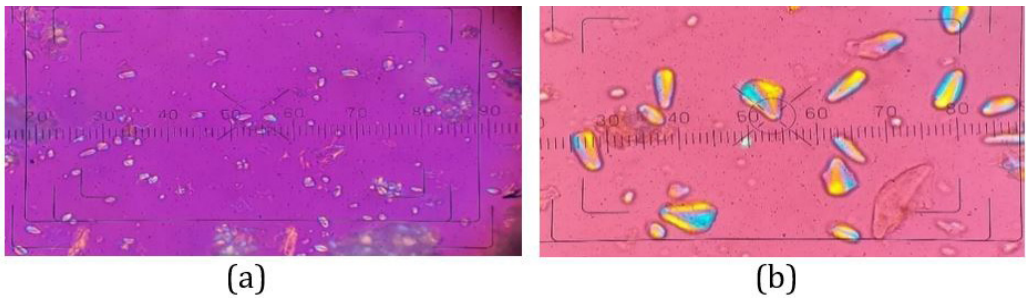


**Figure 5.** Birefringence structure, size, and shape of granule analysis of *Bruguiera* sp's flour (a) and starch (b)

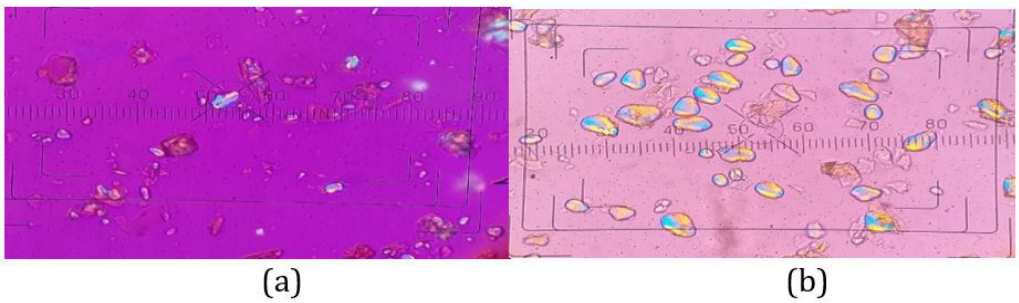


**Figure 6.** Birefringence structure, size, and shape of granule analysis of *Rhizophora* sp's flour (a) and starch (b)


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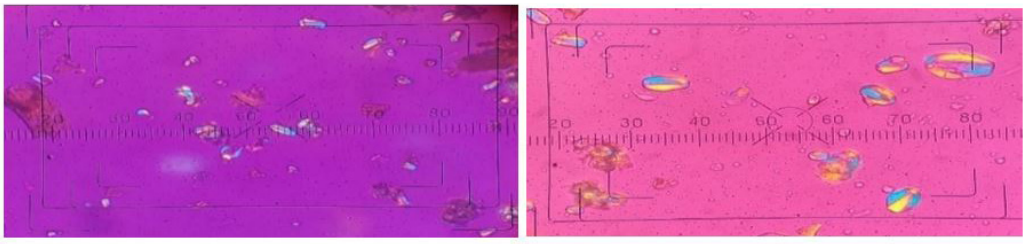


**Figure 7.** Birefringence structure, size, and shape of granule analysis of *Sonneratia* sp's flour (a) and starch (b)

**Table 7**. The values of pasting properties analysis of flour and starch

| Mangrove Species | Flour | | | | | | Starch | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pasting temperature (°C) | Viscosity (cP) | | | | | Pasting temperature (°C) | Viscosity (cP) | | | | |
| | | Peak | Trough | Break down | Final | Set back | | Peak | Trough | Break down | Final | Set back |
| *Avicennia* **sp.** | 72 | 101 | 102 | 0 | 188 | 86 | 50.2 | 289 | 98 | 191 | 208 | 110 |
| *Bruguiera* **sp.** | 82.5 | 820 | 471 | 349 | 702 | 231 | 51.25 | 502 | 142 | 360 | 275 | 133 |
| *Rhizophora* **sp.** | 80 | 49 | 46 | 3 | 67 | 21 | 50 | 50 | 43 | 7 | 59 | 16 |
| *Sonneratia* **sp.** | 78 | 161 | 161 | 0 | 406 | 245 | 56.25 | 156 | 74 | 82 | 98 | 24 |

**Table 8** Initial temperature of gelatinization of several types of natural starch-containing low amylose (waxy)

| Natural starch | Initial temperature of gelatinization (°C) | Reference |
|---|---|---|
| Rice | 58,6 | Waterschoot *et al.* (2014) |
| Rice | 59,6 | Vamadevan *et al.* (2013) |
| Barley | 57,9 | Schirmer *et al.* (2013) |
| Corn | 66,6 | Schirmer *et al.* (2013) |
| Potato | 63,6 | Schirmer *et al.* (2013) |
| Mangrove fruits of *Avicennia* sp. | 50,2 | Results of analysis |
| Mangrove fruits of *Bruguiera* sp. | 51,25 | Results of analysis |
| Mangrove fruits of *Rhizophora* sp. | 50 | Results of analysis |


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### 4.1. Proximate composition of mangrove fruits

Table 2 and Table 3 show the proximate compositions of mangrove fruits and flours.

The highest water content for the fruit of mangroves is owned by the fruit of *Sonneratia* sp. The high moisture content accounts for its short shelf life as it deteriorates quickly after harvest if preservative measures are not employed. This high-water content promotes susceptibility to microbial growth and enzyme activities. This is what causes the fruits of *Sonneratia* sp. damage faster than other fruits. However, the moisture content of mangroves depends on their harvesting time, maturation period, and environmental conditions such as humidity and temperature in the growing period and storage conditions (Crisan & Sands, 1978). The production of mangrove flours aims to increase shelf life without reducing nutritional values. The drying process is one of the crucial stages because it determines the quality and durability of the further processed product from the flours (Erni et al., 2018).

The significant difference in proximate contents for fruits and mangroves flours was caused by the heat absorbed by the material during the drying process. It was affecting the dryness level of the flours. The proximate test of flours changed due to the temperature treatment and drying time. The drying process was carried out at a temperature of 60-70 °C for 4-6 h. The drying process at this temperature was chosen besides reducing the water content of a material, it can also significantly reduce cyanide levels (Jayanegara et al., 2019; Nelfiyanti, 2015; Sulistyawati & Kumalaningsih, 2012) and tannin levels (Nelfiyanti, 2015; Sulistyawati & Kumalaningsih, 2012) in mangroves flours. Moreover, low-temperature drying was chosen to minimize the degradation of phytochemicals (Mahanom et al., 1999, Djaeni & Sari, 2015, Mardiah et al. 2015, park et al. 2021, and A'yuni et al., 2022), amino acids (Park et al., 2021), and giving the best condition of physiological properties of the dry product (Pradana et al., 2019).

In the proximate composition, the mangrove flours produced in this study met the Indonesian commercial flours standard (SNI 7622 2011), with less than 13 % moisture content and at least 7 % for protein content. Overall, total values of both crude protein and crude lipid, when comparing fruits to flours, rose significantly. An increase in protein content after flouring was also reported by Riansyah et al. (2013). This is because the longer time and the higher the temperature used in the drying process causes an increase in lipid and protein content, which is inversely proportional to the value of the water content, which gradually decreases as the temperature and time used during the drying process increase. According to Yuniarti's research (2008), the length of time and high temperature employed in the drying process causes the lipid content in the material to increase and the moisture content to decrease.

### 4.2. Cyanide acid compound in flour

The safe limit for cyanide acid in food is 50 ppm (Baskin & Brewer, 2006). Based on these results, generally, mangroves fruits contain HCN of more than 50 ppm, so it is not safe for direct consumption. Processing steps that can reduce cyanide levels effectively in fruit are required, including drying, boiling, soaking, peeling, starch extraction, and fermentation (silage) (Jayanegara et al., 2019; Muryati & Nelfiyanti, 2015). Some of these food processing techniques can reduce anti-nutritional compounds, improve protein digestion, and increase plant biological value. Furthermore, the process of flours pre-treatment which includes washing, peeling, chopping, drying, and flouring can reduce the cyanide content. The exfoliation process can reduce cyanide by about 50 %. Based on the results, the decrease in HCN content due to the flour pre-treatment process can reduce 50 % to 100 % cyanide levels. This is in accordance with other researchers that a decrease in cyanide levels up to 80-85 % can be done by drying using the sun for 24 hours (Jayanegara et al., 2019; Rukmana, 1997), as well as drying using an oven at a temperature of 60 $^{0}$C for 24 hours (Jayanegara et al., 2019; Sulistyawati & Kumalaningsih, 2012).

We can actually see that after flour preparation, the cyanide acid content decreases. Especially for the flours prepared from *Avicennia* sp., *Bruguiera* sp., and *Sonneratia* sp., they can be used as part of the ingredients since the cyanide levels are acceptable, below 50 ppm. According to Codex Alimentarius Commission (CAC), General Standard For Contaminants And Toxins In Food And Feed CXS 193-1995, Acute Reference


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Dose (ARfD) of cyanide of 0.09 mg/kg body weight. This cyanide-equivalent ARfD applies to foods containing cyanogenic glycosides as the main source of cyanide. Additionally, the Provisional Maximum Tolerable Daily Intake (PMTDI) of cyanide is PMTDI of 0.02 mg/kg body weight. If we make a calculation of PMTDI with a person having 60 kg body weight, then per day, as much as 1.2 mg cyanide is acceptable to be consumed. If we considered the highest cyanide content of 79.65 ppm (Table 4, mangrove *Bruguiera* sp.), this number is not acceptable. Hereby, such processing is needed to reduce this value to an acceptable level. Further pretreatment is needed in addition to heating to reduce or eliminate the HCN content in fruit, including soaking (FAO 1990), withering (Hang & Preston 2005), boiling, steaming, roasting, frying, drying, fermenting, and steam distillation (Montagnac et al. 2009).

### 4.3. Colour Analysis

The positive values for a* and b* coordinates of four mangrove flours indicated that samples had varying red and yellow pigmentation concentrations in their flour. The figure showed that L*, a*, and b* values of *Avicennia* sp. were the lowest than others. It is indicated that *Avicennia* sp. flour is the darkest. *Bruguiera* sp. flour is significantly higher for L* value b* value, which indicated that *Bruguiera* sp. flour is lighter than others.

When compared to other flours, *Bruguiera* sp. flour had considerably higher L* values and Whiteness index. Chroma rose as pigment concentration increased and dropped as the sample became darker. Food samples with identical hue angles and chroma only are distinguished by their L* values (Wrolstad & Smith, 2017).

### 4.4. Starch content

Starch was the source of carbohydrates derived from plants, and had important value for the food industry. It was often used as a thickener, gelling agent, bulking agent, and water retention. The application of starch in food products is usually determined by its properties of gelatinization, pasting, solubility, swelling, and digestibility. Studies on the thermal properties of starch are needed to determine the structure of the starch and how to process and use the starch in its application to foodstuffs (Li et al., 2014). The physical properties of starch included starch paste characteristics, thermal properties, starch granule size, starch granule shape, birefringence structure, crystal type, and degree of starch crystallization. Starch could undergo gelatinization in the presence of water and heated at high temperatures, resulting in the breakdown of starch granules, loss of birefringence structure, and crystallinity. The values of the starch compound could be seen in Table 6.

Among the mangrove fruit flours that are shown in table 6, the highest amylose content was recorded derived from *Bruguiera* sp. (4.21 g/100 g basis flour) and the least from *Sonneratia* sp. (0.61 g/100 g basis flour).

The highest recorded total starch content was recorded with *Bruguiera* sp. (29.17 g/100 g) and the least with *Avicennia* sp. (19.73 g/100 g). The highest amylopectin content was recorded derived from *Bruguiera* sp. (24.96 g/100 g). Higher amylose content lowers the gelatinization profile of starch. Higher the amylose content so that the gel formation would be difficult. Because the amorphous structure formed would increase the gelatinization temperature so gelatinization would walk slowly.

### 4.5. Birefringence structure, size, and shape of starch granule

The birefringence structure was the property of starch granules that could reflect polarized light to form blue and yellow-coloured fields when viewed under a polarizing microscope (Richana & Sunarti, 2004). Starch had birefringence properties defined as the properties of intact starch granules that could form two colours (blue and yellow) crossing on the surface when passed on to polarized light due to differences in the refractive index in the starch granules (Xie et al., 2005). The refractive index is influenced by the molecular structure of amylose in starch (Richana & Sunarti, 2004). The helical form of amylose could absorb some of the light passing through the starch granules. The test was carried out on eight samples, four for flour and four for starch of *Avicennia* sp., *Bruguiera* sp., *Rhizophora* sp., and *Sonneratia* sp. According to (Cready RM, 1970), when water penetrates back and forth into the granules at a temperature of 60-85 oC, the granules will expand rapidly and lose their birefringence

properties. When the starch is partially gelatinized, starch birefringence is still visible in small amounts. This is because it still contains intact starch granules. However, when the starch is completely gelatinized, the birefringence properties will be lost (Anwar et al., 2006). According to Jane & Chen (1992), differences in granule size, amylose content, and amylopectin branching chain length would result in differences in paste properties and gelatinization temperature. The granule analysis of starch of mangrove flours could be seen in figures 3, 4, 5, and 6.

### 4.6. Pasting properties

Based on Table 7 analysis of pasting properties of flour and starch from four mangrove species, it was found that the flour of *Bruguiera* sp. has the fastest time to fully gelatinize compared to others. This is indicated by the peak time, which is 7.5 minutes for flour and 8.3 minutes for starch. To achieve peak viscosity before the starch granules break, *Rhizophora* sp flour takes 11.6 minutes. *Avicennia* sp. flour and *Sonneratia* sp. take the same time to fully gelatinize, which is 13 minutes. For starch, *Sonneratia* sp. takes 8.5 minutes, and again *Avicennia* sp. and *Rhizophora* sp. take the same time, which is 10 minutes. The initial temperature of gelatinization is the temperature at which the starch granules begin to absorb water or can be seen as the viscosity increases. Based on these results, it can be said that the higher the temperature causes the starch granules to be more resistant to heat, thus requiring a higher temperature to start gelatinization. Starch gelatinization temperature indicates the temperature at which natural starch in semi-crystalline form changes to amorphous. The higher the gelatinization temperature, the higher the stability of the starch crystals. Microscopic changes in starch granules during cooking take place quickly and go through 3 stages. The first stage in cold water will occur in water absorption, which is reversible. The second stage occurs at a temperature of about 60 oC when the starch granules begin to expand and absorb large amounts of water to become irreversible. Gelatinization of *Avicennia* sp., *Sonneratia* sp., *Rhizophora* sp., and *Bruguiera* sp. flours are 72, 78, 80, and 82.5, respectively. While the starch gelatinization of *Rhizophora* sp., *Avicennia* sp., *Bruguiera* sp., and *Sonneratia* sp. are 50 oC, 50.2 oC, 51.25 oC, and 56.25 oC, respectively. According to Muhandri (2007), particle size affects the initial and maximum gelatinization temperatures, as well as lowers the maximum viscosity. Based on Table 7, it is found that the large flour particle size has a high gelatinization temperature and a low maximum viscosity.

For starch which has a smaller particle size than flour, it has a low gelatinization temperature with a high maximum viscosity value. This is because the larger particle size has not gelatinized the entire particle size so that the maximum viscosity has not been reached (Muhandri 2007). The composition of amylose and amylopectin can affect the gelatinization temperature of natural starch. According to Rasyda (2021), natural starch with low amylose content (waxy starch) has a lower initial gelatinization temperature than natural starch with medium amylose content. Research results from Hong et al. (2011) showed that waxy rice starch had a lower initial gelatinization temperature than waxy potato starch but higher than waxy corn starch. The amylopectin molecules in waxy starch remain in the granules during the swelling process of the starch granules, but if the heating process is extended, most of the granules will break and the starch suspension will turn into a solution of amylopectin macromolecules (Schirmer et al. 2013). Starch from mangrove flour has a low initial gelatinization temperature because it has low amylose and amylopectin composition. Table 8 shows the initial gelatinization temperature of several types of natural starch-containing low amylose (waxy).

*Avicennia* sp., *Bruguiera* sp., *Rhizophora* sp., and *Sonneratia* sp. have different gelatinization properties. The difference depends on the original structure and composition of amylose amylopectin. *Bruguiera* sp. had the highest peak viscosity and fastest gelatinization time. In food processing, *Bruguiera* sp.'s starch can be used to provide thickness in a short cooking time. *Rhizophora* sp. had the lowest peak viscosity, trough, final viscosity, and setback viscosity than others. In food processing, *Rhizophora* sp.'s starch was not suitable for use as a viscosity-forming material in cold products and semi-solid food products. It belonged to acid hydrolyzed starch with suitable gum-forming agents, candy, and liquid food formulations. *Avicennia* sp. and *Sonneratia* sp. had the longest time to reach peak viscosities, but these flours had the highest viscosity values in the setback phase. For application, they required a long cooking time to give the product


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a good consistency, but this consistency could withstand well at cold temperatures.

Different types of flour have different particle distributions. Particle size plays an important role in flour wetting and water absorption in flour. If the particle size is wider, the surface area will be smaller. This indicates that the water takes longer to be absorbed into the starch particles. On the other hand, smaller particle size will increase the hydration level of flour (Immaningsih, 2012; Mailhot et al., 1988).

Flour derived from *Avicennia* sp., *Bruguiera* sp., *Rhizophora* sp., and *Sonneratia* sp. have different gelatinization properties. The difference depends on the structure and composition of amylose amylopectin. Flour from mangrove fruit has advantages based on indicators of the proportion of amylose content, viscosity, and degree of gelatinization. *Bruguiera* sp. flour has the highest peak viscosity and fastest gelatinization time. *Rhizophora* sp. flour has the lowest peak, trough, final viscosity, and setback viscosity. Flour of *Avicennia* sp. and *Sonneratia* sp. have the longest time to reach peak viscosity, but this flour has the highest viscosity value in the setback phase.

Mangroves are starchy plants that have the potential to be used as industrial raw materials in the form of flour and starch-based products. In the food processing industry, *Bruguiera* sp. has viscosity properties in a short time *Rhizophora* sp. flour is not suitable for use as a thickening agent in cold products and semi-solid food products, but suitable for liquid food formulations that are easily hydrolyzed by acid. *Avicennia* sp. flour and *Sonneratia* sp. require a longer processing time to produce a good consistency in the product, but this consistency only holds up well at cold temperatures. Based on the different characteristics of the four mangrove species, it will produce many uses, namely as raw materials and auxiliary materials in various industries. Therefore, mangrove fruit and its derivative products in the form of flour, starch, starch hydrolyzate, and starch products are superior raw materials for both food and non-food products.

## 5. Conclusions

The proximate compositions either in fruits or flour, for *Avicennia* sp., *Bruguiera* sp., *Rhizophora* sp., and *Sonneratia* sp., were considerably different. The colours and degrees of whiteness were also significantly different for the four mangrove species. From the perspective of cyanide content, all the mangrove fruits do not feel safe for consumption.

In food processing, *Bruguiera* sp.'s starch can be used to provide thickness in a short cooking time. *Rhizophora* sp.'s starch was not suitable for use as a viscosity-forming material in cold products and semi-solid food products. It belonged to acid hydrolyzed starch with suitable gum-forming agents, candy, and liquid food formulations. *Avicennia* sp. and *Sonneratia* sp. required a long cooking time to give the product a good consistency, but this consistency could withstand well at cold temperatures.

## Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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# Proximate biochemical parameters and antioxidant capacity of eight loquat genotypes (*Eriobotrya Japonica Lindl.*) from Zegzel Valley of Morocco

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*Eriobotrya japonica Lindl;* Biochemical; Antioxidant Capacity; Sugar; Carotenoids; Tannin; Vitamin.

The loquat fruit is a very precious product due to its economic and health benefits. For this reason, the evaluation of their biochemical profile and antioxidant activity has become required. Indeed, healthy fruits from eight loquat genotypes belonging to four regions of the Zegzel Valley are analysed. As a result, Moroccan loquat fruit showed a great pomological and biochemical richness. From the eight genotypes, the 'Z1' produced the biggest fruits (54.55 g) while 'TA6' had the highest sugar content (54.85 mg. $g^{-1}$). The genotypes of 'TA5', 'TA6', and 'TA7' showed the highest levels of fructose (31.64 mg. $g^{-1}$), sucrose (21.09 mg. $g^{-1}$), and glucose (10.34 mg. $g^{-1}$) respectively. In addition, the 'T2' showed the highest content of flavonoids (59.58 µg RE. $g^{-1}$), vitamin C (14.19 mg AAE/100g), and organic acids which are malic acid (193.75 mg/100 g), quinic acid (107.25) and succinic acid (12.6 mg/100 g). The total phenolics are abundant in 'TZN1' (186.0.5 µg GAEg-1), while the greatest carotenoid content was revealed in 'TA7' (90.65µg ß-carotene $g^{-1}$). The proteins and tannins content, seem to be similar in all genotypes (0.98 g/100g, 2.94-2.61 µg. $g^{-1}$ respectively). Regarding the DPPH, ABTS, and FRAP tests, the 'Z7' recorded the highest antioxidant capacity compared to the other genotypes. Overall, the quantity of major bioactive compounds and antioxidant capacity varied considerably among the eight genotypes. According to the results obtained in this study, the eight loquat genotypes have a great pomological and biochemical potential that can be exploited in vegetative propagation and improvement programs of this tree.

## 1. Introduction

The loquat (*Eriobotrya japonica* (Thunb.) Lindl.) is a subtropical evergreen tree of the *Rosaceae* family (Sun et al., 2020). The origin of loquat has been approved as the middle and lower valley of the Daduhe River in China (Zhang et al., 1993), but it was subsequently cultivated commercially in many other countries, including Japan, Brazil and northern India (Chen et al., 2009). In the Mediterranean region, Spain and Turkey are the countries where the cultivation of this species has developed successfully over the last 20 years due to the most suitable ecological conditions for the growth of loquat and its early production. (Virginia et

al., 2011). The loquat fruit is widely consumed as fresh fruit or juice for its excellent flavour and abundant nutritional values, which may protect against inflammation, diabetes, cancer, bacterial infections, aging, pain, allergies, and other health problems (Kim and Shin, 2009; Sun et al., 2020; Liu et al., 2016). The loquat has been introduced in Morocco from Algeria by French colonization at the beginning of the century (Rhomari, 2013). The valley of Zegzel, located in the northwestern region of Morocco, accounts for 85% of the country's loquat-growing area. This area produces mainly four varieties, which are Tanaka (Japanese cultivar) and three others known locally as Navela, Muscat, and Mkerkebe. In 2021, the national production of loquat has exceeded 10,000 tons, with a significant improvement in the size and gustative quality of this exceptional local product (RDAO, 2021). Most of the studies carried out on the loquat fruit-focused mainly on its phenotypic characterization, while research on bioactive compounds and antioxidant capacity is restricted. The analysis of the organic acid and sugar composition of loquat cultivars indicated that malic acid is the predominant organic acid, followed by tartaric, quinic, citric, succinic, fumaric, and oxalic acids. In addition, fructose and glucose are the most abundant sugars, and the others are sucrose, maltose, and sorbitol (Serrano et al., 2003; Tian et al., 2007; Amoros et al., 2008; Chen et al., 2009; Pande & Akoh, 2010; Xu et al., 2010). Furthermore, the investigations have demonstrated the high antioxidant capacity of loquat extracts in *vitro* and in *vivo* using multiple antioxidant tests (Liu et al., 2016). Currently, the commercial quality of the Moroccan loquat genotypes is rare and the distinction between most trees is based on the shape of the fruit. In this context, this investigation aims to quantify the main bioactive compounds and to assess the antioxidant capacity of the eight loquat fruits which are chosen according to some agronomic and economic criteria, such as the earliness and tardiness, shape, size, and colour of the fruits and good health of the tree.

## Materials and methods

### 1.1. Plant material

In 2016, a prospection conducted in the regions of Zegzel, Takerboust, Tazaghine, and Taghsrout, belonging to the region of Berkane, allowed to collect eight genotypes of loquat. The code assigned and the geographical origin of each genotype are shown in Figure. 1 and Table 1. The choice of these genotypes is based on size, fruit colour, agronomic characteristics and denominations. Indeed, the mature and healthy fruits were manually pitted, frozen in liquid nitrogen, and stored at -20°C for analysis.

### 1.2.Chemicals

All solvents (methanol, chloroform, ethanol, acetone, acetonitrile), Phenol, sulphuric acid ($H_2SO_4$), glucose, extraction buffer, Bradford reagent, phenolindo- 2,6-dichlorophenol (DPIP), sodium carbonate ($Na_2CO_3$), β-carotene, Folin–Ciocalteu reagent, gallic acid, sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$), sodium hydroxide (NaOH), rutin, vanillin, catechin, ascorbic acid, DPPH, ABTS, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), 5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), standards of organic acids and sugars. All other products used have an analytical grade.

### 1.3. Fruit weight measurement

The weight of 10 loquat fruits from each genotype was measured using an electronic balance (Precisa XB 2200 C, Precisa, UK).

### 1.4. Biochemical analysis

#### 1.4.1. Profile of Sugar

In order to analyse the sugar profile, the protocol described by Chen *et al.* (2002) was applied. Soluble sugars were extracted by grinding 5g of frozen fruit in 5 volumes (w/v) of 12: 5: 3 (v/v/v) methanol/chloroform/water. The extracts were centrifuged at 5000 ×*g* for 5 minutes. The extraction was performed three times. Water and chloroform were then added to bring the final methanol/chloroform/water ratio to 10: 6: 5 (v/v/v) and the chloroform layer was removed. The remaining aqueous alcohol phase was adjusted to pH 7 with NaOH (0.1 mol/l), dried under vacuum and redissolved in distilled water. Sugars dissolved in water were analysed by high-performance liquid chromatography (HPLC) (Hewlett-Packard series 1100; Hewlett-Packard, Wilmington, DE, USA). Sugars were isolated using a Supelco column (Supelcogel TM C-610H column 30 cm × 7.8 mm) and Supelguard (5 cm × 4.6 mm; Supelco, Inc., Bellefonte, PA, USA).

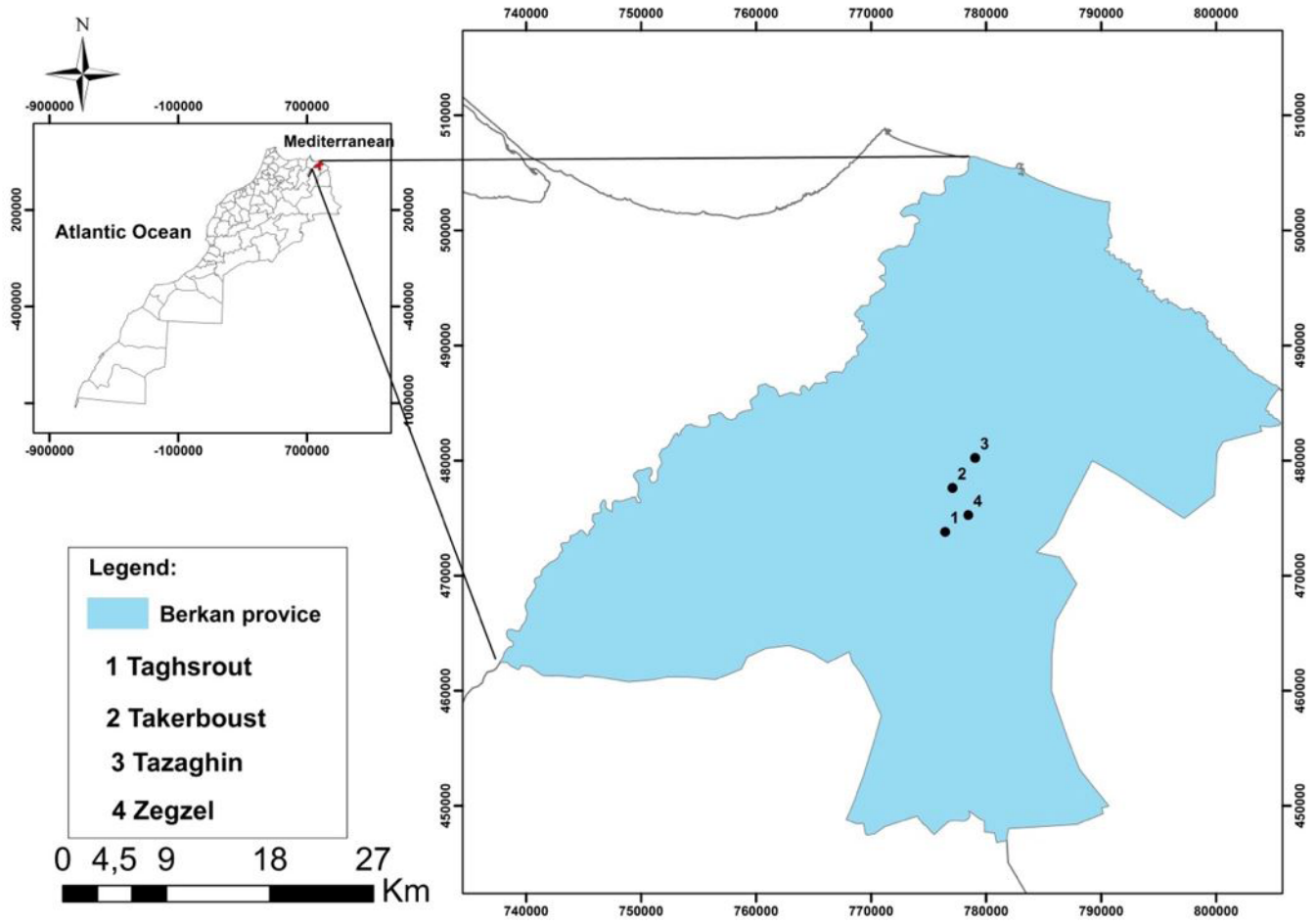


**Figure 1.** Location of sampling areas in North-Eastern Morocco

The column temperature was 90 ◦C and the elution buffer consisted of 0.1% phosphoric acid with a flow rate of 0.5ml mim-1. The absorbance was measured at 210nm using a refractive index detector. Standards of sugars (glucose, fructose, and sucrose) were obtained from Sigma (Poole, UK). Fructose, glucose, and sucrose were identified and quantified by comparison with retention times and integrated peak areas from external standards. The results were expressed as concentrations of mg per g of dry weight.

### 1.4.2. Profile of organic acids

Organic acids in the samples of loquat were quantified according to Hernández *et al.* (2016) protocol. Briefly, 0.5g of each sample lyophilized was subjected to ultra-sonication extraction for 30 min with 5ml of Milli-Q water and centrifugation at 15.000 ×*g* for 20min (Sigma 3–18 K; Sigma. Osterode am Harz. Germany). Then, one millilitre of the hydrophilic extract centrifuged was filtered through a 0.45 µm Millipore filter, and 10µl were injected into high-performance liquid chromatography (HPLC) (Hewlett-Packard series 1100; Hewlett-Packard, Wilmington, DE, USA). Organic acids were isolated using a Supelco column (Supelcogel TM C-610H column 30 cm × 7.8 mm) and Supelguard (5 cm × 4.6 mm; Supelco, Inc., Bellefonte, PA, USA). The elution buffer consisted of 0.1% phosphoric acid with a flow rate of 0.5ml mim-1. and absorbance was measured at 210nm using a diode-array detector (DAD). Standards of organic acids (citric, malic, quinic, and succinic) were obtained from Sigma (Poole, UK). Calibration curves were used for the quantification of organic acids, showing good linearity ($R^2$ = 0.999). Results for organic acids were expressed as concentrations mg /100g of dry weight.

### 1.4.3. Total proteins content analysis

The total protein assay is carried out according to the


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method of Bradford, (1976. In fact, the extraction was carried out by mixing 1g of each lyophilized sample with 5 ml of buffer solution. Subsequently, 0.50 ml from the obtained filtrate was mixed with 2 ml of Bradford reagent. The tubes are incubated for 2 minutes at room temperature. Then the absorbance was read at 595 nm using a spectrophotometer (JASCO V-630). Bovine Serum Albumin (BSA) was used as a standard. The protein contents were expressed in g per 100g of fresh weight.

### 2.4.4. Vitamin C content

The vitamin C content was assessed in fruit juice using a 2,6-dichloroindophenol titrimetric method according to AOAC, (1995). 10 ml of sample were mixed with 40ml of buffer (1g/l oxalic acid plus 4 g/l anhydrous sodium acetate) and titrated against a colorant solution containing 295 mg/l DPIP and 100 mg sodium bicarbonate. A standard curve was generated using concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mg/l of ascorbic acid (BDH. Buffalo. NY. USA). Results were expressed as mg ascorbic acid equivalent (AAE) per 100g of fresh weight.

### 2.4.5. Carotenoid content

For the quantifying of carotenoid content, the method described by Reyes *et al.* (1999) was performed. Indeed, 2g of frozen fruit was homogenized with 25ml of acetone/ethanol (1: 1 v/v) containing 200mg/l butylated hydroxytoluene. The homogenate was filtered through #4 Whatman filter paper, washed in solvent (60 ml), and diluted to 100 ml with the extraction solvent. The extracts were placed in a plastic container with 50ml of hexane. After shaking the mixture was left to stand for 15 minutes before adding 25ml of nanopure water. The container was shaken a second time and the contents were allowed to separate for 30 minutes. Then, the absorbance was measured at 470 nm with a spectrophotometer (ASCO V-630). The carotenoid content was quantified using a standard curve of β-carotene (1–4µg/ml). Results were expressed as µg β-carotene equivalent per g of fresh weight.

### 2.4.6. Extract preparation for phenolic content and antioxidant capacity

The extraction was carried out according to the method described by Swain & Hillis (1959). Indeed, 10g of fruit were homogenized in 25 ml of absolute methanol using a waring blender. The homogenates were maintained at 4°C for 12 h, then centrifuged at 6000 rpm for 20 min and the supernatant is recovered. The extraction of the residue was repeated three times under the same conditions.

### 2.4.7. Total phenolic content

The Folin–Ciocalteu reagent assay was used to determine the total phenolic content (Singleton and Rossi, 1965). A 0.1ml aliquot of the extract was mixed with 5ml of Folin–Ciocalteu reagent (0.2 mol/l). The solution was allowed to stand at 25◦C for 5min before adding 4ml of sodium carbonate solution (150 g/l). The absorbance at 765 nm using a spectrophotometer (ASCO V-630) was measured after the initial mixing and subsequently for up to 90 min until it reached a plateau. Gallic acid was used as a standard for the calibration curve. The results were expressed as µg gallic acid equivalent (GAE) $g^{-1}$ FW.

### 2.4.8. Total Flavonoids content

The total flavonoid content was measured using a colorimetric method developed by Zhishen J *et al.* 1999. Briefly, 2ml of the methanolic extract was mixed with 3ml of distilled water and 0.3ml of sodium nitrate (NaNO2, 0.72 mol/l). After 5 min, 0.6 of aluminium trichloride ($AlCl_3$) at 0.41 mol/l was added. Following 6 min, 2ml of sodium hydroxide (NaOH, 1M) and 2.1ml of distilled water were introduced. The absorbance is measured at 510 nm by using a spectrophotometer (ASCO V-630) and the results are expressed as µg of rutin equivalent (RE). $g^{-1}$ FW.

### 2.4.9. Condensed tannin content

In the presence of concentrated $H_2SO_4$, condensed tannins are transformed into anthocyanidols due to their reaction with vanillin in the methanolic extract (Sun et al., 1998). To measure the carotenoid content, 50ml of methanolic extract was suitably diluted and mixed with 3ml of 4% methanolic vanillin solution and 1.5 ml of H2SO4. After 15 minutes, the absorbance was measured at 500nm using a spectrophotometer (ASCO V-630). Condensed tannins contents were expressed as µg of catechin equivalents per g ac-

cording to the standard curve.

### 2.4.10. Antioxidant capacity analysis

#### DPPH assay

DPPH (2,2-diphenyl-1-picrylhydrazyl radical) radical scavenging activity of loquat extracts was measured according to the method of Braca *et al.* (2001). A quantity of 0.1 ml of fruit extract was added to 3ml of methanolic solution of DPPH (0.1mmol/l). After 30 min in dark, the absorbance at 517 nm was carried out with a spectrophotometer (ASCO V-630). The inhibitory activity (%) was calculated as:

$$\text{Percentage Inhibition (\%)} = \frac{Ab_{593\ \text{sample}}\ \text{nm} - Ab_{593\text{controle}}\ \text{nm}}{Ab_{593\ \text{sample}}\ \text{nm}} \times 100$$

Where:

Ab517 control: control absorbance.

Ab517sample: control sample absorbance.

The Results were expressed as µmol Trolox equivalent. g-1 FW.

#### ABTS assay

The ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation) radical scavenging ability of extracts was determined applying the method described by Re *et al.* (1999). ABTS was produced by reacting an ABTS solution (7 mmol/l) with Potassium persulfate (K2S2O8) (2.45 mmol/l) in the dark for 16 h. The absorbance was adjusted at 734 nm to 0.700 with ethanol. Then 0.2 ml of the appropriate dilution of the extract was added to 2ml of ABTS solution. The absorbance was measured at 734 nm after 15 min with the use of a spectrophotometer (ASCO V-630). The inhibitory activity (%) was calculated as:

$$\text{Percentage Inhibition (\%)} = \frac{Ab_{734\ \text{control}}\ \text{nm} - Ab_{734\ \text{sample}}\ \text{nm}}{Ab_{734\text{control}}\ \text{nm}} \times 100$$

Where:

Ab734 control: control absorbance.

Ab734 sample: control sample absorbance.

Results were expressed as µmol Trolox equivalent $g^{-1}$ FW.

#### FRAP assay

With slight modifications, the FRAP test described by Benzie & Strain, 1996, was applied. The stock solutions included 300 mmol/l acetate buffer (3.1g of $C_2H_3NaO_2 \cdot 3H_2O$ and 16ml of $C_2H_4O2$) at pH 3.6, 10 mmol/l TPTZ solution in 40 mmol/l HCl and 20 mmol/l $FeCl_3 \cdot 6H_2O$ solution. A fresh working solution was prepared by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution, and 2.5 ml of FeCl3· 6H2O solution and then warmed at 37 ◦C before use. Next, 0.15 ml of fruit extract or methanol (reagent blank) was mixed with 2.85 ml of FRAP solution at 37 ◦C for 30 min in the dark (in a water bath). The absorbance was conducted at 593 nm using a spectrophotometer (ASCO V-630). Results were expressed as µmol Trolox equivalents $g^{-1}$ FW.

$$\text{Inhibition (\%)} = \frac{Ab_{517\ \text{control}}\ \text{nm} - Ab_{517\ \text{sample}}\ \text{nm}}{Ab_{517\ \text{control}}\ \text{nm}} \times 100$$

Where:

Ab593 control: control absorbance.

Ab593 sample: control sample absorbance.

Results were expressed as µmol Trolox equivalent g-1 FW

### 2.5. Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) to indicate the signification differences using the Duncan Post Hoc at the 5% level. In addition, the correlation between studied parameters was analysed using the Pearson correlation coefficient (α = 0.05). All analyses were performed using SPSS Ver. 28. software with three replicates for each parameter. Results were reported as mean ± SD.


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## 3. Results

The present study revealed a large variation in fruit weight and biochemical content among eight loquat fruit genotypes.

### 3.1. Fruit weight

The most important commercial criterion for loquats is that of fruit weight. In the present study, with 10 fruits being analysed for each loquat genotype, the highest fruit weight (54.55 g) had been recorded for the Z1 genotype, while the TA6 genotype produced the smallest fruits (28.72 g) (table 1).

### 3.2. Total phenolic, flavonoid, carotenoid, vitamin C, protein, and tannin contents

The total phenolic compounds, flavonoids, carotenoids, condensed tannins, and protein as well as vitamin C contents of the eight genotypes are summarised in Table 2. Phenolic contents among the eight loquat fruit genotypes varied considerably, with the TZN1 and T2 genotypes showing the highest (186.05µg GAE $g^{-1}$) and lowest phenolic contents (92.22µg GAE$g^{-1}$), respectively. Concerning total flavonoids, maximum levels were detected for fruits belonging to genotypes T2 (59.58 µg RE $g^{-1}$) and Z1 (59.00 µg RE $g^{-1}$), whereas minimum levels were detected for genotype TZN1 (57.72 µg RE $g^{-1}$). Carotenoid contents, oscillated from 15.94 µg ß-carotene $g^{-1}$ (Z7) to 90.65 µg ß-carotene $g^{-1}$ (TA7). Regarding vitamin C, the highest and lowest levels had been detected in genotypes T2 (14.19 mg AAE/100 g) and TA7 (6.44 mg AAE/100 g), respectively. The protein and tannin contents exhibited no significant differences between genotypes with values of 0.64-0.98 g/100 g and 2.94-2.61 µg. $g^{-1}$ respectively.

**Table 1.** Loquat genotypes used in this study

| Locality | Code |
|---|---|
| Taghsrout2 | T2 |
| TakerboustA | TA5, TA6. TA7 |
| TazaghinZ | TZN1. TZN2 |
| Zegzel | Z1. Z7. |

**Table 2.** Bioactive compounds of eight loquat genotypes

| Genotypes | Weight fruit (g) | Total phenolic (µg GAE.$g^{-1}$) | Total flavonoids (µg RE $g^{-1}$) | Total carotenoids (µg ß-carotene g-1) | Tannins µg. $g^{-1}$ | Protein g/100g | Vitamin C (mg AAE/ 100g) |
|---|---|---|---|---|---|---|---|
| T2 | 52.35$^{c}$ | **92.22±0.10$^{a}$** | **59.58±0.10$^{e}$** | 78.73±0.00$^{f}$ | 2.89±0.03$^{a}$ | 0.66±0.05$^{a}$ | **14.19±0.98$^{e}$** |
| TA5 | 41 .67$^{b}$ | 150.93±0.16$^{b}$ | 58.59±0.01$^{cd}$ | 65.54±0.00$^{e}$ | 2.75±0.17$^{a}$ | 0.76±0.23$^{a}$ | 11.57±0.33$^{d}$ |
| TA6 | **28.72$^{a}$** | 150.39±0.35$^{b}$ | 58.92±0.03$^{d}$ | 47.45±0.01$^{d}$ | 2.74±0.05$^{a}$ | 0.78±0.26$^{a}$ | 10.03±0.74$^{bc}$ |
| TA7 | 29.95$^{a}$ | 176.79±0.49$^{e}$ | 58.41±0.06$^{bc}$ | **90.65±0.00$^{h}$** | 2.67±0.15$^{a}$ | **0.64±0.08$^{a}$** | **6.44±0.33$^{a}$** |
| TZN1 | 42.09$^{b}$ | **186.05±0.24$^{f}$** | **57.72±0.07$^{a}$** | 80.13±0.01$^{g}$ | **2.94±0.32$^{a}$** | 0.89±0.28$^{a}$ | 8.85±0.95$^{b}$ |
| TZN2 | 47.12$^{bc}$ | 169.36±0.16$^{d}$ | 57.86±0.07$^{a}$ | 49.94±0.01$^{d}$ | 2.89±0.06$^{a}$ | 0.82±0.27$^{a}$ | 10.42±0.28$^{cd}$ |
| Z1 | **54.55$^{c}$** | 164.51±0.16$^{c}$ | 59.00±0.34$^{d}$ | 39.87±0.05$^{b}$ | 2.83±0.11$^{a}$ | 0.97±0.17$^{a}$ | 9.13±0.35$^{bc}$ |
| Z7 | 41.19$^{b}$ | 169.36±0.15$^{d}$ | 58.07±0.17$^{ab}$ | **15.94±0.00$^{a}$** | **2.61±0.04$^{a}$** | **0.98±0.30$^{a}$** | 9.06±0.07$^{bc}$ |

Value in average ± SD. Values marked by the same letter, are not significantly different ($p < 0.05$) using Duncan post hoc test ($p < 0.05$).

### 3.3. Sugar content

Genotype sugar content varied with a range of 54.85–28.03 mg. $g^{-1}$ (Table 3). These amounts had been observed in TA6 and Z1 genotypes, respectively. The loquat fruits revealed high concentrations of fructose, followed by sucrose, and lower concentrations of glucose. The highest levels of fructose (TA5: 31.64 mg. $g^{-1}$), sucrose (TA6: 21.09 mg. $g^{-1}$), and glucose (TA7: 10.34 mg/100 g) had been observed in TA genotypes, while the TZN1 genotype showed the lowest levels for all three sugars (16.15 mg/100 g, 5.61 mg/100 g, and 6.27 mg/100 g respectively) (Table 3).

### 3.4. Organic acid contents

Organic acid profiles of the eight loquat fruit genotypes are presented in Table 4. The total amount of organic acids exhibited a large variation from 168.09 mg/100 g (TZN1) to 326.24 mg/100 g (T2). In addition, the HPLC analyses revealed high variation in the levels of four organic acids, with malic acid (100.74–193.75 mg/100 g) being the most prevalent, followed by quinic (51.28–107.25 mg/100 g), citric (12.64–15.36 mg/100 g) and succinic acids (0.67–12.6 mg/100 g). The T2 genotype showed the greatest quantity of malic, quinic, and succinic acids while the TZN1containted the highest level of citric acid.

### 3.5. Antioxidant activity

The antioxidant capacity of the different fruit genotypes, as determined by three different methods, is shown in Table 5. According to DPPH assays, the Z7 and TA7 genotypes showed the highest (53.94 63 µmol TE. $g^{-1}$) and lowest (12.63 63 µmol TE. $g^{-1}$) antioxidant activities, respectively. Similarly, the ABTS method indicated high antioxidant activity for Z7 fruits (58 63 µmol TE. $g^{-1}$), while the lowest activity had been recorded for TZN1 fruits (27.63 63 µmol TE. $g^{-1}$). Readings from the FRAP method ranged from 16.06 µmol TE /g for the TZN2 genotype to 130 µmol TE /g for the Z7 genotype. All three methods thus reported higher antioxidant levels for the Z7 genotype compared to that of the other loquat fruit genotypes.

### 3.6. Correlations between analysed parameters

Correlations among all biochemical parameters are summarised in Table 6. For the DPPH assay, a strong and positive correlation with polyphenols ($r^2$ = 0.75), flavonoids ($r^2$ = 0. 72) and condensed tannins ($r^2$ = 0. 72) had been revealed. In contrast, no correlation between vitamin C and the DPPH assay could be detected. Furthermore, total phenolics showed a strong correlation with flavonoids ($r^2$ = 0.671), while carotenoid content positively correlated with flavonoids ($r^2$ = 0.31). The other parameters did not reveal significant correlations.

## 4. Discussion

Weight and biochemical analyses of the eight fruit genotypes revealed a great biochemical richness of the Moroccan loquat. The fruit weights recorded in the present study (54.55–28.72 g) were similar to the finding of Durgac et al., (2006) (22.55–29.54 g), higher than those reported by Xu & Chen, (2010) and Zhang *et al.* (2015) (19.1-39.3g and 24.24-42.19g respectively), but lower than fruit weight revealed in Italian loquat, ranging from 38.4 to 74.2 g (Insero et al., 2003).

The assessment of the biochemical composition of the fruit is a very important criterion, especially for the identification of nutritional value and the antioxidant effect, which is due mostly to the phenolic compounds (Mansouri et al., 2005). The Moroccan loquat showed phenolic content levels of 92.22– 186.05µg GAE $g^{-1}$ which is weak in comparison to that showed by Zhang *et al.* (2015) (9.90–13.73 mg GAE.$g^{-1}$) and Xu & Chen, (2011) (204.5–572.3µg GAE.$g^{-1}$). However, the total flavonoid levels (57.72–59.58 µg RE $g^{-1}$) had been similar to previous results obtained by Xu & Chen, (2011) (21.2-77.5 µg RE. $g^{-1}$). In contrast, fruit carotenoid contents (5.94– 90.65µg ß-carotene $g^{-1}$) were lower than the values reported by Xu & Chen, (2011), for Chinese loquats (23.4–496.3 µg β-carotene $g^{-1}$). Moreover, vitamin C levels noted in the present study (6.44–14.19mg AAE /100 g) had been more important than those recorded by Hasegawa et al., (2010) in Brazilian loquat (5.28–8.20mg/100 g) and also by Xu & Chen, (2011) in Chinese loquat (19.2–10.3 µg AAE $g^{-1}$) cultivars.

Sugar content is an important quality characteristic of fresh loquat fruits (Xu & Chen, 2011). Furthermore, the reducing sugars glucose and fructose together


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**Table 3.** Sugar content of eight loquat genotypes (mg. $g^{-1}$)

| Genotype | Sucrose | Glucose | Fructose | Total sugar |
|---|---|---|---|---|
| **T2** | 8.47±0.18$^{b}$ | 7.15±0.98$^{ab}$ | 29.01±0.52$^{d}$ | 44.63±0.33$^{b}$ |
| **TZN1** | **5.61**±0.52$^{a}$ | **6.27**±0.17$^{a}$ | **16.15**±0.31$^{a}$ | **28.03**±0.62$^{a}$ |
| **Z1** | 19.41±0.65$^{f}$ | 8.49±0.21$^{cd}$ | 25.34±0.01$^{b}$ | 53.24±0.27$^{d}$ |
| **TA7** | 16.47±0.72$^{d}$ | **10.34**±0.03$^{e}$ | 27.18±0.41$^{c}$ | 53.99±0.42$^{d}$ |
| **TA5** | 10.79±0.35$^{c}$ | 8.76±0.75$^{cd}$ | **31.64**±0.08$^{e}$ | 51.19±0.38$^{c}$ |
| **TA6** | **21.09**±0.66$^{g}$ | 9.01±0.62$^{cd}$ | 24.75±0.66$^{b}$ | **54.85**±1.03$^{d}$ |
| **Z7** | 17.72±0.52$^{de}$ | 9.51±0.38$^{de}$ | 24.36±0.47$^{b}$ | 51.59±0.55$^{c}$ |
| **TZN2** | 17.96±0.85$^{e}$ | 7.91±0.52$^{bc}$ | 25.35±0.33$^{b}$ | 51.22±0.11$^{c}$ |

Value in average ± SD. Values marked by the same letter, are not significantly different ($p < 0.05$) using Duncan post hoc test ($p < 0.05$). (Bold values are minimum and maximum)

**Table 4.** Organic acid composition of eight loquat genotypes in mg/100g DW

| Genotype | Citric acid | Malic acid | Quinic acid | Succinic acid | Total |
|---|---|---|---|---|---|
| **T2** | **12.64**±0.27$^{c}$ | **193.75**±0.58$^{g}$ | **107.25**±0.25$^{g}$ | **12.6**±0.25$^{c}$ | **326.24**±0.33$^{h}$ |
| **TZN1** | **15.36**±0.59$^{e}$ | **100.74**±0.55$^{a}$ | **51.28**±0.01$^{a}$ | 0.71±0.11$^{a}$ | **168.09**±0.10$^{a}$ |
| **Z1** | 12.94±0.04$^{cd}$ | 160.13±0.83$^{d}$ | 74.12±0.33$^{d}$ | **0.67**±0.03$^{a}$ | 247.86±0.95$^{c}$ |
| **TA7** | 13.63±0.57$^{d}$ | 160.52±0.52$^{d}$ | 73.44±0.48$^{cd}$ | 11.6±0.21$^{bc}$ | 259.19±0.13$^{e}$ |
| **TA5** | 0.896±0.01$^{a}$ | 187.79±0.10$^{f}$ | 105.46±0.14$^{f}$ | 12.58±0.62$^{c}$ | 306.73±0.41$^{g}$ |
| **T6** | 0.95±0.04$^{a}$ | 152.85±0.13$^{c}$ | 73.34±0.35$^{c}$ | 0.88±0.10$^{a}$ | 228.02±0.16$^{c}$ |
| **Z7** | 11.7±0.51$^{b}$ | 140.8±0.62$^{b}$ | 63.06±0.17$^{b}$ | 0.76±0.01$^{a}$ | 216.32±0.18$^{b}$ |
| **TZN2** | 11.65±0.33$^{b}$ | 172.55±0.35$^{e}$ | 82.61±0.13$^{e}$ | 11.05±0.06$^{b}$ | 277.86±0.02$^{f}$ |

Value in average ± SD. Values marked by the same letter, are not significantly different ($p < 0.05$) using Duncan post hoc test ($p < 0.05$). (Bold values are minimum and maximum)

**Table 5.** Antioxidant activity of eight loquat genotype as µmol TE. $g^{-1}$

| Genotypes | ABTS | DPPH | FRAP |
|---|---|---|---|
| TZN1 | **27.63**±0.30$^{a}$ | 28.71±0.30$^{c}$ | 58.32±0.41$^{d}$ |
| TZN2 | 41.62±0.41$^{c}$ | 33.31±0.03$^{e}$ | **16.06**±0.41$^{a}$ |
| T2 | 45.74±0.42$^{d}$ | 30.85±0.31$^{g}$ | 42.57±0.68$^{b}$ |
| Z7 | **58**±0.17$^{e}$ | **53.94**±0.14$^{d}$ | **130.21**±0.03$^{h}$ |
| Z1 | 39.9±1.02$^{c}$ | 26.08±0.24$^{b}$ | 61.46±0.31$^{e}$ |
| TA5 | 44.76±0.24$^{d}$ | 28.47±0.34$^{c}$ | 105.68±0.47$^{f}$ |
| TA6 | 44.65±0.55$^{d}$ | 37.06±0.18$^{f}$ | 108.82±0.65$^{g}$ |
| TA7 | 32.83±0.49$^{b}$ | **12.63**±0.25$^{a}$ | 28±0.44$^{b}$ |

(Bold values are minimum and maximum)

**Table 6.** Person's correlation among bioactive compounds and antioxidant activity

| | DPPH | Polyphenols | Flavonoides | Carotenoids | Tannins |
|---|---|---|---|---|---|
| **DPPH** | 1.00 | | | | |
| **Polyphenols** | **0.759** | 1.00 | | | |
| **Flavonoids** | **0.723** | **0.671**$^{**}$ | 1.00 | | |
| **Carotenoids** | **0.222** | 0.202 | **0.312**$^{**}$ | 1.00 | |
| **Tannins** | **0.720** | -0.040 | -0.002 | 0.003 | 1.00 |
| **Vitamin C** | *-0.03* | -0,091 | -0,046 | -0,054 | *<.0001* |

** Correlation is significant at the 0.01 level (2-tailed), * Correlation is significant at the 0.05 level (2-tailed).

with sucrose constitute the majority of the soluble solids (Nunes et al., 1995). The main sugar in the Moroccan loquats had been fructose (16.15– 31.64 mg. $g^{-1}$), followed by sucrose (5.61– 21.09 mg. $g^{-1}$) and glucose (6.27–10.34 mg. $g^{-1}$). These results are in agreement with the levels of sugar revealed by Hasegawa *et al.* (2010) in Brazilian loquat cultivars, which are published in a range of 0.89-1.82; 2.70-4.96 and 0.15-1.51 g/100g respectively. In Chinese loquat, Xu & Chen (2011) reported comparable values to the present results (fructose: 35.9- 54.2 mg. $g^{-1}$, sucrose: 1.59 -5.02 mg. $g^{-1}$ and glucose:27.4-53.6 mg. $g^{-1}$). As well as sugar, organic acids play a crucial role in fruit taste and flavor, which are important indicators of fruit quality (Liu et al., 2016).

The organic acid levels of Moroccan loquats analysed in the present study are the following: malic with 100.74–193.75 mg/100 g, quinic with 51.28–107.25 mg/100 g, citric with 12.64–15.36 mg/100 g, and succinic with 0.67–12.6 mg/100 g. These findings are lower than the values revealed by Toker et al., (2012) in Turkish loquat (malic: 364.93–842.49 mg/100 g, citric: 6.23–14.76 mg/100 g, and succinic: 10.38–30.79 mg/100 g). Also, Hasegawa *et al.* (2010) showed a high value in Brazilian loquat cultivars (malic: 587.97–988.05 mg/100 g, citric: 31.68–150.14 mg/100 g, and succinic: 13.78–24.71 mg/100 g). Regarding antioxidant activity, it is generally advised to combine at least two tests to obtain an accurate view of a food's total antioxidant capacity (Perez-Jimenez et al., 2008).

In the present study, the antioxidant activity of eight loquat genotypes had been assessed with DPPH, ABTS, and FRAP methods which recorded levels of 12.63–53.94 µmol/g, 27.63–58µmol/g and 16,06–130µmol /g respectively. These findings were in agreement with previous results which revealed a DPPH antioxidant level of 30.34 µmol TE $g^{-1}$ and ABTS antioxidant capacity of 30.54 µmol TE $g^{-1}$ (Ahumada *et al.* 2017). Many reports have highlighted the potential antioxidant activity of phenolic compounds in fruits, vegetables, beverages, and grains (De Ancos et al., 2000; Zielinski et al., 2000). Yet, several studies have found that the antioxidant activity of plant extracts may not be limited to phenolics, but that activity may be due to other secondary metabolites such as volatile oils, carotenoids, and vitamins (Javanmardi et al., 2003; Chanwitheesuk et al., 2005).

Regarding the correlation analyses, the antioxidant activities results showed a significant and positive correlation of DPPH assay with polyphenols, flavonoids, and condensed tannins. These correlations were in agreement with the finding that revealed positive and significant correlations of DPPH with total phenolics ($r = 0.706$) and flavonoids ($r = 0.759$) (Xu & Chen, 2011). Furthermore, total phenolics showed a strong correlation with flavonoids ($r^2 = 0.671$), which is similar to the result detected by Xu & Chen, (2011) ($r = 0.924$, $P < 0.01$). Indeed, several studies have shown a strong positive correlation between total polyphenol content and total flavonoids (Ercisli *et al.* 2012). In addition, the results recorded a significant correlation between carotenoid content and flavonoids, while Xu & Chen, (2011) found that no correlation was observed between carotenoids and the variables studied. Similarly, Gardner *et al.* (2000) demonstrated that the contribution of carotenoids to antioxidant potential was negligible. The other parameters studied did not show a significant correlation.

This variation in biochemical composition of Moroccan loquat may be due to genotypes, age of the tree, ecological factors, cultivation techniques, harvest date, and analysis conditions (Amoros et al., 2003; Chen et al., 2009; Xu & Chen, 2011).

## 5. Conclusion

The present investigation showed that the amount of major bioactive compounds and antioxidant capacity vary significantly among the eight genotypes except for the tannins and protein contents. According to the level of biochemical parameters studied in loquat, these genotypes should be considered the as first genotypes to be manipulated for propagation. Thus, Moroccan loquat can be considered a good source of natural compounds with antioxidant activity that can be used in therapeutic and condiment functional foods.

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## Conflict of interest

The authors declare no conflicts of interest.


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## UN Biodiversity Conference: COP15 in Montréal

One hundred fifty government leaders first signed the Convention on Biological Diversity (CBD) at the 1992 Rio Earth Summit. Its main objectives are the conservation of biological diversity, the sustainable use of the components of biological diversity, and the fair and equitable sharing of the benefits arising out of the utilization of genetic resources.
The 15th UN Conference of the Parties (COP15), Convention on Biological Diversity (CBD), is taking place from December 7 – 19, 2022, in Montréal, Canada. CBD is a global meeting that gathers international governments from around the world. The leaders will set out agreements to achieve new goals and develop an action plan for nature over the next decade. The main focus of COP15 will be on reducing the biodiversity and natural resources loss worldwide. The Government of Canada is seeking a successful meeting and collaboration for an ambitious Post-2020 Global Biodiversity Framework. One of the important goals includes targeting 30% of lands and oceans conserved by 2030. Protecting nature requires serious collaboration between the Indigenous Peoples, and the land's original guardians, as well as innovations and initiatives.

To have an overview of Canada's actions for the Fifteenth Conference of the Parties (COP15) to the United Nations Convention on Biological Diversity: https://www.canada.ca/en/services/environment/wildlife-plants-species/biodiversity/cop15/canada-cop15.html

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## Sharm El-Sheikh Climate Change Conference (Cop) November 2022

From the 6th to the 20th of November, the global leaders gathered in Sharm El-Sheikh, Egypt, for the 27th UN Climate Change Conference of the Parties. CATF was on the ground, pushing leaders to put promises into practice now to achieve a zero-carbon future for all.

Heads of state and government, civil society, industry, and finance gathered from all around the world to push actions towards achieving more goals of the Paris Agreement and the UN Framework Convention on Climate Change.

The government step up action on agriculture and food security at cop27. Food and Agriculture for Sustainable Transformation initiative (FAST) was launched on 12 November by more than 20 Agriculture Ministers and the Egyptian cop27 presidency.

To have a deeper insight into the conference, you can visit the UN Framework Convention on Climate Change UNFCCC: https://unfccc.int/cop27#news

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## A daily cup of tea could help you to enjoy better health late in life

A cup of tea a day could help you enjoy better health late in life. But tea is one of many things that will benefit your diet.
The secret lies in flavonoids, which are natural substances exist in found in black and green tea as well as many other foods and beverages such as, apples, nuts, citrus fruit, berries and more. Flavonoids are known for their health benefits. However, new research done by Edith Cowan University (ECU) showed that these substances might hold more benefits than previously thought. The study included 881 elderly women (median age of 80). The results showed that the women were far less likely to have an extensive build-up of abdominal aortic calcification (AAC) if they consumed a high level of flavonoids in their diet.

Researchers at the ECU Nutrition and Health Innovation Research Institute reported that there are types of specific food that contain high amounts of flavonoids.
"In most populations, a small group of foods and beverages -- uniquely high in flavonoids -- contribute the bulk of total dietary flavonoid intake," said the study lead Ben Parmenter.
"The main contributors are usually black or green tea, blueberries, strawberries, oranges, red wine, apples, raisins/grapes and dark chocolate."
Tea was the main resource of the flavonoids in the study. However, people can still get the flavonoid benefits even without drinking tea.
"Out of the women who don't drink black tea, higher total non-tea flavonoid intake also appears to protect against extensive calcification of the arteries," he said.
"This implies flavonoids from sources other than black tea may be protective against AAC when tea is not consumed."

These finds are important as non-tea drinkers could also benefit from flavonoids in their diet.
"In other populations or groups of people, such as young men or people from other countries, black tea might not be the main source of flavonoids," he said.
"AAC is a major predictor of vascular disease events, and this study shows intake of flavonoids, that could protect against AAC, are easily achievable in most people's diets."

For more details about the study, you can read the paper '' Higher habitual dietary flavonoid intake associates with less extensive abdominal aortic calcification in a cohort of older women"

1. Benjamin H. Parmenter, Catherine P. Bondonno, Kevin Murray, John T. Schousboe, Kevin Croft, Richard L. Prince, Jonathan M. Hodgson, Nicola P. Bondonno, Joshua R. Lewis. **Higher Habitual Dietary Flavonoid Intake Associates With Less Extensive Abdominal Aortic Calcification in a Cohort of Older Women**. Arteriosclerosis, Thrombosis, and Vascular Biology, 2022; 42 (12): 1482
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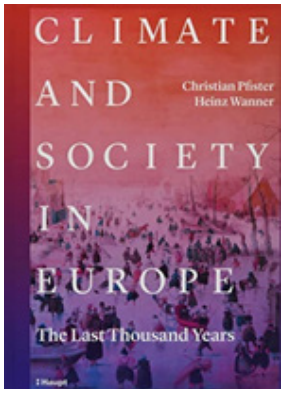


# Climate and Society in Europe: The Last Thousand Years

A review by Diana Ismael

Authors (Eds.): Christian Pfister, Heinz Wanner
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*The book Climate and Society in Europe:* The Last Thousand Years, was written by two of the leading climate scientists in Europe. The authors, through an appropriate interdisciplinary approach, explore the past, present and future climatic variability analysing also its impact on environmental contexts, on historical events and on the becoming of European societies in the last millennium.

The book consists of eleven chapters that combine between two distinct approaches of the natural sciences and the social sciences, follow the common thread through specific best-case examples.

Chapter 1, the introduction, explores the different approaches of the natural sciences and human sciences in studying the climate, and explains how both approaches can counterpart each other to a more understanding of the relationships between climate change and human societies in the last millennium.

In its second chapter, the book dealt with the story of the Austrian valley of Ötztal of Iceman Ötzi. This was hidden for more than 5250 years and then found in 1991. The book highlighted the case study of the Holocene climate variability, which is characterized by **two initial millennia of fast warming followed by four millennia of higher temperatures and humidity, and a progressively accelerating cooling and drying for the past six millennia.** These changes are driven by variations in the obliquity of the Earth's axis. The chapter focused on the last 1000 years of the Holocene up to the modern anthropogenic warming.

Volcanic eruption was the focus of the third chapter. Volcanic eruption has a huge impact on both climate change and human societies. The chapter explore the case study of the Indonesian volcano Tambora. This incident, in 1815, had massive impacts of not only regional but also global climate system and was the main driver behind the great famine in Western and Central Europe.

*From weather narratives to climate science*, chapter four, the book goes through the last 1000 years to understand more about the time evolution. It began with the Greek science, mediated by Islamic world. Back then, the religious leaders started to obtain better perception about the cosmos. The investigation and explanation of perception of time started to be preserved in the society archives. This archiving act went through a long path of characterizing the events as eschatological, apocalyptic or divine punishments, until it arrived a point when the events were being appropriately calibrated and standardized. Nowadays, we have the modern satellite studies to deal with more sophisticated models.

Chapter five, *reconstructing past climate*, explores the reliable global records of the climate (proxy data) that were found in the nature and societies old archives. These data, allows the scientists to obtain a better overview on the changing in the temperature and precipitation indices before the new developed measurements started to be used.

In *the European climate—present and past chapter,* the spatial dynamics of Europe's current climate is explored deeply using a set of proxy data that shows only limited space–time resolution. The results of studying this data combine the findings of the simulation and the latest Earth System Model (ESM) such as greenhouse, human and volcanic emissions.

Chapters seven and eight deals with the data from the nature archive over the last 1000 year. These data show how the climate over this period. The data focus precisely on ma-

jor factors such as solar activity and volcanic eruption that lead to infinite changes. Moreover, the chapters present a clear image on how the seasonal temperature changed over the millennium the preceded the rapid warming in 1988.

Regarding weather, climate, and the human world, chapter nine goes through the interaction relationship between the climate, the natural environment and the human history. The chapter analysis the clear impact of the climate change on the population demographic and its economy before the emergence of the modern industry.

In chapter ten that deals with the European climate over the last millennium the fluctuation of the climate change from medium to long-term is highlighted. The chapter sheds the light on the period from the High Medieval Period (HMP) until The Boreal Little Ice Age (BLIA) as a period of a long cold winter. In addition, it addresses the impact of this period on the environmental and social life.

Final chapter is no less interesting as it outlines the transition from the slow to the rapid warming. It also highlights the first discovery of the negative impact of the greenhouse emissions. The chapter present the greenhouse effect in a simple less technical method focusing on future scenarios of the climate change impact on the planet.

The book is highly recommended not only for scholars but also for any reader who is interested in studying the climate and its history and is curious to find out how much climate change has had an impact on the history of human societies in Europe.

**About the author:**

Diana Ismael is a sensory specialist with a PhD in Food and Sensory Science/Consumer Behavior from Kassel University, Germany. Her research focuses on understanding the intention-behaviour gap in organic food consumption. Currently, she works as the Managing Editor at the Future of Food Journal: Journal on Food, Agriculture & Society.


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