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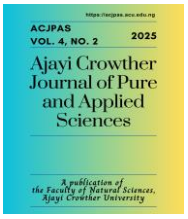

Article

Multidrug-resistant microorganisms associated with slightly spoilt oranges

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Abstract

Slightly spoilt oranges are a breeding ground for microbial growth, potentially leading to foodborne illnesses and the development of antimicrobial resistance. Antimicrobial resistance is known to pose serious human, animal, and environmental public health threats. This study therefore aimed at isolating microorganisms associated with slightly spoilt oranges as well as determining the multidrug-resistant microorganisms present in the slightly spoilt oranges. Microbial and physicochemical parameters were determined using standard methods. Slightly spoilt orange recorded 60.4 % moisture content as against the healthy range of 80-89 %. Five bacterial species were isolated, out of which four were *Bacillus* spp. *Bacillus* sp. code Os 3 had the highest frequency of occurrence (33.3 %). Four different fungal species of *Chrysonilia* were isolated from the slightly spoilt oranges. Three of the *Bacillus* spp. were multidrug-resistant, and the fungal isolates were basically resistant to two out of three antifungal agent employed at different concentrations. This may pose a human health threat if these slightly spoilt oranges are continually consumed. Overall, this study reveals that these slightly spoilt oranges are unfit for consumption as multidrug-resistant microorganisms of public health risk were associated with them.

Keywords: Antimicrobial resistance, multidrug-resistant, physicochemical parameters, public health, slightly spoilt orange

1. Introduction

One of the most significant fruit crops in the world is citrus. Orange is a specific type of citrus fruit mainly known as *Citrus sinensis*, or sweet orange, it is one of the most significant fruit crops in the world belonging to the *Rutaceae* family. It is mostly grown in tropical and subtropical regions throughout more than 130 countries. Sweet oranges, which are sold as fresh fruit or processed juice, make up almost 60% of all citrus production. Mandarins are grown for the fresh market and account for over 21% of all citrus fruit production. Citrus's complicated genetics and reproductive biology (apomixis, partial pollen and/or ovule sterility, cross- and self-incompatibility, and significant heterozigosity) make conventional breeding by hybridization difficult [1].

Though orange is actually a hybrid between the mandarin (*Citrus reticulata*) and the pomelo (*Citrus maxima*). Orange tree fruit can be consumed fresh or processed to extract its juice or aromatic peel. "Ya'yan itace" in Hausa, "Oroma" in Igbo, "eso osan" in Yoruba, and "Sokoro" in Ibibio are the local names for it. The word orange comes from the Sanskrit word for "orange tree," which itself comes from a Dravidian root word called "bitter orange."

Microorganisms may infect orange fruits as a result of improper handling procedures, storage conditions, distribution, marketing strategies, vendor unhygienic conditions, and transportation. Number of contaminants on a plant can vary from a few hundred to thousands, or millions, of organisms per centimeter square of surface, depending on the plant and its surroundings

[2]. For example, an orange fruit that has been thoroughly cleaned may have 400–700 particles per square centimeter, whereas an orange fruit that has not been cleaned will have thousands [2]. Orange fruit spoilage may also be microbiological in nature, caused by bacteria and fungi (moulds). By growing in oranges and generating compounds that change the fruit's colour, texture, and odour, these microbes contaminate them and render them unfit for human consumption.

Food may be exposed to spoilage bacteria on the seed, during field crop growth, harvesting, postharvest handling, storage, and distribution [3]. The rotting organisms found on harvesting equipment, in packing houses, storage facilities, and on food contact surfaces throughout the distribution chain are the same kinds of soil-borne bacteria that are found on produce [4]. Bacteria, which include species of Bacillus and Staphylococcus, are the cause of bacteria spots, bacteria specks, bacteria soft-rot, and bacterial canker. The type of microorganisms linked to degradation is significantly influenced by the extrinsic and predominant intrinsic characteristics in orange [3].

It is noteworthy that oranges (*Citrus sinesis*) are also prone to fungal contamination especially when exposed to tropical humid climates. This suggests that consumers of these fruits and their byproducts may be at health risk. Foodborne pathogens cause thousands of diseases, and because of their detrimental impact on human health, they constitute a threat to the overall economy [5].

It is necessary to adhere to stringent cleanliness, good agricultural practices, and good manufacturing methods during cultivation, harvest, storage, transportation, and marketing in order to avoid orange rotting and the related negative health effects [6]. In order to suppress and regulate the growth of pathogenic microorganisms in orange fruit, growers can utilize biological control methods, chemical control methods that involve the use of fumigants and fungicides, and cultural preventive control methods [7]. The issues of growing, harvesting, storing, and transporting orange fruits must be addressed in order to slow their deterioration. By performing the aforementioned procedures under the proper conditions necessary to prevent the survival of spoiling microorganisms, this issue can be resolved [7].

The incidence of antimicrobial resistance in food is a global issue. Because these organisms have been isolated from a wide variety of human meals, it poses a serious threat to public health. Information about antibiotic-resistant microorganisms is relevant for assessing the scope of the issue and setting baselines for intervention [8]. This study therefore aimed at isolating microorganisms associated with slightly spoilt oranges as well as determining the multidrug-resistant microorganisms present in the slightly spoilt oranges.

2. Materials and Methods

2.1 Study Area

The area of study was in Oyo town, Oyo state, in the South-western part of Nigeria. The town which is situated on latitude 8^{o} 00 North of the equator and longitude 4^{0}00 East, has an average temperature range between 25°C (77.0°F) and 35°C (95.0°F), covering an area of 28,456 square kilometers, whose landscape consists of old rocks and dome-shaped hills gently rising from about 500 meters in the southern part and reaching a height of about 1,219 meters above the sea level in the Northern part.

2.2 Sample Collection and Physicochemical Analysis

2.2.1 Sample Collection

The oranges were purchased randomly from Ajegunle market, Oyo town. The samples were aseptically collected in sterile foil paper for microbiological analysis and were immediately transported to the laboratory.

2.2.2 Physicochemical Analysis of the Sample

The physicochemical parameters of the samples such as moisture content, protein, ash, fibre, calcium, total carbohydrate etc. were determined following standard methods described by AOAC [9], Kirk and Sawyer [10] and James [11].

2.3 Isolation and Culture Methods

2.3.1 Media Preparation

The media used were Orange Serum Agar (OSA), Hays Agar (HA), Nutrient Agar (NA), and Potato Dextrose Agar (PDA). The NA and PDA were prepared following the manufacturer's instruction and then autoclaved at 121^{o}C for 15 minutes. The OSA which was used as a control medium and was prepared using the following composition; Peptone - 1g, Yeast extract -0.3, Dextrose - 0.4, Agar – 7g, Orange serum – 50 ml, Distilled water - 100 ml, Dipotassium phosphate 0.3g and then autoclaved at 121^{o}C for 15 minutes.

The HA was used to isolate organisms from the already spoilt oranges, this serves as a suitable medium for spoilage organisms. The HA, a composition of; Yeast extract - 0.25, clean potable water-100 ml, salt solution A which contains 10 % of 1g each of K_2HPO_4 and KH_2PO_4 in 10 ml of distilled water, salt solution B which contains 2 % of $MgSO_4$, 0.1 each of $FeSO_4$ $7H_2O$, $MnSO_4$, $4H_2O$ and NaCL in 50 ml of distilled water was prepared and then autoclaved at 121^{0}C for 15 minutes.

2.3.2 Isolation of Microorganisms

Isolation of the microorganisms (fungi and bacteria) was carried out by aseptically collecting 1 ml orange serum samples from both healthy and slightly spoilt oranges. These were serially diluted into four test tubes that were appropriately labelled. One mL each of the serum samples was dispensed into sterile test tubes containing 9 mL sterile distilled water, a serial dilution of four folds was carried out. One mL of the 1st and 2nd dilutions were pipette into sterile petri dishes using the pour plate method. The media used were Nutrient Agar (NA) (NA, Oxoid Ltd., Basingstoke, UK) Potato dextrose Agar (PDA, Oxoid Ltd., Maschester, UK), Hays Agar (HA). These were all prepared according to manufacturers' instructions and referenced guidelines.

The prepared agar (NA, HA and PDA) were allowed to cool to about 45 ^{o}C – 50 ^{o}C, the mouth of the flask was flamed, then about 15 ml of the agar medium was poured aseptically into the petri dishes containing the samples. The plates were then swirled carefully and gently on the work bench for uniform distribution, after which they were allowed to solidify, followed by incubation of the plates invertedly at 37 ^{o}C for 24 hours.

The NA plate and the HA plate were incubated at 37 ^{o}C for 24 hours, while the PDA plates were incubated at 27 ^{o}C for 72 hours. Orange Serum Agar served as a control media, 1 ml each of the healthy oranges was dispensed into petri dishes containing OSA, on solidifying, and the plates were then incubated at 37 ^{o}C for 24 hours. The microbial load on each of the plates were estimated using a colony counter.

2.3.3 Maintenance of Pure Culture

The pure cultures of the organisms were maintained on NA slants and PDA slants for bacterial and fungal isolates respectively and preserved at 4 ^{o}C.

2.4 Identification of Bacterial and Fungal Isolates

The individual bacterial colonies were purified and identified using morphological and biochemical techniques, and distinct colonies were subcultured until pure cultures were obtained and transferred onto slant bottles containing freshly prepared agars [12]. The probable identities of the organisms were carried out and further confirmed on the National Center for Biotechnology Information (NCBI) platform (https://www.ncbi.nlm.nih.gov/) using the biochemical characteristics of the isolates, such as oxidase, catalase, and substrate utilization tests. The fungal isolates were identified based on their

colonial morphology, colour and morphology of the sporulating structures. Glass slides preparations were done using lactophenol blue [13]. The slides were afterward mounted on the microscope focused and viewed.

2.5 Antibiotics Susceptibility Test

The bacterial isolates were tested for their susceptibility to conventional antibiotics using the Kirby Bauer Disc Diffusion method according to the guidelines of the Clinical Laboratory Standards Institute [14]. The inoculum was prepared for each bacterial isolate by adjusting the turbidity to 0.5 McFarland standard. The inoculum was swabbed on the surface of Muller Hinton agar plate using a swab stick for each of the isolates. The antibiotics were placed on the surface of the agar plate using sterile forcep and was incubated at 37 ^{o}C. After 24 hours, the zones of inhibition were measured and recorded appropriately. The antibiotics used were: Ceftazidime (30 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Chloramphenicol (12.5 µg), Tetracycline (30 µg), Imipenem (10 µg), Meropenem (10 µg), Oxacillin (1 µg), Cotrimoxazole (25 µg), Augmentin (20 µg), Vancomycin (30 µg) and Erythromycin (15 µg). The zones of inhibition (ZOI) were measured and the isolates were classified as sensitive, intermediate or resistant according to CLSI tables and guidelines [15].

2.6. Antifungal Assay

The fungal isolates were tested for their minimum inhibitory concentration, the isolates were suspended in 5 ml of sterile water, the inoculum afterwards were swabbed on the surface of the PDA plates using a swab stick. A 6 mm cork borer was then used to bore holes on the agar plates, after which antibiotics were dispensed into the bored holes. The antibiotics used were: Nystatin (6.5mg/5ml), Griseofulvin (125mg/5ml) and Fluconazole (50mg/5ml) and the plates were subsequently incubated for 72 hours. After 72 hours the zone of inhibition was measured.

3. Results

3.1 Physicochemical Analysis

The result of the physicochemical analysis affirmed the healthy orange satisfactory for human consumption while the slightly spoilt orange unsuitable for human consumption. The healthy orange smelt fresh with a sweet aroma, while the slightly spoilt had an unpleasant odour. The moisture content of the healthy orange was reported as 82.9 % but for the slightly spoilt orange it was 60.4%. The total carbohydrate as monosaccharide for the healthy orange was 8.3 and the slightly spoilt orange reported as 3.5. Quantitatively, vitamin A for the healthy orange is positive and that of the slightly spoilt orange is negative. The protein % of the healthy orange was reported as 1.2 % and for the slightly spoilt orange it was 2.4 (Table 1).

3.2 Microbial Analysis

The result of the isolation using the different media gave a good outlook on the diversity of microorganisms isolated, on the OSA medium, which served as control, there was no growth whatsoever of any microorganisms from the healthy orange samples, but that of the spoilt samples showed visible sign of microbial growth, though few colonies were seen on the plates inoculated with the healthy sample (control) on NA. The growth on PDA for the slightly spoilt samples showed similar trend to that of the HA medium, Therefore, the total microbial counts in Cfu/ml (bacteria and fungi respectively) from slightly spoilt orange samples obtained from Ajegunle market, Oyo town, Oyo state for the purpose of this report were recorded for NA and HA plates (Table 3).

The frequency of occurrence of the bacterial isolates showed that one of the bacterial species with the code Os 3 had the highest frequency of occurrence (33.3 %) (Table 4). The characterization of fungal isolates from the slightly spoilt oranges showed that some of the isolates were cream in colour, dry in texture, umbonate in elevation and irregular in form. Some were white in colour, dry in texture, raised in elevation and round in form. Another isolate was white in colour, dry in texture, raised in elevation and round in form, isolates Os1 and Os5 were identified as the same species (Table 5).

3.3 Antibiotics Susceptibility Test for Bacterial Isolates

The antibiotic sensitivity pattern for the isolates from the slightly spoilt oranges, proved that some of the isolates were resistant while some others were susceptible to the antibiotics used (e.g plate 1a). The result also showed the MAR index of the isolates with three (3) of the isolates having an index of 0.2 and above (Table 6).

3.4 Antifungal Assay/Minimum Inhibitory Concentration assay

The result of the antifungal assay showed that some of the different species of *Chrysonilia* were resistant at different levels of concentrations, so also for those that were susceptible, for instance, the second *Chrysonilia* sp. on the table 7 was resistant at three concentrations of griseofulvin used (25, 6.25 and 3.13 mg/ml), but susceptible at 12.5 mg/ml concentration. The antifungals used were griseofulvin, nystatin and fluconazole, the assays were carried out using different concentrations of the agents (Table 7).

Table 1: Physicochemical Analysis of Healthy and Slightly Spoilt Oranges

Test	**Result**	
	Healthy orange	**Slightly spoilt orange**
Description	Round, orange-coloured fruit having a characteristic sweet aroma	Soft, deep, orange-coloured fruit, having an unpleasant odour
Moisture content %	82.9	60.4
Total Carbohydrate (as monosaccharide)	8.3	3.5
Vitamin C, mg/100g	56	48
Vitamin A, (Qualitative)	Positive	Negative
Calcium, mg/100g	49	40
Protein,%	1.2	2.4
Fibre,%	5.8	3.6
Ash,%	1.1	1.2
Total inedible waste,%	10.7	32.5

Table 2: General Microbial Analysis on Orange Samples

Test (Cfu/mL)	**Healthy orange**	**Slightly spoilt orange**
Total viable count	8	1400
Yeast/Mould count	NIL	800
Total coliforms count	NIL	NIL

Table 3: Total Heterotrophic Microbial Count on Slightly Spoilt Oranges Samples

Samples	Microbial group/count (Cfu/ml)	
	Heterotrophic bacteria count (NA)	Heterotrophic fungal count (HA)
Os 1	3.0×10^2	1.4×10^2
Os 2	3.1×10^2	3.0×10^1
Os 3	2.4×10^2	1.5×10^2
Os 4	2.7×10^2	1.0×10^2
Os 5	2.0×10^2	1.0×10^2

KEY: Os - Orange sample, NA - Nutrient agar, HA - Hays agar

Table 4: Frequency of Occurrence of Bacterial Isolates from Slightly Spoilt Orange Samples

Isolates	% of Occurrence
Os 1 *Bacillus* sp.	2(22.2)
Os 2 *Staphylococcus* sp.	1(11.1)
Os 3 *Bacillus* sp.	3(33.3)
Os 4 *Bacillus* sp.	2(22.2)
Os 5 *Bacillus* sp.	1(11.1)
Total	9(100)

Table 5: Fungal Identification of Isolates from Slightly Spoilt Oranges

Isolates	Characterization	Probable organisms
Os 1	Colonies are white in appearance, dry in texture, raised in elevation and irregular in form.	*Chrysonilia* sp.
Os 2	Colonies are grey in appearance, dry in texture, raised in elevation and raised in form.	*Chrysonilia* sp.
Os 3	Colonies are cream in appearance, dry in texture, umbonate in elevation and round in form	*Chrysonilia* sp.
Os 4	Colonies are cream in appearance, dry in texture, raised in elevation and irregular in form.	*Chrysonilia* sp.
Os 5	Colonies are white in appearance, dry in texture, circular in elevation, and irregular in form.	*Chrysonilia* sp.

Table 6: Antibiotic Sensitivity Pattern of Isolates from Slightly Spoilt Oranges

Sample Code	IMI	MEM	COT	GEN	OXA	CAZ	AUG	CIP	ERY	VAN	CHL	MAR INDEX	R%	PROBANLE ORAGANISM
Os 1	**S**	**S**	**S**	**S**	**I**	**R**	**S**	**S**	**S**	**R**	**S**	**0.2**	**18.2**	*Bacillus* sp.
Os 2	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**S**	**0.1**	**9.1**	*Staphylococcus* sp.
Os 3	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**S**	**S**	**S**	**0.2**	**18.2**	*Bacillus* sp.
Os 4	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**0.1**	**9.1**	*Bacillus* sp.
Os 5	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**S**	**R**	**S**	**S**	**0.3**	**27.3**	*Bacillus* sp.

KEY:
R indicates Resistance
S indicates Susceptible
CHL indicates Chloramphenicol
IMI indicates Imipenem
ERY indicates Erythromycin
CIP indicates Ciprofloxacin
VAN indicates Vancomycin
MEM indicates Meropenem
COT indicates Cotrimoxazole
GEN indicates Gentamicin
OXO indicates Oxacillin
CAZ indicates Ceftazidine
AUG indicates Augmentine

Table 7: Minimum Inhibitory Concentration Assay for Antifungal Drugs Against Isolates from Slightly Spoilt Oranges

Isolate	Antibiotics											
	Griseofulvin	**(mg/ml)**			**Nystatin**	**(mg/ml)**			**Fluconazole**	**(mg/ml)**		
	25	12.5	6.25	3.13	1.3	0.65	0.33	0.163	10	5	2.5	1.25
Chrysonilia sp.	- (R)	46 (S)	40 (S)	46 (S)	40(S)	40(S)	48(S)	50(S)	42(S)	46(S)	42(S)	- (R)
Chrysonilia sp.	- (R)	42(S)	-(R)	- (R)	50(S)	40(S)	40(S)	50(S)	-(R)	-(R)	-(R)	46(S)
Chrysonilia sp.	- (R)	48 (S)	40(S)	46(S)	46(S)	50(S)	40(S)	48(S)	-(R)	-(R)	-(R)	46 (S)
Chrysonilia sp.	46 (S)	38(S)	40 (S)	44(S)	60 (S)	62(S)	46(S)	48(S)	- (R)	-(R)	46(S)	48 (S)

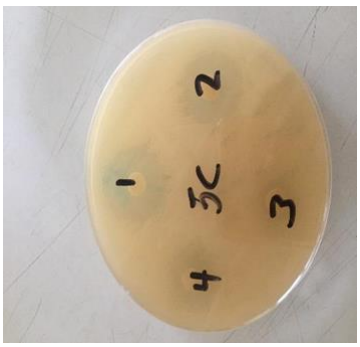

PLATE 1a: Zone of Inhibition against *Bacillus* sp. (**Os 5)**

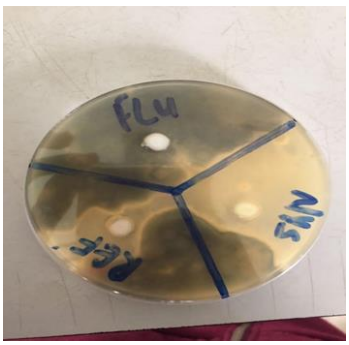

PLATE 1b: Zone of Inhibition against *Chrysonilia* sp.

4. Discussion

The physicochemical analysis proved that the uncontaminated healthy orange is satisfactory for human consumption as it has natural minerals and vitamins necessary for human growth and development, whereas the slightly spoilt orange is unsatisfactory for consumption. Majority of the physicochemical parameters of slightly spoilt orange showed that they had lower values compared to the healthy counterpart. For instance, the fiber content was observed that slightly spoilt orange had lower fiber content, this could be a result of the invasion and utilization by foodborne pathogens.

Microorganisms were isolated from the orange samples used for this study. Oranges naturally contain microorganisms, but they can also be introduced by external factors such soil, water, wind, insects, animals, and human contact. Oranges may become contaminated when being grown, harvested, transported, and/or processed into final goods. *Bacillus* spp. have been dominantly found to be associated with spoilt fruits [16]. Food poisoning from certain *Bacillus* species can result in nausea, vomiting, and abdominal pain, among other forms of intoxication [17]. Additionally, they may result in anthracis [17].

Chrysonilia sp. isolated in this study, though not known as a primary fungal pathogen that causes severe infections in humans. Fruit rotting caused by fungi is also acknowledged as a possible health risk to both humans and animals. Their ability to produce mycotoxins—naturally occurring toxic chemicals with an aromatic structure—that can cause mycotoxicosis in humans after consumption or inhalation is the reason for this. Their toxicity levels and modes vary [18]. Eighty-three percent of the citrus fruit analyzed in a study by Al-Hindi *et al*. [19] displayed fungal development at levels ranging from 25 to 100%. Fungal degradation in storage and its relationship to shops (local storage locations) have been investigated in orange [19].

The antibiotics susceptibility test revealed that three of the isolates which are *Bacillus* spp, showed the highest level of resistance to the antibiotics compared to others. It is worth noting that researchers such as Jia *et al*. [20] have also isolated resistant *Staphylococcus* species in ready to eat fruits.

The antifungal assay was carried out to evaluate which of the isolates is resistant or susceptible to the antifungals used and at what concentrations. Most of the isolates were resistant to some concentrations of Fluconazole and Griseofulvin but were all susceptible to Nystatin. However, [21] in their study found that *Chrysonilia* sp. was sensitive to antifungals such as Ketonazole (KCA), Miconazole (MCN) and Econazole (ECN) with inhibition areas exceeding 30mm; less sensitive to amphotericin B, with 24 mm inhibition area and showed resistance to Nystatin (NY), Flucytosine (FY), Metronidazole (MTZ), and Griseofulvin (GF). Apart from Nystatin, the other antifungal agents showed concentration dependent activity, where *Chrysonilia* spp. were resistant to the antibiotics at high concentrations but susceptible at lower concentrations. Other studies have shown the possibility of concentration dependent activity, where the microbes exhibit this phenomenon [21].

5. Conclusion

Microorganisms are naturally present on the surface of oranges, and some can even grow inside the fruit tissue and can also be brought in by outside elements (water, soil, insect, animals, wind and human handling). They can become contaminated during growing, harvesting and transportation and/or processing into finished products. It can be inferred from this study that the isolated organisms are likely associated with the spoilage of oranges, since most of the healthy oranges had no visible microbial growth on the cultured medium. High numbers of these microorganisms in raw consumed orange could lead to the consumer's illness with attendant symptoms and consequences of the particular or combined microbial presence. It also indicated that fungi were involved in the spoilage of the fruits. Three of the *Bacillus* spp. were multidrug-resistant, and the fungal isolates were basically resistant to two out of three antifungal agents employed at different concentrations. This may pose a human health threat if these slightly spoilt oranges are continually consumed. Overall, this study reveals that these slightly spoilt oranges are unfit for consumption. Mechanical injuries such as bruises or cuts that occur during harvesting or post-harvesting, grading and packing could provide infection

sites for spoilage pathogens, hence these should not be kept but discarded, extensive education on this matter is crucial. This study is only preliminary; extensive molecular research on the associated microorganisms as well as investigation into the possible long-term risks of diseases via the ingestion of these drug resistant microbes in animal models is advocated.

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Conflict of Interest
The author declared no conflict of interest in the manuscript.

Authors' Declaration
The author(s) hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Author Contributions

Conceptualization – BMP; Design – B.M.P; Supervision – B.M.P; Resources – R.A.; Materials – R.A.; Data Collection and/or Processing – R.A.; Analysis and/or Interpretation - B.M.P; R.A; Literature Search -B.M.P., R.A; Writing – B.M.P., R.A.; Critical Reviews – B.M.P., R.A.

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