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Review

Production of food colourants by filamentous fungi

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Food colourants are pigments or dyes added to food to maintain, intensify or add colour to foods. Although, the initial natural sources of food colourants were plants and animals, these sources have become inadequate due to increase in demand. This led to the use of synthetic colourants, some of which have harmful effects on human. Filamentous fungi are good sources of colourants since they are capable of synthesizing large quantities of pigments with different colour sheds. Various genera of filamentous fungi such as *Monascus, Penicillium, Talaromyces* and *Fusarium,* have been used for colourant production. Some fungal pigments also have antimicrobial, antioxidant and cholesterol lowering effects. However, some fungi co-produce pigments with mycotoxins such as citrinin. It is therefore necessary to select non-citrinin producing fungal strains or employ culture conditions that limit citrinin biosynthesis. Production of fungal pigments is affected by some nutritional and environmental factors such as carbon and nitrogen sources, pH, temperature, light, moisture, agitation speed and dissolved oxygen concentration. This article highlights major species of pigment-producing filamentous fungi, antimicrobial activities of fungal pigments, and control of pigment and mycotoxin co-production by fungi. The nutritional and culture parameters that affect pigment production by the fungi are discussed in details.

Key words: Food colourant, pigments, fungi, antimicrobial substances, antioxidants, culture conditions.

INTRODUCTION

Food colourants are pigments or dyes added to food to achieve some objectives which include to (i) maintain the original colour of the food substance; (ii) intensify the original colour; (iii) add colour to a colourless food and (iv) preserve the food material (Barrows et al., 2003). The major objectives of adding food colourants are to improve the quality and make the food more attractive to consumers. With a steady increase in human population and civilization, there is an increased demand for food with long shelf life and other attractive qualities. In the early period, the sources of food colourants were mainly plants and animals. However, with the increase in demand for food colourants, these natural sources of colourants became inadequate and synthetic food colourants were introduced into the market. Synthetic food colourants were widely used but people have become more conscious of the health implications of synthetic colourants in food, cosmetics and pharmaceuticals (Arnold et al., 2012; Stevens et al., 2013, 2014). People's interest in natural sources of food colourants have been revitalized and research in ways of expanding sources of natural food colourants have become greatly

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revolutionized. The high demand for natural food colourants is reflected in the global market size which has been increasing steadily. It was estimated at 1.15 billion US dollars in 2007 (Mappari et al., 2008) and projected to reach 1.7 billion US dollars in 2020 (Singh and Tyagi, 2015). Rohan (2012) has projected the value to reach 2.3 billion US dollars in 2019. The increasing demand for and steady increase in the market size of natural food colourants has led to investigations into microbial pigments as natural sources of food colourants. Although, many groups of microorganisms such as fungi, algae, lichen and bacteria have been explored for colourant/pigment production, the present review will focus on food-grade colourant production by filamentous fungi.

One of the very interesting and useful features of fungi is their ability to produce secondary metabolites that are very useful to man. Some of the fungal secondary metabolites include antibiotics such as penicillin (Laich et al., 2002; Berg, 2010; Asnaashari et al., 2012), cephalosporin (Skatrud et al.,1989; DeModena et al., 1993; Muñiz et al., 2007; Rodríguez-Sáiz, 2009), cyclosporine (Moussaïf et al.,1997; Lee et al., 2008; Azam et al., 2012; Sharmila et al., 2012) and statin drugs (Singh and Pandey, 1999; Manzoni and Rollini, 2002; Singh and Pandey, 2013). Pigments are another group of fungal secondary metabolites with various useful applications. Fungal pigments are natural colourants and have several advantages over their synthetic counterparts. Natural colourants are biodegradable and environmentally friendly. Most of them are non-toxic, and can be produced using cheap raw materials. Different colour shades are produced by varying the culture conditions (Shi et al., 2015) while most of them are stable over a wide range of light intensity, temperature and pH (Mappari et al., 2005). However, for any pigment to be used as food colourant, it must be safe for human consumption.

The use of fungal pigments as food colourant has been in practice early in history even before 1884 when the French Botanist Tieghem characterized the fungus *Monascus* (Tieghem, 1884). Among the fungi, members of the Class, Ascomycetes are the most widely studied group for pigment production. Some of the fungal pigments that have been approved and currently used include Arpink red from *Penicillium oxalicum*, riboflavin from *Ashbya gossipii*, lycopene and Beta-carotene from *Blakeslea trispora* and *Monascus pigments* (Dufosse et al., 2013).

Aside from adding desired colours to foods, fungal pigments have other attractive qualities such as anti-mutagenic and antimicrobial (Visalakchi and Muthumary, 2010; Geweely, 2011; Teixeira, 2012); antioxidants (Shcherba et al., 2000; Cassia et al., 2005; Li et al., 2009; Gessler 2013); anti-cancerous and anti-obesity activities(Visalakchi and Muthumary, 2010; Feng et al., 2012). Production of fungal pigments is affected by both nutritional and environmental factors. Some review articles on production and uses of fungal pigments (Dufosse et al., 2014; Chen et al., 2015; AbdelGhany, 2015; Vendruscolo et al., 2015) have shown that fungi are major sources of renewable and reliable natural food colourants. However, commercial production of these pigments requires good understanding of the factors that affect their production.

In this review, the antimicrobial activities of fungal pigments, as well as production and control of pigments and mycotoxins co-production by fungi were highlighted. Important species of fungi used for pigment production were listed and nutritional and culture parameters that affect production of pigments by fungi were discussed in details.

ANTIMICROBIAL ACTIVITIES OF FUNGAL PIGMENTS

In addition to giving or maintaining desired colours in food, some of the food grade pigments produced by fungi can also be used as food preservatives, since some of them have been shown to have antimicrobial properties. For example, pigments produced by *Monascus purpureus* have been reported to inhibit the growth of both fungi such as *Aspergillus*, *Trichoderma, Mucor*, *Penicillium* and *Fusarium* species and some bacteria such as *Bacillus, Pseudomonas, Escherichia* and *Streptomyces* species (Ungureanu and Ferdes, 2010). The level of activity depends on the solvent used for pigment extraction and Gram positive bacteria seem to be more susceptible than the Gram negative bacteria (Ungureanu and Ferdes, 2010). Pigments from other fungi such as *Monodictys castaneae* SVJM139 (Visalakchi and Muthumary, 2010); *Sporobolomyces* sp. (Manimala and Murugesan, 2014), *Fusarium* sp. (Geweely, 2011; Mani et al., 2015), *Aspergillus* sp. (Geweely, 2011; Teixeira et al., 2012) and *Penicillium* species (Geweely, 2011; Teixeira et al., 2012) have also been reported to have antimicrobial activities against various species of fungi and bacteria. Activity of fungal pigments against other cells has also been investigated. Although, Teixeira et al. (2012) reported that pigment extracts from *Penicillium siplicissimum* DPUA1379 and *Penicillium janczewskii* DPUA 304 had very high degree of mortality to *Artemia salina* larvae, Jongrungrungchok et al. (2004) reported that monasone A which is the major metabolite of *Monascus kaoliang* showed no activity against malaria parasite (*Plasmodium falciparium*) and antitubercular activity against *Mycobacterium tuberculosis* H37Ra. Monasone also showed no cytotoxicity against BC breast cancer and Kb human epidermoid carcinoma of cavity cell lines. The antimicrobial activities of pigments produced by *Monascus* species can be enhanced by addition of some L- and D-amino acids to the culture medium during production. The minimum inhibitory concentration of

pigment derivatives with L-Phe, D-Phe, L-Tyr and D-Tyr against some Gram positive and negative bacteria, as well as some filamentous fungi such as *Aspergillus niger, Penicillium citrinum* and *Candida albicans* were enhanced from 32 mg/L (for the normal pigments) to 4-8 mg/L when L- and D-amino acids derived pigments were used (Kim et al., 2006). Whether or not the quantities of colourants added to produce the desired colour intensity are sufficient to prevent contamination depends on the type of pigment as well as the nature of food. This is because the antimicrobial activities vary with the type of pigment while different foods require different concentrations of pigments to produce the desired colour intensity. Zong-Xin and Dong-dong (2010) reported addition of 0.006% of *Monascus* red pigments, 0.003% carmines and 0.001% of fancy red (giving a total of 0.01%) to Sophia ham while Cheng-yun and Wen-Ping (2008) reported addition of 0.03 g of *Monascus* pigment per kg of sausage. El-Kholie et al. (2012) reported even a very high concentration of 0.8 g of *Monascus* pigments per kilogram of beef burger. Rojsuntornkitti et al. (2010) noted that addition of 0.1- 0.4 g of red rice powder to 100 g of Thai sausage was able to control the growth of *Salmonella, Staphylococcus aureus* and *Clostridium perfringens*.

Citrinin as a mycotoxin co-produced with pigments

One major concern in the use of fungal pigments as food colourants is that some species have been reported to produce some toxic compounds (mycotoxins) such as aflatoxin, ochratoxin, fumocin and citrinin (Chen et al., 2015). These mycotoxins have very negative effect on human health when consumed. For example, citrinin chemically known as (3R,4S)-8-hydroxy-3,4.5-trimethyl-6-oxo-4, 6-dihydro-3H-isochromene-7 -carboxylic acid is hepato and nephritoxic and it has some antibiotic activity against Gram positive bacteria. Citrinin was initially isolated from *Penicillium citrinum* and subsequently it has been found to be produced by a variety of fungi including *Aspergillus niveus, Aspergillus ochraceus*, *Aspergillus oryzae*, *Aspergillus terreus*, *Monascus ruber*, *Monascus purpureus* and *Penicillium camemberti* (Abou-Zeid, 2012). They are produced both in complex and chemically defined media (Hajjaj et al., 1997; Mossini and Kemmelmeier, 2008). Citrinni production is inherent in some strains and there is no relationship between pigment and citrinin synthesis (Pisareva et al., 2004; Carvalho et al., 2005).

Production of citrinin in *Monascus* spp. can be controlled by optimizing the fermentation conditions such as media components, aeration, pH and temperature (Hajjaj et al., 1999, Zhang et al., 2013), or screening for citrinin-free strains, and through genetic regulation (Carvalho et al., 2005; Chen and Hu, 2005; Jia et al., 2010; Feng et al., 2014; Kang et al., 2014; Chen et al., 2015). Abou-Zeid (2012) reported that aqueous extracts obtained from Neem (*Azadirachta indica* A. Juss) and other medicinal plants were able to reduce growth and citrinin production by *Penicillium citrinum* under *in vitro* conditions in liquid media. Hajjaj et al. (2000b) also reported that addition of medium chain fatty acids to the medium stimulated peroxisome proliferation. These peroxisomes produced hydrogen peroxides which degraded the citrinin or its intermediate in the fatty acid pathway, and thus resulted in very little or absence of citrinin in the pigment. Citrinin and other polyketides are at least partially synthesized by multifunctional enzymes called polyketide synthases (PKSs). The genes encoding these enzymes have often been reported to localize in an adjacent region or to form a gene cluster (Brown et al., 1999; Kennedy et al., 1999). Shimizu et al. (2005, 2007) reported that *pksCT* gene was responsible for citrinin biosynthesis in *M. purpureus*. Thus, it is possible to regulate citrinin production through genetic manipulation. For example, it has been reported that *Aspergillus oryzae* transformants containing only the CT gene cluster produced minimal quantities of citrinin, but introducing an additional activator gene (ctnA) enhanced the transcriptional level of each biosynthetic gene in the cluster, thereby elevating citrinin production more than 400-fold (Sakai et al., 2008). It has been reported that citrinin biosynthesis by *M. ruber* originates from a tetraketide instead of pentaketide as has been shown for *Aspergillus* and *Penicillium* species (Hajjaj et al., 1999). Production of the polyketide red pigments and citrinin by the fungus may therefore be regulated at the tetraketide branch point to avoid co-production of the red pigments with the mycotoxin. In summary, although some of the fungal genera used in the production of food grade colorants produce toxins there are some safe strains among them that are carefully selected either using molecular tool or through manipulation of culture conditions. For example, Frisvard et al. (2013) used chemotaxonomic tools to identify *Talaromyce astroroseus* which does not co-produce toxin with pigments. Reduction in citrinin production can be achieved by various strategies depending on the fungal strain used and the production process employed. The following strategies can be used to control coproduction of citrinin with fungal pigments:

1. Use of metabolic regulation at the tetraketide branch point (Hajjaj et al., 1999)
2. Addition of plant extract (Reddy et al., 2010; Abou-zeid, 2012)
3. Addition of medium chain fatty acids to the culture medium (Hajjaj et al., 2000b)
4. Selection of citrinin-free strains (Wang et al., 2004; Mapari et al., 2009).
5. Selection of appropriate culture system and control of culture conditions such as pH, temperature, and C/N ration (Zhang et al., 2013; Hajjaj et al., 2015). Even after

Table 1. *Monascus* species used for pigment production.

Fungi	Colour of pigment	References
Monascus purpureus strain FTC 5391	Red pigment	Musaalbakri et al., 2006
Monascus pilosus strain C1.	Yellow and red pigments	Pisareva and Kujumdzieva, 2010)
Monascus purpureus CMU001	Red pigment	Mimnoi and Lumyong, 2011
Monascus ruber	Red pigment	Fabre et al., 1993, Hajjaj, 2000a, Meinicke et al., 2012
Monascus pilosus MS-1.	Pigment	Feng et al., 2015
Monascus.	Red pigment	Lin and Demain 1994, 1995; Carels and Shepherd.1997
Monascus purpureus CCT3802	Red pigment and citrinin	Orozco and Kilikian, 2008
Monascus purpureus TISTR 3002; 3090; 3180; 3385	Pigments	Kongruang, 2010)
Monascus purpreus ATCC1603	Pigments	Baneshi et al., 2014
Monascus purpureus atcc 36928	Red pigment	Pereira et al., 2008
Monascus ruber ICMP 15220	Biopigment	Said et al., 2010
Monascus ruber strain HS. 4000	Pigment and citrinin	Yang et al., 2014
Monascus ruber	Pigments	Bühler et al., 2015
Monascus sp strain M9	Monascin and ankaflavin	Wang et al., 2015
Monascus purpureus	Red and yellow pigments	Hamdi et al., 1996; Lee et al., 2002; Babitha et al., 2007; Babitha et al., 2008; Velmurugan et al., 2010a; Mukherjee and Sigh, 2011
Monascus purpureus strain FTC 5391	Red pigment	Musaalbakri et al., 2006
Monascus mutant	Yellow pigment	Yongsmith et al., 2013)
Monascus purpureus	Red and yellow pigments	Hamdi, et al., 1996; Lee et al., 2002; Babitha et al., 2007; Babitha et al., 2008; Velmurugan et al., 2010; Mukherjee and Sigh 2011
Monascus purpreus ATCC1603	Pigments	Baneshi et al., 2014
Monascus ruber ICMP 15220	Red colorant	Said et al., 2010
Monascus purpureus KACC 42430	Pigments	Velmurugan et al., 2011
Monascus purpureus 192F	Monascin, ankaflavin, rubropunctatin, monascorubrin, and monascorubramine)	Chen and Johns, 1993
Monascus sp. J 101	Monascus pigments	Kim et al., 2002

Table 2. *Penicillium* species used for pigment production.

Organism	Colour of pigment	References
Penicillium aculeatum ATCC 10409	Yellow pigments	Afshari et al., 2015
Penicillium herbarum	Pigments	Geweely, 2011
Penicillium purpurogenum DPUA 1275	Red colourant	Santos-Ebinuma et al., 2013; Ventura et al., 2013
Penicillium purpurogenum GH2	*Pigments*	Méndez et al., 2012
Penicillium purpurogenium	Pigments	Geweely 2011
Penicillium sp.	Red pigment	Gunasekaran and Poorniammal, 2008
Penicillium sp (DLR-7)	Red pigment	Chintapenta et al., 2014

using these strategies to reduce mycotoxin production, it is very important to separate the toxins from the colourants and ensure that the colourants do not contain any toxin before use in food industries.

FUNGI SPECIES USED FOR PIGMENT PRODUCTION

Many species of fungi have been used for pigment production. As shown in Tables 1 to 3, *Monascus*, *Penicillum* and *Fusarium* species are the most extensively studied but there are also many other species that produce various colors of pigments. The type (colour) of the pigments is not species-specific as many strains are able to produce the same colour while a single species is capable of simultaneously producing various colours under the same culture condition. Although, the chemical nature of some of the pigments is known, most other pigments are not yet characterized, and in some papers, even the colour of the pigments was not stated.

Table 3. Other fungi species used for pigment production.

Fungi	Colour of pigment	Reference
Alternaria alternate	Pigments	Geweely, 2011; Sharma, 2012
Alternaria sp.	Yellow pigments	Mawthols et al., 2005
Aspergillus flavus	Pigments	Geweely, 2011
Aspergillus niger	Pigments	Mawthols et al., 2005
Emericella nidulans	Red and yellow pigments,	Velmurugan et al., 2010
Fusarium aquaeductuum	Carotenoid	Rau and Rau-Hund ,1977
Fusarium bulbigenium	Naphthaquinone pigments	Medentsev et al., 2005
Fusarium decemcellulare F.-1179	Naphthaquinone pigments	Medentsev et al., 2005
Fusarium graminearum	Naphthaquinone pigments	Medentsev et al., 2005
Fusarium moniforme	Pigments	Geweely, 2011
Fusarium moniliforme KUMBF1201	Pigments	Pradeep et al., 2013
Fusarium verticillioides	Red and yellow pigments	Velmurugan et al., 2010
Fusarium sp.		Mawthols et al., 2005
Isaria farinose	Red water soluble Pigment	Velmurugan et al., 2010b
Monodictys castaneae SVJM139	Pigments with antimicrobial activity	Visalakchi and Muthumary 2010
Neurospora crassa	Carotenoid	Rau and Rau-Hund ,1977; Iigusa et al., 2005
Paecilomyces sp.	Pigments	Cho et al., 2002
Pyrenochaeta terrestris	Pigments	Gunasekaran and Weber 1981
Pycnoporus spp HEMIM-55 and 80.	Pigments	Hernández et al., 2014
Rhodotorula glutinis DFR PDY	Carotenoids	Latha and Jeevaratnam, 2010 (Non-filamentous)
Scytallidium cuboideum	Red and blue	Tudor et al.,2013
Talaromyces amestolkiae GT11	Red and yellow	General et al., 2014
Talaromyces astroroseus	Red pigments	Frisvad et al., 2013
Talaromyces purpurogenus	Red, orange and yellow pigments	Ogbonna et al., 2013; Ugwu et al., 2014
Trametes versicolor.	Red and blue	Tudor et al., 2013
Trichoderma virens,	Red	Sharma, 2012
Xanthophyllomyces dendrorhous DSM 5626	Astaxanthin	Stachowiak, 2013
Xylaria polymorpha	Red and blue	Tudor et al., 2013
Epicoccum nigrum	Natural colorant	Mapari et al., 2008

FACTORS THAT AFFECT PIGMENT PRODUCTION BY FUNGI

Production of pigments by fungi is affected by the type and concentrations of nutrients such as carbon, nitrogen and some micronutrients, as well as some environmental and physicochemical conditions such as pH, temperature, dissolved oxygen concentration, agitation speed, light and moisture content. These factors are discussed below.

Effect of carbon sources on pigment production by filamentous fungi

Organic carbon sources are the main sources of carbon and energy for growth and metabolite production by heterotrophic microorganisms such as fungi. Fungi species are able to metabolize various types of carbon sources as both carbon and energy sources. However, the optima organic carbon source for growth and pigment production depends on the fungi. It has been reported that *Penicillium* spp. can grow and produce pigments in submerged cultures containing glucose, fructose, dextrose, lactose, sucrose, maltose, mannose, galactose, soluble starch, xylose or glycerol but glucose was the best (Gunasekaran and Poorniammal, 2008). However, the optimum carbon source may vary even among the same strain. For example, potato dextrose broth was reported to be the best for *Penicillium* strain DLR-7 (Chintapenta et al., 2014) while sucrose was reported to be the best for *Penicillium purpurogenum* DPUA 1275 (Santos-Ebinuma et al., 2013).

In the case of *Monascus* species, Pisareva and Kujumdzieva, (2010) compared various sugars and alcohols as the carbon source and reported that glucose was the best carbon source for both growth and pigment production by *Monascus pilosus* strain C1, and that there were no growth and no pigment produced by this strain in

media that contained lactose and galactose as carbon sources. Also, Musaalbakri et al. (2006) reported that for *Monascus purpureus* strain FTC 5391, among glucose, potato starch and rice starch, glucose was the best carbon source for red pigment production by monospore strain 3 but for monospore strain 4, the highest red pigment concentration was obtained when potato starch was used as the carbon source while rice starch was the optimum carbon source for red pigment production by monospore strain 5. On the other hand, Chen and Johns (1993) reported that maltose was better than glucose when peptone was used as the nitrogen source for *Monascus purpureus* 192F. While soluble starch was used for *Paecilomyces sinclairii* (Cho et al., 2002), potato dextrose agar or broth was the best for *Fusarium moniliforme* KUMBF1201 (Pradeep et al., 2013*)* and glucose or sucrose was the best for *Isaria farinosa* (Velmurugan et al., 2010).

Agricultural feedstocks such as grated jackfruit seeds (Subhasree et al., 2011), rice grains, corn meal, mugbean, soybean, potato, sweet potato, cassava tubers, peanut meal, coconut residue have also been investigated for growth and pigment production by *Monascus* species (Nimnoi and Lumyong, 2011; Yongsmith et al., 2013) but the results showed that supplementation with mono and di-saccharides such as glucose, fructose, lactose, galactose and sorbose improved growth and pigment productivity from agricultural feedstocks (Subhasree et al., 2011). It is also important to note that the optima carbon source for growth may not be the optima for pigment production. For example, Nimnoi and Lumyong (2011) reported that potato dextrose broth was the best for pigment production but maximum cell biomass was obtained when *Monascus purpureus* CMU001 was cultivated on glucose. On the whole, it appears that the best carbon source depends on the strain, other media components and the target pigment.

The optimum concentrations of these carbon sources vary widely from 250 g/L for *M. purpreus* ATCC1603 (Baneshi et al., 2014) to 40 g/L of glycerol for *Monascus pilosus* MS-1 (Feng et al., 2015), 40- 70 g/L of glycerol for *Monascus ruber* (Meinicke et al., 2012), 20 g/L of glucose when peptone was used as the nitrogen source (Gunasekaran and Poorniammal, 2008), 20 g/L of xylose for *Penicillium* (Chintapenta et al,. 2014) to 50 g/L of sucrose for *Penicillium purpurogenum* (Santos-Ebinuma et al., 2013). For the same strain, the optimum carbon source concentration may even depend on the type of pigment. In the case of *M. purpureus* 192F, for example, the yellow pigment (monascrubrimin) and red pigments (monascrubramine) production were favoured by low initial glucose concentration of 20 g/L. However, ankaflvin production was favoured by higher initial glucose concentrations (Chen and Johns, 1993). In the case of *Paecilomyces sinclairii*, much lower soluble starch concentration of 15 g/L was reported to be the optimum (Cho et al., 2002).

Effect of nitrogen source on pigment production by fungi

Just like carbon source, nitrogen source is required by various species of fungi for growth and synthesis of both primary and secondary metabolites. Different kinds of compounds have been investigated as nitrogen source for pigment production by various fungi. The effects of both inorganic and organic nitrogen sources on cell growth and pigment production by various species of fungi have been investigated. The various results imply that inorganic compounds are not good nitrogen sources for pigment production by filamentous fungi. For example, Lin and Demain (1995) reported that ammonium nitrate was not good for pigment production by resting cells of *Monascus* sp. as it had inhibitory effect on the action of pigment synthase(s). Also, in comparison with ammonium sulphate and ammonium nitrate, peptone and especially yeast extract stimulated production of both red and yellow pigments by *M. purpureus*. Velmurugan et al. (2010) also reported that for *Isaria farinosa*, the optimum nitrogen sources for pigment production were yeast extract, meat extract, peptone and monosodium glutamate. On the other hand, Carels and Shepherd (1997) reported that organic nitrogen sources stimulated growth but did not favour pigment production by *Monascus* sp.

Although amino acids are considered growth factors that stimulate growth of many species of microorganisms, it does appear that they are not good nitrogen sources for pigment production by filamentous fungi. Lin and Demain (1994) noted that many amino acids such as leucine, valine, lycine and methionine had negative effect on pigment production by *Monascus* sp. They further explained that leucine enhanced the decay of pigment synthase(s) which brought about poor pigment production. Furthermore, Chintapenta et al. (2014) reported that in the case of *Penicillium* strain (DLR-7), basic amino acids such as arginine and lysine did not support pigment production. However, they noted that acidic amino acids such as aspartic acid and glutamic acid enhanced pigment production.

It does appear that the type of nitrogen source affects the pH and thus the colour of the produced pigments. In the case of *Monascus,* Carels and Shepherd (1997) reported that when yeast extract or nitrate was used as the nitrogen source, the pH of the medium was 6.5 and red pigment was produced. On the other hand, when ammonium sulphate or ammonium nitrate was used as the nitrogen source, the pH of the medium was very acidic (2.5) and the produced pigment was orange in colour. Shi et al. (2015) also reported that *Monascus* sp. produced predominantly yellow pigments in a medium containing peptone but when ammonium sulphate was used as the nitrogen source, red pigment predominated. Pisareva and Kujumdzieva (2010) also reported that physiologically alkaline nitrogen sources such as sodium glutamate favoured pigment production by *M. pilosus* strain C1, while physiologically acidic nitrogen sources

Table 4. Optima pH for pigment production by some species of fungi.

Organism	Optimum pH	References
Trametes versicolor	4.5	Tudor et al. (2013)
Xylaria polymorpha	4.5-5.0	Tudor et al. (2013)
Fusarium	3.5	Mawthols et al. (2005)
Alternaria.	5.5-6.5	Mawthols et al. (2005)
Isaria farinosa	5.0	Velmurugan et al. (2010)
Monodictys castaneae SVJM139	5.0	Visalakchi and Muthumary (2010)
Penicillium purpurogenum GH2	5.0	Méndez et al. (2012)
Pyrenochaeta terrestris	6.5	Gunasekaran and Weber (1981)
Paecilomyces sinclairii	6.0	Cho et al. (2002)
Fusarium moniliforme KUMBF1201	5.5	Pradeep et al. (2013)
Talaromyces amestolkiae GT11	7.0	General et al. (2014)

such as urea were better for cell growth.

Effects of pH on pigment production by filamentous fungi

Hydrogen ion concentration is a very important factor that affects metabolite production by fungi. The pH of the environment affects most aspects of the production process such as the cellular metabolism and nutrient absorption and utilization by the organism. As a reaction to a stressed environment, some lignicolous fungi respond with pigment formation that helps to isolate and protect their mycelia from other fungi, while some species produce pigments regardless of the changes in the conditions in the environments in which they grow. The effects of pH depend on the species of fungi as well as on the type of pigments. Chen and Johns (1993) reported that cell growth and ankaflavin production by *M. purpureus* were favoured at pH 4.0, whereas production of other pigments by the organism was relatively independent on pH. However, it seems that for most of the species, acidic pH favours pigment production. Although, the optimum pH for some strains such as *M. purpreus* ATCC1603 can be as low as 3 (Baneshi et al., 2014), for the majority of strains the optimum pH ranges from 4 to neutral as shown in Table 4. However, alkaline pH has also been reported to be favourable for pigment production by some fungi. For example, Mawthols et al. (2005) reported that the optimum pH for *A. niger* was 8.5 while Gunasekaran and Poorniammal (2008) reported that biomass and pigment production by *Penicillium* sp. was best at pH 9.0.

It is important to note, however, that the optimum pH for pigment production may not be the same for cell growth. For example, Hernández et al. (2014) reported that the optimum pH values for growth and pigment production by two strains of *Pycnoporus* were 5.5 and 6.5, respectivel while Afshari et al. (2015) noted that for *Penicillium aculeatum* ATCC 10409, the highest concentration of yellow pigment was obtained with an initial pH value of 6.5 while the maximum biomass concentration was obtained at pH value of 8. Furthermore, Orozco and Kilikian (2008) reported that the highest pigment production by *M. purpureus* CCT3802 was obtained when the growth phase was at pH 5.5 and the production phase at pH 8.5. They reported that pH also affects pigment secretion by fungi, and that the alkaline pH of 8.5 reduced the intracellular pigments from 75 to 17% of the total pigment. Furthermore, various reports have indicated that pH affects the colour of pigments produced. Cho et al. (2002) reported that the pigment colour was strongly dependent on the pH of the solution. They reported that the colour of the pigment produced by *Paecilomyces sinclairii* was red at pH 3-4, violet at pH 5–9 and pink at pH 10-12. Also Chintapenta et al. (2014) reported pH 3.0 as the optimum for red pigment production by *Penicillium* strain (DLR-7) while at pH 2.0, yellow fluorescent pigment was produced instead of red and spores were completely absent. Tudor et al. (2013) further reported that *Scytallidium cuboideum* produced maximum red pigment at pH 6.0 and blue pigment at pH 8.0. Shi et al. (2015) also noted that for *Monascus* sp. orange colour was produced at a pH of 2.5 - 4.0 but at a pH of 6.5, the colour of the pigments depended on the type of nitrogen source used. Furthermore, *Fusarium* species produce naphthoquinone pigments which are known for their wide range of biological activities including phytotoxicity, insecticidal, antibacterial and fungicidal properties. It has been reported that at pH less than 4.0, naphthasarins which is composed of fusarubin, javanicin and hostricoidin were produced but at pH of 8.0, only the dimeric naphthoquinone (aurofusarin) was produced (Baker and Tatum, 1998).

Effect of temperature on pigment production by filamentous fungi

In addition to nutrition, physicochemical factor such as

Table 5. Optima temperature for pigment production by some species of fungi.

Organism	Optimum temperature (°C)	References
Penicillium aculeatum ATCC 10409	30	Afshari et al. (2015)
Talaromyces amestolkiae GT11	28	General et al. (2014)
Penicillium purpurogenum GH2.	24	Méndez et al. (2012)
Paecilomyces sinchlairii	25	Cho et al. (2002)
Penicillium strain (DLR-7)	25	Chintapenta et al. (2014)
Monascus ruber	30	Said et al. (2010)
Monascus purpureus CMU001	30	Nimnoi and Lumyong (2011)
Monascus purpreus ATCC1603	25	Baneshi et al. (2014)
Isaria farinosa	27	Velmurugan et al. (2010)

temperature is a major factor that affects pigments and other metabolite production by fungi. Reports on the effects of temperature on cell growth and pigment production by various species of fungi indicate that the optimum temperature ranges from 24 to 30°C regardless of the strain (Table 5). However, Babitha et al. (2007) reported that high temperature greater than 45°C resulted in production of high concentration of yellow pigments by *Monascus* sp. Generally, it is known that temperature affects the membrane fluidity and thus, the uptake of nutrients and excretion of products by microorganisms.

Effect of agitation and dissolved oxygen concentration on pigment production by filamentous fungi

Agitation and aeration are very important factors in submerged cultivation of fungi. Aside from improving mass transfer in culture, agitation also helps to prevent sedimentation of the cells and maintain homogenous condition inside the bioreactor. However, agitation is associated with high hydrodynamic stress which often has negative effects on the growth and metabolite formation by filamentous cells. Various reports have shown that dissolved oxygen concentration and agitation speed affect growth and pigment production by different species of fungi. Hamdi et al. (1996) reported that under low dissolved oxygen concentration, *M. purpureus* first converts the carbon source to ethanol and later convert the ethanol to red pigments. Thus it is necessary to maintain aerobic condition (pCO2 = 10%) for efficient production of pigment by *M. purpureus*. Pereira et al. (2008) reported that in the case of *M. purpureus* ATCC 36928, maintaining aerobic condition resulted in high pigment production but low citrinin concentration. At the optima dissolved oxygen concentration and agitation speed of 60% and 600 rpm, respectively, the pigment concentration was maximum while citrinin concentration decreased to half of the maximum concentration. Yang et al. (2014) also demonstrated that aeration can be used to maximize pigment production while keeping citrinin production low. Using *Monascus ruber* strain HS 4000, they showed that by maintaining the flask shake speed at 150 rpm in the first 48 h, and then increasing it to 250 rpm between 48-108 h, and finally reducing it to 200 rpm between the 108th and 120th h, pigment production was very high while citrinin concentration was lower than the values obtained at constant shake speed of 250 rpm. In the case of packed bed solid state culture of *M. ruber* ICMP 15220, forced aeration was necessary for efficient pigment production but at aeration rates higher than 0.5 L/min, pigment production decreased due to water loss from the bed (Said et al., 2010). It is also important to note that the optima agitation speed for cell growth may be different from the optima value for pigment production. For example, Gunasekaran and Poorniammal (2008) reported that an agitation speed of 200 rpm was the optimum for red pigment production by *Penicillium* sp. in shake flask culture. However, in flask cultures, Afshari et al. (2015) reported that shake speed of 100 rpm was the optimum for the growth of *Penicillium aculeatum* ATCC 10409 but pigment production was higher at 150 rpm. This was consistent with the work of Velmurugan et al. (2010) which showed that agitation speed of 150 rpm was the optimum for red water soluble pigment production by *Isaria farinosa.*

Effect of light on pigment production by filamentous fungi

Fungi, like other living organisms, respond to light during growth and metabolite production. The effects of light on pigment production by fungi have been studied by many researchers. Buhler et al. (2015) reported that during cultivation of *M. ruber*, growth and pigment production were inhibited in Petri dishes and baffled flasks exposed to direct illumination. Velmurugan et al. (2010b) also noted that growth and pigment production by *M. purpureus, Isaria farinosa*, *Emericella nidulans*, *Fusarium verticillioides* and *Penicillium purpurogenum* were higher under dark condition than when exposed to lights of various wavelengths. In the case of *M. purpureus*, Babitha

et al. (2008) reported that incubation in total darkness increased red pigment production but illumination resulted in total suppression of pigment production. On the other hand, Wang et al. (2015) reported stimulatory effects of low light intensities on pigment production by *Monascus* species. In their report, when the culture was illuminated at constant intensity of 100 lux, monascin and ankaflavin production increased with exposure time while at constant exposure time of 15 min/day, light intensity of 200 lux gave higher pigment concentration than 100 lux. However, both monascin and ankaflavin production decreased when exposed to light intensities higher than 300 lux.

The effect of light on growth and pigment production by fungi may depend on the light wavelength. Bühler et al. (2015) reported that during cultivation of *M. ruber*, both pigment and biomass were higher under red light than in dark but highly inhibited by direct exposure to white light. Wang et al. (2015) also reported that monascin production increased by about 15 to 27% when grown under blue light of different intensities and durations while Velmurugan et al. (2010) reported that even yellow light inhibited both pigment and biomass production by *M. purpureus*.

Furthermore, the effect of light illumination depends on the target pigment. Generally, caretenoids synthesis is stimulated by light illumination. Stachowiak (2013) reported that the highest asthaxanthin yields by *Xanthophyllomyces dendrorhous* DSM 5626 was obtained in cultures at 600 lux while Rau and Rau-Hund (1977) reported that illumination of dark grown *Fusarium aquaeductuum* and *Neurospora crassa* resulted in increased carotenoid synthesis. The stimulatory effect of light on carotenoid production is attributed to the effect of oxygen radicals. Iigusa et al. (2005) reported that when *N. crassa* was treated with a high concentrations of oxygen gas and H_2O_2 to release radical oxygen species, an enhanced light-induced carotenoid accumulation and the expression of gene related to light-inducible carotenogenesis was observed.

Effect of moisture content on pigment production in solid state culture

In the case of solid state production of pigments, the moisture contents of the substrate had profound effects on cell growth and pigment production. However, the optimum moisture content depends on the species of fungi as well as on the nature of the substrate. Tudor et al. (2012) reported that *Trametes versicolor* and *Xylaria polymorpha* were stimulated to form pigments at moisture contents below 28 and 38% in *Acer saccharum* (sugar maple) and *Fagus grandifolia* (American beech), respectively. However, *Inonotus hispidus* and *Polyporus squamosus* were stimulated to produce pigments at moisture contents above 22-28% and 34-38% in beech and sugar maple, respectively. *Fomes fomentarius* and *Polyporus brumalis* produced maximum pigmentation in beech at 26-41% and at 59-96% moisture content in sugar maple. *Scytalidium cuboideum* pigmented both wood species at moisture content above 35%. Even for the same *M. purpureus*, the optimum moisture contents varied between 42 and 60% (Lee et al., 2002; Babitha, 2007; Velmurugan et al., 2011; Yongsmith et al., 2013). On the other hand, Said et al. (2010) reported that maximum pigment concentration and productivity by *M. ruber* ICMP 15220 were obtained under an initial moisture content of 70%. They further explained that low initial moisture content of 45% resulted in a very low biomass and pigment production.

CONCLUSION

1. Fungal pigments are not only used as food colouring agents, they can also serve as food preservatives.
2. Although, some fungi co-produce the mycotoxin citrinin with pigments, citrinin biosynthesis in fungi can be controlled through strain selection, modification of the culture conditions, use of medicinal plant extracts and genetic modification of fungal strains.
3. *Monascus* spp. was the first and mostly studied fungal species for food colourant production but presently many other fungal species including *Penicillium*, *Fusarium*, *Aspergillus*, *Talaromyces* and *Paecilomyces* have been employed in the production of fungal pigments.
4. Both simple and complex carbohydrates have been used as organic carbon sources for pigment production by filamentous fungi. However, glucose has been the most frequently utilized organic carbon source for the production of fungal pigments.
5. Research results have demonstrated that organic nitrogen sources are preferred to inorganic ones for pigment production by most species of fungi. It has been reported that the optimum nitrogen source for cell growth may differ from that for pigment production and generally physiologically alkaline nitrogen source were better for pigment synthesis while biomass production preferred acidic nitrogen source by many strains of fungi.
7. The optimum pH values for pigment production by most fungal strains ranged between 4 and 7. However, a few strains have their optimum pH as low as 3 and others as high as pH 9. The optimum pH also depends on the target pigment.
8. The optimum temperature for pigment production by most species of fungi ranged from 24 to 30°C
9. The optimum agitation speed for pigment production ranges from 150 to 200 rpm. However, the optimum agitation speed for cell growth was reported to be about 100 rpm.
10. Generally, pigment production was higher in dark. Although low light intensity of some wavelengths stimulated pigment production, high intensity of white

light inhibited pigment production by many strains of fungi. On the other hand, synthesis of carotenoid pigments was reported to be stimulated by low light illumination.

11. Moisture content is an important parameter for pigment production in solid substrate. The optimum moisture content ranged from 22- 60% and moisture requirements varied from species to species. In some extreme cases, moisture content requirement as high as 96% was reported in some fungal species.

Conflict of Interests

The authors have not declared any conflict of interests.

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Antibacterial properties of wild edible and non-edible mushrooms found in Zimbabwe

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Mushrooms have been used extensively in traditional medicine as antimicrobial, antiviral and antitumor agents. Infectious diseases remain a major threat to human health, due to global antimicrobial resistance. This has led to an increase in the search for new and potent antimicrobial substances. The aim of the present study was to investigate the antimicrobial activity of the aqueous (cold and hot) and organic solvents (methanol, ethanol and acetone) extracts of ten mushroom species collected from the woodlands in Zimbabwe against common local bacterial isolates *Escherichia coli, Salmonella typhi, Staphylococcus aureus* and *Streptococcus pneumoniae* using agar disc diffusion method. The crude extracts of the mushrooms exhibited antibacterial properties to all the bacteria tested. Extracts obtained from ethanol were the most effective tested against bacteria (36.5%), followed by methanol (30.8%) and acetone (30.8%). Aqueous extracts exhibited the lowest effect on bacterial growth inhibition (1.9%), despite including the extract with the highest inhibitory activity (14 mm). The acetone extract of *Cantharellus symoensii* had the second highest inhibitory value of 11.5 mm followed by the methanol extract from *Cantharellus miomboensis* and the ethanol extracts of *Ganoderma lucidum* and *C. symoensii* with values 11.0, 10.67 and 10.0 mm, respectively. *Cantharellus heinemannianus* and *C. symoensii* had the highest effect on inhibition of bacteria as indicated by the different extracts showing high inhibitory properties ranging from 8-14 mm [15.4% (8) each] followed by *G. lucidum* [13.5% (7)], while *Boletus edulis, Coprinus* sp. and *Trametes strumosa* had the least [5.8% (3) each]. The positive results of screening local mushrooms for antibacterial activity forms the basis for further phytochemical studies and development of antimicrobial agents against common human bacterial and fungal infections.

Key words: Antibacterial activity, *Cantharellus* species, *Salmonella typhi*, organic extracts, aqueous extracts.

INTRODUCTION

The emergence of drug resistance globally, is currently presenting a large and growing problem in infections that account for most of Africa's disease burden, including tuberculosis (TB), respiratory and diarrheal diseases (Okon et al., 2013; Padmavathy et al., 2014; Sangeeth et al., 2014). In addition to the multi-drug resistance problem,

the nosocomial infections (healthcare-associated infections) are associated with high mortality. This has necessitated a need for a continuous search and development of novel antimicrobial substances from different biological sources to minimize the threat of further antimicrobial resistance (Padmavathy et al., 2014; Shah et al., 2014).

Mushrooms have been recognized as functional foods and as a source for the development of medicines and nutraceuticals (Alves et al., 2012). Basidiomycetes, to which mushrooms belong, are a group of higher fungi with distinctive fruiting bodies and reproductive structures. Some mushrooms are edible, while others are extremely poisonous. There are about 140 000 species of mushrooms and of these, only 22 000 are known, while only a small percentage (5%) has been investigated (Faridur et al., 2010). Mushrooms have been prescribed for treatment of various human diseases such as gastro-intestinal disorder, bleeding, high blood pressure and various microbial infections (Akyuz, 2010; Gbolagade and Fasidi, 2005). Many varieties of mushrooms have been identified as major sources of biologically active natural products, such as oxalic acid and sulphated lentinan from *Lentinula edodes*, triterpenes and ganodermin, an antifungal protein, both from *Ganoderma lucidum*, polysaccharopeptides from *Coriolus versicolor*, water-soluble lignins from *Inonotus obliquus* and velutin, a ribosome inactivating protein from *Flammulina velutipes* (Chaudhary and Tripathy, 2015; Collins and Ng, 1997; Lindequist, 2005; Moon and Lo, 2014; Wang and Ng, 2006). These compounds may be sources of natural antibiotics and may have immunomodulatory, cardio-vascular, antifibrotic, anti-inflammatory, antidiabetic, antioxidant, antiviral, antimicrobial and antitumor properties (Alves et al., 2012; Gan et al., 2013; Geethangili et al., 2013; Ramesh and Pattar, 2010; Tehrani et al., 2012; Wang and Ng, 2004).

In recent years, a number of studies were conducted in various countries to determine the potential therapeutic properties of mushrooms. The reported bioactivities from mushrooms include antibacterial, antifungal, antioxidant and antiviral properties (Padmavathy et al., 2014; Reis et al., 2011). The species C*antherellus, Lentinus, Russula, Agaricus* and *Pleurotus* are examples of mushrooms that have shown antimicrobial properties against *Bacillus* species, *Enterococcus*, *Streptococcus*, *Staphylococcus* and *Micrococcus* species (Alves et al., 2012; Khan and Tania, 2012; Pushpa and Purushothama, 2010). Crude organic and aqueous extracts from *Ganoderma* have been reported to inhibit *in vitro* growth of *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Neisseria meningitides*, *Alcaligenes faecalis* and *Proteus vulgaris,* bacteria known to cause wound infections, intestinal and urinary-genital tract infections and skin infections (Shikongo et al., 2013). The European *Ganoderma* has been reported to inhibit growth of most bacteria especially methillicin-resistant *S. aureas* (Linderquist et al., 2005).

Zimbabwe is rich in mushroom diversity. However, the potential of mushrooms as source of new drugs is still largely unexplored (Sharp, 2011, 2014). Despite many studies on potential therapeutic properties of different mushroom species globally, little or no work has been carried out on the antimicrobial activities of mushrooms in Zimbabwe. In addition, there are several wild edible species of mushrooms which are yet to be exploited in Zimbabwe. Thus, the main aim of this work was to investigate the antimicrobial potential of different extracts of ten selected wild edible and non-edible mushrooms found in Zimbabwe.

MATERIALS AND METHODS

Collection of samples

A total of ten different mushrooms, both edible and non-edible, were collected from the local woodlands of Zimbabwe (Table 1). Identification of the mushrooms (Figure 1) was done on the basis of morphological characteristics, including colour of the mushroom cap and spore print. Final identification was done by comparing the visual appearance and the recorded characters of mushroom species with standard mushroom collection guides by Sharp (2011) and Ryvarden et al. (1994).

Test microorganisms

A total of four bacteria, *E. coli, S. typhi, S. aureus* and *S. pneumoniae* were used in this study. *E. coli* and *S. aureus* were obtained from the Cimas Medical Aid Society laboratory, *S. pneumoniae* from Lancet laboratory and *S. typhi* from the University of Zimbabwe. The bacterial strains tested were isolated from local patients.

Preparation of mushroom crude extracts

The fresh mushrooms were sliced into thin strips and sun dried for 7 days. Dried mushrooms were ground to powder using an electrical grinder (Siebtechnik steel pulverizer 2, 376, GmbH). Dried mushroom powder was mixed with 15 ml of distilled cold water, absolute methanol, ethanol or acetone in 50 ml tubes. The samples were placed in an incubator shaker for 24 h at 150 rpm and 25°C. Hot water extracts were obtained by boiling the mushrooms in 15 ml of distilled water for 10 min and then allowing the suspension to cool to room temperature. Al l the suspensions were then filtered

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Figure 1. Some of the mushrooms that were collected. A - *Cantharellus heinemannianus*, B - *Boletus edulis*, C - *Cantharellus symoensii*, D - *Ganoderma lucidum*, E - *Coprinus* sp., F- *Lactarius kabansus.*

Table 1. Different types of mushrooms collected locally.

Latin name	Local Shona name	Edibility
Amanita zambiana	Nhedzi	Edible
Amanita sp.	-	Non-edible
Boletus edulis	Dindindi	Edible
Cantharellus miomboensis	Chihombiro	Edible
Cantharellus symoensii	Firifiti	Edible
Cantharellus heinemannianus	Tsvuketsvuke	Edible
Coprinus sp.	-	Non-edible
Ganoderma lucidum	Howa danda	Non-edible
Lactarius kabansus	Nzeveyambuya	Edible
Trametes strumosa	Howa danda	Non-edible

using Whatman no. 1 filter paper, dried under a stream of cold air and reconstituted to 10 mg/ml in sterile distilled water for water extracts or dimethyl sulfoxide for the rest of the extracts. A total of fifty different extracts were obtained. All the reagents used in the extractions were of analytical grade.

Determination of total phenolic content

Total phenolic content in each mushroom extract was determined using the Folin and Ciocalteu (FC) reagent method with gallic acid as the standard according to Gan et al. (2013) and Sun et al. (2014), with modifications. Briefly, 40 µl of each sample was diluted to 200 µl using distilled water or dimethyl sulfoxide and mixed with 200 µl of Folin and Ciocalteu's phenol reagent, diluted 1:9 ml in distilled water. After 6 min, 200 µl of 7.5% sodium carbonate was added to the mixture and adjusted to 2 ml with distilled water. The reaction was kept in the dark for 60 min after which the absorbance was measured at 725 nm using a spectrophotometer (Spectronic^R 20 Genesys™, Spectronic Instruments). Distilled water and dimethyl sulfoxide were used as blanks.

Determination of antibacterial activity

Antibacterial effect of the mushroom extracts on *E. coli, S. typhi, S. aureus* and *S. pneumoniae* was determined using the agar disc diffusion method. Briefly, a suspension containing $1x10^6$ cfu/ml of bacteria was inoculated into Mueller Hinton Agar (Mast Group Ltd., Merseyside, U.K.). The discs (6 mm) were dipped in 200 µg of mushroom extract, dried and placed on the inoculated agar. Negative controls were prepared with the same solvents used to dissolve the sample extracts. Kanamycin 50 µg/disc and vancomycin 30 µg/disc were used as positive controls for the tested bacteria. After 2 h, incubation at 4°C, inoculated plates were incubated at 37°C for 18 h. At the end of the incubation period, the inhibition zones were measured.

Table 2. Total phenolic content of mushrooms extracted using different solvents.

Mushroom type	Total phenolic content (mg GAE/ 100 g dry weight)				
	Methanol	Ethanol	Acetone	Cold water	Boiling water
Amanita zambiana	59.03±10.36^{a}	31.72±4.77^{b}	33.85±7.81^{b}	122.86±4.71^{c}	35.43±1.85^{b}
Amanita sp.	120.11±10.12^{a}	37.48±5.15^{b}	16.95±1.77^{b}	308.85±14.52^{c}	319.89±8.83^{c}
Boletus edulis	341.47±16.31^{a}	78.77±3.46^{b}	25.43±2.91^{c}	336.28±3.54^{a}	503.70±20.65^{d}
Cantharellus miomboensis	37.85±11.33^{c}	30.08±4.28^{c}	99.88±2.08^{b}	56.86±1.86^{a}	38.10±2.83^{a}
Cantharellus symoensii	35.13±7.32^{a}	14.95±2.07^{b}	7.89±0.34^{b}	132.53±7.50^{c}	87.52±3.56^{d}
Cantharellus heinemannianus	44.57±10.72^{b}	17.70±8.67^{a}	20.53±2.68^{a}	74.20±4.60^{c}	67.46±4.56^{c}
Coprinus sp.	35.33±10.76^{b}	5.75±2.36^{a}	11.13±3.11^{a}	176.20±10.58^{c}	159.01±6.30^{c}
Ganoderma lucidum	109.82±9.15^{b}	70.35±3.02^{a}	4.78±0.17^{c}	131.41±5.06^{d}	62.08±2.67^{a}
Lactarius kabansus	80.77±15.39^{a}	39.32±16.05^{b}	15.80±1.36^{b}	225.31±5.11^{c}	203.79±14.58^{c}
Trametes strumosa	6.60±.37^{a}	3.61±1.23^{a}	5.48±0.19^{a}	17.71±1.13^{b}	27.14±1.76^{c}

Data expressed as mean ± SD; n = 150, 36 df. Values in the same row that do not share a common superscript are significantly different at p‹ 0.05.

Statistical analysis

Experimental values are given as means ± standard deviation (SD). Graph-pad prism was used to analyse the data. Statistical significance was determined by both one and two way variance analysis (ANOVA). All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Total phenolic composition

The results of the total phenolic composition of the different mushrooms from the crude extracts are shown in Table 2. With a few exceptions, extracts from cold and boiled water gave the highest levels of total phenolics (17.71 – 503.70 mg GAE/100 g dry mushroom), followed by methanolic extracts (6.60 – 341.47 mg GAE/100 g dry mushroom), while acetone extracts overly gave the lowest values (4.78 – 99.88 mg GAE/100 g dry mushroom). However, most of the yields from the acetone and ethanol extracts were not significantly different (4.78 – 99.88 mg GAE/100 g dry mushroom and 3.61 – 78.77 mg GAE/100 g dry mushroom, respectively). Statistical analysis by two way ANOVA showed that there is significant difference in the effect of solvents in extracting total phenols (4 df, F = 7.815, P-value = 0.000122) and that the total phenolic composition is also dependant on the mushroom type (9 df, F = 4.984, P-value = 0.000224). The high values in water extracts could be explained by the high polarity of water as compared to the other organic solvents, hence, more compounds dissolving in water. From the 10 different mushroom types studied, *Boletus edulis* was observed to have the highest total phenolic compounds (25.43 – 503.70 mg GAE/100 g dry mushroom) followed by *Amanita* sp. (16.95 – 319.89 mg GAE/100 g dry mushroom). Similar trends, where cold water extracts gave high total phenolic yields followed by hot water extracts, while acetone extracts gave the least yields, were as observed by Wang and Xu (2014).

Antibacterial activity

The antibacterial activities of methanol, ethanol, acetone, cold and hot water extracts of ten different mushrooms, against the four bacterial types tested are shown in Tables 3 to 7, respectively. The results showed that all the mushrooms exhibited inhibitory activities against at least one of the bacteria tested, as shown by the clear zone of inhibition around the tested mushroom extracts. The different mushroom extracts exhibited various degrees of inhibition of bacterial growth (6.3 – 14 mm diameter). It has been reported that mushroom species possess different constituents and in different concentration which account for their differential antimicrobial activity (Akyuz et al., 2010; Padmavathy et al., 2014). The highest *in vitro* antibacterial activity was shown by the cold water extract of *C. miomboensis* against *S. typhi* (14 mm zone of inhibition). This was followed in order by the acetone extract of *C. symoensii*, the methanol extract from *C. miomboensis* and the ethanol extracts of *G. lucidum* and *C. symoensii* with values 11.5, 11.0, 10.67 and 10.0 mm, respectively. *C. miomboensis*, *C. symoensii*, *Amanita* sp. and *B. edulis* all had the highest number of total extracts inhibiting at least one of the bacteria (12 each) closely followed by *C. heinemannianus* and *A. zambiana* (10 each), while *Coprinus* sp. had the least (6). *C. miomboensis*, *C. heinemannianus*, *C. symoensii*, *Amanita* sp., *A. zambiana*, *Lactarius kabansus* and *B. edulis* all had inhibitory effect on all the four bacteria tested. *C. heinemannianus* and *C. symoensii* had the highest effect on inhibition of bacteria as indicated by having the most extracts which had high inhibitory properties ranging from

Table 3. Antibacterial activities of methanol extracts of mushrooms on test organisms.

Mushroom type	Zone of inhibition diameter (mm)			
	Gram positive bacteria		Gram negative bacteria	
	Staphylococcus aureus	***Streptococcus pneumoniae***	***Escherichia coli***	***Salmonella typhi***
Amanita zambiana	7.0 ±0.00	9.0 ± 0.20	7.0 ± 0.0	8.5 ± 0.0
Amanita sp.	-	9.33 ± 1.16	8.8 ± 0.0	7.83 ± 0.29
Boletus edulis	-	7.5 ± 0.87	8.0 ± 0.0	7.33 ± 0.29
Cantharellus miomboensis	6.5 ± 0.0	6.84 ± 0.29	-	11.0 ± 2.0
Cantharellus symoensii	7.23 ± 0.25	8.14 ± 0.90	7.6 ± 1.15	9.5 ± 0.5
Cantharellus heinemannianus	-	8.67 ± 0.76	8.5 ± 1.73	8.0 ± 0.0
Coprinus sp.	-	8.0 ± 0.0	-	7.33 ± 0.58
Ganoderma lucidum	-	8.33 ± 1.16	-	8.0 ± 0.0
Lactarius kabansus	-	9.33 ± 1.16	-	7.5 ± 0.5
Trametes strumosa	-	7.43		8.33 ± 0.29

(-): No inhibition. Each value is expressed as mean ± SD (n = 3).

Table 4. Antibacterial activities of ethanol extracts of mushrooms on test organisms.

Mushroom type	Zone of inhibition diameter (mm)			
	Gram positive bacteria		Gram negative bacteria	
	Staphylococcus aureus	***Streptococcus pneumoniae***	***Escherichia coli***	***Salmonella typhi***
Amanita zambiana	7.33 ± 0.58	7.83 ± 0.76	7.2 ± 0.0	8.67 ± 0.76
Amanita sp.	8.23 ± 1.25	7.07 ± 0.12	8.0 ± 0.0	9.0 ± 1.0
Boletus edulis	7.5 ± 0.5	6.6 ± 0.0	8.67 ± 0.58	7.5 ± 0.0
Cantharellus miomboensis	8.16 ± 0.29	7.67 ± 0.76	7.77 ± 0.25	9.17± 0.29
Cantharellus symoensii	8.2 ± 0.76	8.94 ± 0.31	7.4 ± 0.17	10.0 ± 0.0
Cantharellus heinemannianus	8.83 ± 0.29	8.4 ± 0.53	-	8.18 ± 0.58
Coprinus sp.	-	8.0 ± 0.0	-	8.0 ± 0.00
Ganoderma lucidum	-	8.0 ± 0.0	7.67 ± 1.16	10.67 ± 1.16
Lactarius kabansus	7.83 ± 1.04	8.5 ± 0.87	-	8.0 ± 0.5
Trametes strumosa	-	7.33 ± 0.76	-	6.5 ± 0.0

(-): No inhibition. Each value is expressed as mean ± SD (n = 3).

Table 5. Antibacterial activities of acetone extracts of mushrooms on test organisms.

Mushroom type	Zone of inhibition diameter (mm)			
	Gram positive bacteria		Gram negative bacteria	
	Staphylococcus aureus	***Streptococcus pneumoniae***	***Escherichia coli***	***Salmonella typhi***
Amanita zambiana	-	7.5 ± 0.0	-	9.0 ± 0.0
Amanita sp.	6.67 ± 0.29	7.67 ± 0.76	6.3 ± 0.0	9.0 ± 1.0
Boletus edulis	7.0 ± 0.0	7.33 ± 0.29	7.83 ± 0.58	8.17 ± 0.76
Cantharellus miomboensis	7.67 ± 0.29	6.67 ± 0.29	7.73 ± 0.25	8.67 ± 0.29
Cantharellus symoensii	8.67 ± 0.58	8.17 ± 0.29	-	11.5 ± 1.0
Cantharellus heinemannianus	8.07 ± 0.12	7.0 ± 0.0	-	9.17 ± 0.76
Coprinus sp.	-	7.0 ± 0.0	-	7.5 ± 0.5
Ganoderma lucidum	-	8.27 ± 0.64	8.0 ± 0.0	8.33 ± 0.76
Lactarius kabansus	-	8.43 ± 0.81	7.5 ± 0.5	9.5 ± 0.5
Trametes strumosa	9.5 ± 1.8	8.17 ± 0.76	-	7.5 ± 0.5

(-): No inhibition. Each value is expressed as mean ± SD (n = 3).

Table 6. Antibacterial activities of cold water extracts of mushrooms on test organisms.

Mushroom type	Zone of inhibition diameter (mm)			
	Gram positive bacteria		Gram negative bacteria	
	Staphylococcus aureus	***Streptococcus pneumoniae***	***Escherichia coli***	***Salmonella typhi***
Amanita zambiana	-	-	-	-
Amanita sp.	-	-	-	-
Boletus edulis	-	-	-	7.33 ± 0.29
Cantharellus miomboensis	-	-	-	14.0 ± 1.0
Cantharellus symoensii	-	-	-	-
Cantharellus heinemannianus	-	-	-	-
Coprinus sp.	-	-	-	-
Ganoderma lucidum	-	-	-	-
Lactarius kabansus	-	-	-	7.5 ± 0.0
Trametes strumosa	-	-	-	-

(-): No inhibition. Each value is expressed as mean ± SD (n = 3).

Table 7. Antibacterial activities of hot water extracts of mushrooms on test organisms.

Mushroom type	Zone of inhibition diameter (mm)			
	Gram positive bacteria		Gram negative bacteria	
	Staphylococcus aureus	***Streptococcus pneumoniae***	***Escherichia coli***	***Salmonella typhi***
Amanita zambiana	-	-	-	-
Amanita sp.	-	-	7.0±0.0	-
Boletus edulis	-	-	-	-
Cantharellus miomboensis	-	-	-	-
Cantharellus symoensii	-	-	6.5±0.0	-
Cantharellus heinemannianus	-	-	6.5±0.0	-
Coprinus sp.	-	-	-	-
Ganoderma lucidum	-	-	-	-
Lactarius kabansus	-	-	-	-
Trametes strumosa	-	nt	-	-

(-): No inhibition. nt: not tested. Each value is expressed as mean ± SD (n = 3).

8-14 mm [15.4% (8) each] followed by *G. lucidum* [13.5% (7)], while *B. edulis, Coprinus* sp. and *Trametes strumosa* had the least [5.8% (3) each]. This shows that *C. heinemannianus*, *C. symoensii* and *G. lucidum* extracts contain compounds that are highly potent against the bacteria studied than the rest of the mushroom extracts found in this study.

In similar studies carried out by Quereshi et al. (2010), methanol, ethanol, acetone and cold water extracts of *G. lucidum* from India showed antimicrobial activity against the *S. aureus, S. typhi* and *E. coli* bacterial culture collections. From this study, the methanol extract showed no inhibition to *S. aureus* and *E. coli*, while the ethanol and acetone extracts inhibited growth of both *E. coli* and *S. typhi* but did not inhibit growth of *S. aureus*. The water extracts showed no inhibition to all the bacteria tested. Ethanol extracts of *G. lucidum* from Turkey inhibited growth of *E. coli* while the methanol extract showed no inhibition (Celik et al., 2014). In another study, acetone and ethanol extracts of *Cantharellus cibarius* collected in Turkey, exhibited antibacterial activity against *E. coli* and *S. aureus* but showed no inhibition against *S. typhi* (Dulger et al., 2004). Results of a study in Nigeria showed that methanol and ethanol extracts of *Cantharellus cibarius* from Nigeria inhibited *E. coli* and *S. typhi* growth but showed no inhibition against *S. aureus* and *S. pneumoniae* (Aina et al., 2012). Similarly, results obtained from this study show that methanol, ethanol and acetone extracts of the three *Cantharellus* species studied exhibited various degrees of inhibition against the four bacteria tested. This shows that different species of mushrooms exhibit different antimicrobial activity due to a number of factors such as the presence of different antimicrobial components, type of the extracting medium,

geographical location of the mushroom and the type of organism being tested.

Extracts obtained from ethanol gave the highest number of bacterial growth inhibition (33), followed by acetone (31) and methanol (28). In addition, ethanolic extracts showed the strongest antibacterial activity (8-14 mm) among the five extracts against the bacterial strains, followed by methanol and acetone. Water extracts exhibited the lowest number of antibacterial activity, despite having the extract with the highest inhibitory effect. This indicates, that the active compounds from the mushrooms studied which inhibit the growth of susceptible bacteria, may dissolve better in the organic solvents than in the aqueous solvents. These results are consistent with already reported literature that extracts from organic solvents give more consistent antimicrobial activity than water extracts (Kamra and Bhatt, 2012; Tiwari et al., 2011). It is interesting to note that, although cold water and hot water extractions gave highest values of total phenolic compounds in Table 2, these had the least effect on most bacteria. This shows that the antibacterial activity in the mushroom extracts depends not only on the presence of phenolic compounds but also on the presence of various secondary metabolites. Ethanol, acetone and methanol extracts were all effective against all the four bacteria indicating the broad spectrum of antibacterial activity of the extracts. However, Gram negative bacteria were slightly more susceptible to the extracts than Gram positive bacteria (52 and 46 extracts, respectively). Many antibiotics are designed to attack the integrity of the cell wall by preventing cell wall synthesis, therefore killing the cell. Although, all bacteria have an inner cell wall, Gram negative bacteria have a unique outer membrane which prevents certain drugs and antibiotics from penetrating the cell. Thus, antibiotics that affect the cell wall will impair Gram positive bacteria and not Gram negative bacteria. The results obtained in this study suggest that the antibacterial extracts may act by affecting not just the cell wall, but other cell growth mechanisms like protein synthesis, bacterial DNA replication and transcription. Among the four bacteria tested, *S. typhi* was the most susceptible bacteria as indicated by its highest number of inhibitions as well as the highest number of most potent extracts in the 8-14 mm diameter range. A decline in the number of multi-drug resistant clinical isolates (*S. typhi*) has been reported (Madhulika et al., 2004). Thus, the study shows that the *S. typhi* isolate studied, may be a phage type that is susceptible to most antibiotics.

The antibacterial activity of the ethanolic, methanolic and acetone extracts against *E. coli, S. typhi, S. aureus* and *S. pneumoniae* is of great importance in the human healthcare system. *S. pneumoniae* is the most common cause of community acquired pneumonia (CAP) in children while *E. coli* accounts for more than 70% of the infections of the urinary tract worldwide (Blossom et al., 2006; Sangeeth et al., 2014). *S. typhi* is the cause of typhoid fever, which was recently epidemic in Zimbabwe. *S. aureus* is the most common cause of bacterial infections and abscesses of skin, joints and bones (Stanely et al., 2013). Resistance to antibiotics has been reported in *S. aureus*, *S. pneumoniae, S. typhi* and *E. coli* (Blossom et al., 2006; Okonko et al., 2009; Rowe et al., 1997; Sangeeth et al., 2014; Stanely et al., 2013). All the bacterial strains used were clinical isolates from individuals in Zimbabwe. *E. coli* and *S. aureus* are mostly encountered in urinary tract infections while isolated cases of *S. typhi* are common. Thus, the antibacterial activity found in the mushroom extracts can be further investigated for future use in the development of therapeutic agents to treat infections caused by these bacteria.

Conclusion

Wild edible and non-edible mushrooms can be used as agents in the development of new drugs for bacterial infections. This study indicated that the antibacterial effects of mushrooms vary depending on the type of mushroom, the solvent medium used and the type of organism tested. *C. heinemannianus* and *C. symoensii* had the highest effect on inhibition of bacteria as indicated by the different extracts showing high inhibitory properties ranging from 8-14 mm [15.4 (8) each], followed by *G. lucidum* [13.5% (7)], while *Boletus edulis, Coprinus* sp. and *Trametes strumosa* had the least effect [5.8% (3) each]. Extracts obtained from ethanol were the most effective tested against bacteria (36.5%), followed by methanol (30.8%) and acetone (30.8%) and lastly, aqueous extracts (1.9%). Thus, of the five solvents tested, ethanol, methanol and acetone were determined to be the solvents of choice for isolation of antibacterial compounds from the majority of mushrooms studied. However, identification of the phyto-constituents responsible for the antibacterial activity is required for large commercial production.

Conflict of interest

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Efficacy of *Rhodopseudomonas* containing multi-microbe probiotic on growth performance, mortality and cecal microflora in broilers

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Combinations of probiotic microorganisms are the study of interest due to beneficial impact through synergistic actions. Several researchers including our previous result found positive effects of various combinations of probiotic inclusion in broiler diet. In the current study, the efficacy of combinations of probiotic organisms by adding *Rhodopseudomonas* spp. was the primary interest on growth performance, mortality, immunity and cecal microflora in broilers. According to the completely randomized design, three hundred day-old Ross broiler chicks (initial BW, 45.08 ± 0.79 g) were randomly allocated to three dietary treatments with ten replications of 10 birds per replicate. Broilers were reared for 5 weeks, where experimental diets were provided for starter (0 to 3 weeks) and finisher (4 to 5 weeks) period. The dietary treatments were, CON = control (corn-soybean meal based basal diet); ABT = basal diet + chlortetracycline-HCl; RCMP = basal diet + multi-microbe probiotic, (*Bacillus* spp. + *Lactobacillus* spp. + *Saccharomyces* spp. + *Rhodopseudomonas* spp.). Present study revealed that, dietary supplementation of RCMP significantly improved overall average daily gain and feed conversion ratio compared to CON (P<0.05). Mortality rate was significantly reduced in RCMP and ABT supplemented group compared to CON (P<0.05). Serum immunoglobulin level was upgraded after RCMP and ABT supplementation relative to CON but remained nonsignificant (P>0.05). Moreover, cecal pathogenic *Escherichia coli* and *Salmonella* was significantly suppressed in RCMP and ABT supplemented group compared to CON (P<0.05); while number of beneficial microorganisms were higher in ABT and RCMP relative to CON but not significant (P>0.05). Thus, dietary supplementation of *Rhodopseudomonas*-based multi-microbe probiotic (RCMP) has the potentiality to be used as effective feed additives in broilers.

Key words: Broilers, multi-microbe probiotic combinations, immunity, mortality, performance.

INTRODUCTION

The use of antibiotics as prophylactic and growth promoting compounds has long been practiced in commercial poultry farming. However, increased antibiotic use has led to the development of antibiotic resistant microorganisms, production losses and increased risk of infection (Bager et al., 1998). In addition, tremendous use of antibiotic cause imbalance of the gut microflora causes health hazards, and antibiotic residues influence the

environment; and owing to these adverse effects, antibiotic growth promoters have been banned in many countries all over the world (Edens, 2003). Consequently, the entire poultry industry has been under pressure to identify viable alternatives to antibiotics. Probiotics, prebiotics and herbal feed additives and their different combinations became viable alternatives to antibiotic growth promoters in the poultry industry (Patterson and Burkholder, 2003; Kim et al., 2016).

Probiotics are commonly known as the mono or mixed cultures of living microorganisms that exerts beneficial effects on the host by balancing indigenous microbial population (Fuller, 1992; Havenaar et al., 1992). They have beneficial effects on growth performance (Kabir, 2009), health of the host (Guarner et al., 2003), increase nutrient digestibility (Mountzouris et al., 2010), modulate intestinal microflora (Teo and Tan, 2007), and facilitate development of humoral immunity (Waititu et al., 2014). There are variety of microrganisms widely used in the animal as well as poultry nutrition including *Bacillus, Lactobacillus, Lactococcus, Bifidobacterium, Enterococcus, Streptococcus, Pediococcus, Aspergillus, Candida* and *Saccharomyces* species, a variety of yeast species, and undefined mixed cultures (Patterson and Burkholder, 2003; Kabir, 2009). *Rhodopseudomonas* are non-sulphur phototrophic organisms under the family Athiorhodaceae, found in soils and many types of marine environments, currently, grew attention along with other probiotics in aquaculture, agriculture, biofuel production and waste management (Lee et al., 2008; Qi et al., 2009). Single strain use of probiotics are beneficial where Sanders and Veld (1999) proposed that multi-strain and multi-species probiotics are more effective; Timmerman et al. (2004) reported effectiveness of multi-microbe probiotic due to successful colonization. Xu et al. (2014) reported beneficial effects of single use of *Rhodopseudomonas* in broilers while Zhou et al. (2010) reported that *Rhodopseudomonas* with other multi-strain probiotic products have growth promoting efficacy in fish and plants. But there have been limited attempts to develop *Rhodopseudomonas* based multi-microbe probiotic products for livestock and poultry production. Beneficial effects of different combinations were found effective (*Bacillus*, *Lactobacillus*, *Saccharomyces, Enterococcus, Streptococcus* and *Clostridium* spp.) on broiler performance and immunity (Kim et al., 2012; Bostami et al., 2015). To the best of our knowledge, there were no previous reports on combination of different microorganisms along with *Rhodopseudomonas* species with other probiotic microorganisms, whether they exert positive or negative impact on broiler performance. It was hypothesized that, addition of purple non-sulphur microorganism *Rhodopseudomonas* with *Bacillus*, *Lactobacillus* and *Saccharomyces* probiotic bacteria would synergistically improve the growth performance, immunity; reduce the mortality and modulate the cecal microflora through microbial balance and nutrient utilization in broilers. Therefore, the present study was conducted to investigate the efficacy of *Rhodopseudomonas* containing multi-microbe combinations (RCMP) on growth performance, mortality, immunity and cecal microflora in broilers.

MATERIALS AND METHODS

The protocol for this experiment, use and care of broilers were carried out in accordance with the guidelines of the Animal Care and Use Committee of the Sunchon National University, Suncheon, Republic of Korea.

Birds, diet, experimental design and experimental care of birds

Broiler chicks for conducting the experiment were obtained from a local commercial hatchery (Yang Ji Company, Cheonan, Choongnam, South Korea). Three hundred day-old mixed sex Ross broiler chicks (initial BW, 45.08 ± 0.79 g) were randomly allocated to three dietary treatments. The dietary treatments were, CON = Control (Corn-soybean meal based basal diet); ABT = Basal diet + Chlortetracycline-HCl; and RCMP = Basal diet + Multi-microbe probiotic combinations (*Bacillus subtilis* + *Lactobacillus acidophilus* + *Saccharomyces cerevisiae* + *Rhodopseudomonas capsularis*); where each treatment had ten replications of 10 birds per replicate. Broilers were reared for a total of 5 weeks, where experimental diets were provided for two stages, namely the starter (0 to 3 weeks) and finisher (4 to 5 weeks).

Broilers were kept in a closed and well ventilated house with having well-arranged wire-floored cages of 100 cm long, 80 cm wide and 40 cm high, having a floor space of 800 cm^2/bird. The cages had a linear feeder in the front and a nipple drinker in the back to provide *ad libitum* feed and water throughout the entire experimental period. Temperature was maintained at 33°C from day 0 to 7, after which it was gradually reduced to 27°C at a rate of 3°C per week and then maintained at this temperature until the end of the experiment. The relative humidity (RH) was maintained at around 50% and continuous lighting was provided throughout the experimental period. The lighting regime for the study consisted of 23L: 1D for the entire experimental period.

A commercial corn soybean meal-based diet was used as the basal diet, which was formulated to meet the nutrient requirements of Ross broiler chickens following National Research Council guidelines (NRC, 1994).The starter diets were offered from 0 to 3 weeks and finisher from 4 to 5 weeks. The *Rhodopseudomonas* containing multi-microbe probiotic combinations (RCMP) and antibiotic were added at 0.1% (W/W) with basal diet in powdered form by replacing the equal amount of basal diet. Diets were supplied to the birds on weekly basis, where firstly total feed was

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Table 1. Feed ingredients and chemical compositions of the basal diets.

Items	Starter diet (0 to 3 weeks)	Finisher diet (4 to 5 weeks)
Ingredients (%, as fed basis)		
Corn grain	57.58	60.64
Soybean meal	26.80	24.90
Corn gluten	5.00	3.50
Soybean oil	2.20	2.20
Animal fats	4.50	5.00
Common salt	0.25	0.25
Dicalcium phosphate	2.14	2.00
Limestone	0.92	0.88
Vitamin-mineral premix [1]	0.30	0.30
Choline	0.08	0.07
L-lysine HCl (78%)	0.24	0.16
DL-Methionine	0.20	0.10
Calculated composition (% DM)		
ME (MJ/kg)	13.03	13.27
Moisture	12.07	13.08
Crude Protein	20.89	19.12
Ether extract	4.65	2.43
Crude Fiber	4.42	3.71
Crude Ash	5.63	5.61
Calcium	1.05	0.81
Available phosphorus	0.55	0.45
Lysine	1.42	1.10
Methionine	0.49	0.45

[1]Vitamin-mineral mixture provided the following nutrients per kilogram of diet: Vitamin A, 15,000 IU; vitamin D_3, 1,500 IU; vitamin E, 20.0 mg; vitamin K_3, 0.70 mg; vitamin B_{12}, 0.02 mg; niacin, 22.5 mg; thiamine, 5.0 mg; folic acid, 0.70 mg; pyridoxine, 1.3 mg; riboflavin, 5 mg; pantothenic acid, 25 mg; choline chloride, 175 mg; Mn, 60 mg; Zn, 45 mg; I, 1.25 mg; Se, 0.4 mg; Cu, 10.0 mg; Fe, 72 mg; Co, 2.5 mg (Bayer Korea Ltd., Dongjak-Ku, Seoul, Korea).

weighed for each treatment and then replace the basal diet at 0.1% to add similar amount of antibiotic and multi-microbe probiotic for the ABT and RCMP, respectively. Finally total feed was mixed properly and then supplied to the birds for each treatment and replications. *Rhodopseudomonas* containing multi-microbe probiotic combinations (RCMP) and feed sample was homogenized and analysed for microbial populations and chemical compositions. Dietary dry matter (DM), crude protein (CP), calcium, phosphorus and other chemical compositions were analysed and calculated according to the procedures described by Association of Official Analytical Chemists (AOAC, 2000). The ingredients and nutritional composition of the experimental basal diet are presented in Table 1.

The probiotics used in the present study were provided by the Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea and Probion, Woogene B&G Co. Ltd., Seoul, South Korea. *Rhodopseudomonas capsularis* cells were grown under natural illuminations for 96 h in outdoor culture. In short, *R. capsularis* cells were collected by centrifugation (10,000×*g*) and then the residual cell mass mixed with the corn meal in the ratio of 1:5. After mixing properly, it was dried in a forced-air drying oven at a temperature of 60°C and finally stored in 4°C maintaining the temperature. A culture broth (CB) was prepared and then autoclaved before use. The culture broth medium was prepared with corn steep liquor (6%), molasses (4%), yeast extract (0.30%), KH_2PO_4 (0.50%) and K_2HPO_4 (0.25%). After autoclaving 2 L of culture broth and each microbial culture of 2 ml (*Lactobacillus*, *Bacillus*, *Saccharomyces*) was added and mixed properly and then subjected to fermentation for 48 h. The microorganisms grown on the culture broth were then sprayed after drying at the temperature of 40°C for 72 h, on the corn soybean meal (1:1). A two-step fermentation method was accomplished using a commercial fermenter (W-1000; Wonbalhyo Industry Co., Icheon, South Korea). Firstly, 0.5% *L. acidophilus* KCTC 3111 and 0.5% *B. subtilis* KCTC 3239 were added to the solid substrate media and fermented at 40°C by the repetition of 5 h of anaerobic and 3 h of aerobic conditions for about 48 h. Second fermentation was performed by adding 0.5% *S. cerevisiae* for 72 h at a temperature of 40°C under aerobic condition. Following completion of the fermentation process, the probiotic products were then dried to less than 15% of moisture at the temperature of 80°C for 48 h using a drying oven. Then the product was stored at 4°C for further use. The *Rodopseudomonas* spp., *Lactobacillus* spp. *Bacillus* spp. and *Saccharomyces* spp. were mixed properly and for the determination of the number of cells, 1 g was taken and diluted with sterilized distilled water (10 ml) at room temperature. After about 10 min, 1 ml of the dilution was serially diluted 10-fold in NaCl (8.5 g/kg) solution and then cultured in the agar media. The culture plate was then

Table 2. Microbial population and composition of *Rhodopseudomonas* containing multi-microbe probiotic combinations (RCMP).

Microflora and strain	Concentration (cfu/g)
L. acidophilus (KCTC 3111)	3.2×10^7
B. subtilis (KCTC 3239)	2.6×10^7
S. cerevisiae (KCTC 7915)	6.2×10^9
R. capsularis (KCTC 2583)	2.5×10^8
Chemical composition	**(%)**
Moisture	40.36
Crude protein	10.23
Crude fat	2.21
Crude fiber	11.33
Crude ash	10.13
Nitrogen free extract	22.52

KCTC, Korean Collection for Type Cultures.

incubated at the temperature of 37°C for 24 to 48 h and the number of colonies were counted. The chemical compositions of *Rhodopseudomonas* containing mixed microbial combinations (RCMP) were determined by the method of AOAC (2000). The microbial concentrations and the chemical composition of RCMP were shown in Table 2.

Measurement of broilers performance and mortality

Body weight and feed intake of all birds were measured on pen basis (replications of treatments) every week. Feed intake was measured according to residual feed. Based on the weekly data of body weight and feed intake, average daily gain (ADG) and average daily feed intake (ADFI), and feed conversion efficiency (FCR) was calculated for all treatments. ADG, ADFI and FCR were calculated for starter (0 to 3 weeks), finisher (4 to 5 weeks) and overall period (0 to 5 weeks). The mortality of birds was recorded on daily basis.

Blood analysis

At the end of the feeding trial (5^{th} week), three birds were randomly selected from each replication of all treatments in order to perform immunological analyses. Birds of the similar weight of the replications of treatments were selected for blood collections; where blood samples were collected from selected birds' brachial vein. After collection, blood samples were quickly transferred into centrifuge tubes that were centrifuged for 15 min at 1610× *g* in a cold chamber (4°C). Sera were then carefully removed to plastic vials and stored at -20°C for immunoglobulin (Ig) analysis. Concentrations of serum IgG, IgM and IgA were assayed using Chicken IgG (Cat. No. E30-104), IgM (Cat. No. E10-101) and IgA (Cat. No. E30-103) ELISA Quantitation Kits (Bethyl Laboratories Inc., Montgomery, TX, USA), respectively according to the manufacturer's instructions. Each sample was run in duplicate. The absorbance of each well was measured within 30 min by using a micro-plate auto-reader (Thermo Lab Systems, Helsinki, Finland) at 450 nm.

Cecal microbial analysis

Three birds were randomly selected from each pen in order to perform microbial analysis. Selected birds were slaughtered, and cecal contents were collected to measure the microfloral count. Feed was withdrawn 12 h before slaughtering. Approximately 1 g of cecal content was aseptically collected into a 2-mL safe-lock Eppendorf tube (Thermo Fisher Scientific Inc., Seoul, South Korea) and immediately preserved at -40°C for subsequent microbial analysis. After thawing, 1 g of the cecal sample was serially diluted with 9 mL of 0.9% sterile saline (1:10 dilution) and thoroughly mixed. Viable bacterial counts from the cecal samples were conducted by plating serial 10- fold dilutions in duplicate into different agar plates to isolate different bacteria. The culture media used for *E. coli* was MacConkey Sorbitol agar; for *Lactobacillus* spp. was Mann, Rogosa & Sharpe agar; and for *Bacillus* spp. was Mannitol-Egg Yolk-Polymyxin agar; and for *Salmonella* spp. was Salmonella Shigella Agar. Supernatant (100 µl) was smeared onto agar plate and incubated anaerobically at 37°C for 24 to 48 h. After incubation, microbial colonies were counted and expressed as log10 CFU/ml.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using the General Linear Model Procedures (GLM) of SAS (2003) with diet as the main effect. The pen was used as the experimental unit to analyse growth performance, whereas individual chicks were used as the experimental unit for analysis of blood and cecal microflora. Statistically significant effects were further analysed and means were compared using Duncan's multiple range tests (DMRT) when necessary. Probability value $P<0.05$ was considered as statistically significant.

RESULTS

Growth performance and mortality of birds

Average daily gain (ADG) was significantly improved during starter (0 to 3 weeks), finisher (4 to 5 weeks) and overall period (0 to 5 weeks) in the RCMP and ABT supplemented group ($P<0.05$); but RCMP and ABT did not differ significantly ($P>0.05$) (Table 3). In addition,

Table 3. Effect of *Rhodopseudomonas* containing multi-microbe probiotic combinations (RCMP) on broiler growth performance.

Parameters	Period	Dietary treatments			SEM	P-value
		CON	ABT	RCMP		
IBW (g/bird)		44.75	44.91	44.84	0.389	0.962
FBW (g/bird)		2061.48[b]	2257.16[a]	2289.60[a]	32.174	0.001
ADG (g/bird)	0-3 weeks	44.58[b]	48.50[a]	49.47[a]	0.812	0.005
	4-5 weeks	77.18[b]	85.27[a]	86.14[a]	1.527	0.005
	0-5 weeks	57.62[b]	63.21[a]	64.14[a]	0.923	0.001
ADFI (g/bird)	0-3 weeks	59.66	61.61	62.15	0.827	0.143
	4-5 weeks	145.44	148.67	149.48	2.563	0.539
	0-5 weeks	92.33	94.18	94.05	0.842	0.281
FCR (Feed/Gain)	0-3 weeks	1.34	1.27	1.26	0.034	0.276
	4-5 weeks	1.89[a]	1.75[b]	1.74[b]	0.043	0.061
	0-5 weeks	1.60[a]	1.49[b]	1.47[b]	0.027	0.009

[a,b] Means with different superscripts within the same row are significantly different (P<0.05). SEM = Standard error of mean. CON = Control (Corn-soybean meal based basal diet); ABT = Basal diet + Chlortetracycline-HCl; RCMP = Basal diet + Multi-microbe probiotic combinations (*B. subtilis* + *L. acidophilus* + *S. cerevisiae* + *R. capsularis*).

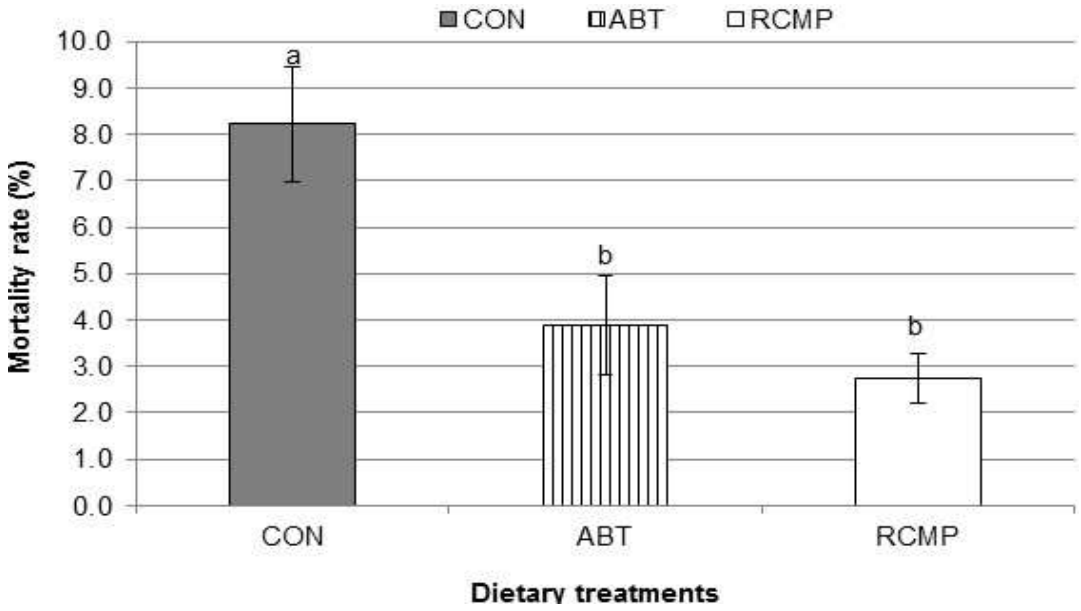

Figure 1. Effect of *Rhodopseudomonas* containing multi-microbe probiotic combinations (RCMP) on mortality of broilers. [a,b] Means with different superscripts within the similar bar are significantly different (P<0.05). Error bar indicated standard error. CON = Control (Corn-soybean meal based basal diet); ABT = Basal diet + Chlortetracycline-HCl; RCMP = Basal diet + Multi-microbe probiotic combinations (*Bacillus subtilis* + *Lactobacillus acidophilus* + *Saccharomyces cerevisiae* + *Rhodopseudomonas capsularis*).

average daily feed intake (ADFI) was statistically similar among the dietary treatments during starter, finisher and overall period (P>0.05). Furthermore, feed conversion ratio (FCR) was improved in RCMP and ABT during overall period compared to CON (P<0.05). Where during finisher period FCR tended to be improved in the RCMP and ABT relative to control (P<0.10).

The mortality of the broilers was lower in ABT (53%) and RCMP (67%) groups over the phases (0 to 5 weeks) (Figure 1) compared to CON (P<0.05); however, no difference was observed between RCMP and ABT during entire experimental period (P>0.05).

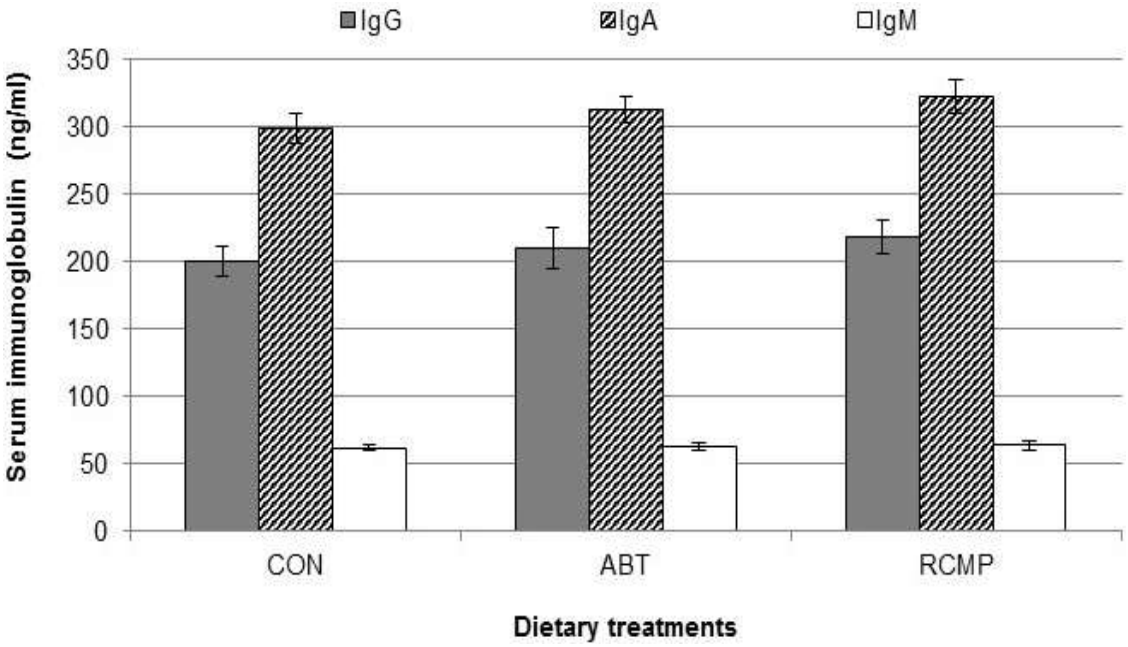

Figure 2. Effect of *Rhodopseudomonas* containing multi-microbe probiotic combinations (RCMP) on serum immunoglobulins of broilers. [a,b] Means with different superscripts within the similar bar are significantly different (P<0.05). Error bar indicated standard error. CON = Control (Corn-soybean meal based basal diet); ABT = Basal diet + Chlortetracycline-HCl; RCMP = Basal diet + Multi-microbe probiotic combinations (*B. subtilis* + *L. acidophilus* + *S. cerevisiae* + *R. capsularis*).

Serum immunoglobulins of broilers

It was observed from the present study that, there were higher value of the serum immunoglobulins, IgG, IgA and IgM of the birds (Figure 2) in RCMP and ABT supplemented groups in comparison to CON group, but there was no significant differences among the dietary treatments (P>0.05).

Cecal microbiology of broilers

As shown in the Table 4, the pathogenic *E. coli* and *Salmonella* was suppressed in the RCMP and ABT supplemented groups (P<0.05). There were no significant differences observed for the number of pathogenic *E. coli* and *Salmonella* between RCMP and ABT (P>0.05). However, the non-pathogenic bacteria (Lactobacillus and Bacillus) were numerically increased in RCMP and ABT supplemented group but did not differ significantly (P>0.05).

DISCUSSION

In the current study dietary supplementation of RCMP (*Bacillus*, *Lactobacillus*, *Saccharomyces,* and *Rhodopseudomonas*) improved the growth performance of broilers during 0 to 5 weeks of experimental period (Table 3). This is in agreement with Mountzouris et al. (2007) where growth promotion was reported with combination of multi-microbe probiotic (*Lactobacillus, Bifidobacterium, Enterococcus,* and *Pediococcus*). Photosynthetic bacteria, *Rhodopseudomonas* species stimulate the growth of fish (Zhang et al., 1988); and broiler (Xu et al., 2014). Zhou et al. (2010) reported that along with other beneficial microbes, *Rhodopseudomonas* species are able to improve the growth performance in fish through enhancement of immunity and health status. Improved body weight gain in the RCMP group of present study could be attributed to a better microbial environment in the gut, which in turn might have enhanced digestion, absorption and utilization of nutrients (Panda et al., 2000). Combination of probiotic microorganism exerts multifunctional effects such as enhancement of growth performance through synergistic (assisting the beneficial microbes) and antagonistic actions (inhibiting the pathogenic microbes) (Timmerman et al., 2004; Qi et al., 2009).

Generally, probiotic organisms help to maintain the intestinal microfloral balance by increasing digestive enzyme activity which consequently helps to improve feed intake and digestion (Jin et al., 1997). However, in the present study, feed intake did not differ significantly

Table 4. Effect of *Rhodopseudomonas* containing multi-microbe probiotic combinations (RCMP) on cecal microflora in broilers.

Microorganism (log_{10}CFU/g)	Dietary treatments			SEM	P-value
	CON	ABT	RCMP		
Bacillus	7.57	7.69	7.78	0.11	0.531
Lactobacillus	7.12	7.56	7.66	0.36	0.583
E. coli	7.47[a]	6.55[b]	6.34[b]	0.24	0.021
Salmonella	6.59[a]	6.21[b]	6.13[b]	0.06	0.001

[a,b] Means with different superscripts within the same row are significantly different (P<0.05). SEM = Standard error of mean. CON = Control (Corn-soybean meal based basal diet); ABT = Basal diet + Chlortetracycline-HCl; RCMP = Basal diet + Multi-microbe probiotic combinations (*B. subtilis* + *L. acidophilus* + *S. cerevisiae* + *R. capsularis*).

among the dietary treatments. Although the combinations of multi-microbe probiotic were different; Kim et al. (2012) observed significant improvements in feed conversion ratio (FCR) in response to *Bacillus, Lactobacillus,* and *Saccharomyces* supplemented diet, which supports the present findings of Ross broilers with combination of multi-microbe probiotic (*Bacillus* spp. + *Lactobacillus* spp. + *Saccharomyces* spp. + *Rhodopseudomonas* spp.). However, Salim et al. (2013) observed no effect on feed efficiency of broilers and this is consistent with starter period of the present study. It has been suggested that probiotic can promote feed efficiency by increasing the bioavailability of dietary micronutrients, modulating intestinal microflora, enhancing immuno-modulation and improving the health (Kabir, 2009; Yang et al., 2012). *Saccharomyces* can increase vitamin absorption, synthesis of enzymes, and protein metabolism (Crumplen et al., 1989); while *Rhodopseudomonas* can produce acetic, butyric and lactic acid, and extracellular polymeric substances and enzymes (Merugu et al., 2012). It has been postulated that the combination of *Rhodopseudomonas* spp. with other probiotic bacteria created probiotic niches, optimal pH, and successful colonization, and improved feed efficiency of broilers hence the observed better performances in the present study. The significant improvement of FCR in the RCMP supplementation in the current study additionally might be attributed to the purple non sulphur bacteria (*Rhodopseudomonas* spp.) as a source of single cell protein (for the poultry industry) (Salma et al., 2007); which have more digestible bacterial cell wall, and are rich in proteins, carotenoids, biological cofactors, and vitamins (Kobayashi and Kurata, 1978).

Mortality rate was reduced in the RCMP supplemented group (Figure 1) and this concurs with Khaksefidi and Rahimi (2005) who found reduced mortality rate in Ross broilers diet supplemented with multi-microbe probiotic. *Bacillus* can produce iturin and surfactin, and enzyme (Ferrari and Schmidt, 1993); *Lactobacillus* can produce lactic acid and bacteriocines (Yamato et al., 2003); have the antibacterial potential and produce fermentative enzymes. *Bacillus* and *Lactobacillus* inhibits the putrefactive bacterial enzymes and reduce mortality (Jin et al., 2000). The latter could be ascribed as the reason behind the reduced mortality in the current study. Zhou et al. (2010) reported that *Rhodopseudomonas* and *Bacillus* could enhance immunity and health status through secretion of immune substances. Combinations of probiotic strains can increase beneficial health effects owing to their synergistic and biotherapeutic effects (Musa et al., 2009). It could be inferred that the combination of the beneficial microorganisms exerted similar actions leading to reduced mortality in the RCMP groups in the present study.

Dietary supplementation of RCMP showed an increased trend of immunoglobulin status (IgG, IgA and IgM) in broilers in the present experiment (Figure 2). Several researchers have reported that probiotics in broilers enhanced humoral immune response (Koenen et al., 2004; Salim et al., 2013). Haghighi et al. (2005) reported that administration of a multispecies probiotic (*Lactobacillus, Bifidobacterium, Streptococcus*) enhanced the serum antibodies to several foreign antigens in case of chickens. Probiotics are involved in protection against a variety of pathogens in chickens (*Escherichia coli*, *Campylobacter* and *Salmonella*) and can reduce the mortality of birds (Cross, 2002; Gunal et al., 2006); which might be the ultimate fate of improvement of the immunoglobulin status. The gut and its resident microbiota play an essential role in shaping the immune system (Waititu et al., 2014); while probiotics promote such types of biological shaping of the immune system (Salminen et al., 1996).

The pathogenic *E. coli* and *Salmonella* were suppressed significantly, while the beneficial *Bacillus* and *Lactobacillus* were increased non-significantly in the RCMP supplemented group (Table 4). Consistent with present findings, Ceylan et al. (2003) and Ghadban et al. (1998) reported that multi-microbe probiotic could reduce pathogenic bacteria count (*Salmonella* and *E. coli)*. Supplementation of multi-microbe probiotic (*Lactobacillus*, *Bacillus* and *Clostridium*) could fortify beneficial micro-organisms, such as *Lactobacillus* and *Bifidobacterium* (Teo and Tan, 2007; Mountzouris et al., 2010). In addition,

probiotic helps in regulation of microbial homeostasis in the intestine which led to balancing the microorganisms in the gastrointestinal tract (Jin et al., 1997; Lee et al., 2010). This reduction could also be attributed to competitive exclusion, where the adhesion of beneficial bacteria to the intestinal mucosa prevents attachment by pathogenic bacteria (Kabir, 2009). Facilitation of antibody production (Ng et al., 2009), and secretion of biochemical substances by the probiotic organisms inhibit the growth and development of pathogenic bacteria (Patterson and Burkholder, 2003). *Rhodopseudomonas* provide the organic acids and energy resource for beneficial gut microflora and might exert proliferative effects on colonocytes; and antibacterial actions for pathogenic microorganisms (Guarner et al., 2003; Merugu et al., 2012). It could be inferred that the combination of the probiotic microbes exerted profound actions leading to a reduction in the pathogenic bacteria in the RCMP supplemented group in the current study.

Conclusion

The results of the current study suggested that supplementation with *Rhodopseudomonas*-based multi-microbe probiotic (RCMP) (*L. acidophilus* + *B. subtilis* + *S. cerevisiae* + *R. capsularis*) significantly improved the growth performance, reduced mortality; while significantly suppressed pathogenic *E. coli* and *Salmonella* in broilers. Thus, *Rhodopseudomonas*-based multi-microbe probiotic (RCMP) has the potential to be used as growth promoters in broiler nutrition.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Isolation and characterization of some gut microbial symbionts from fungus-cultivating termites (*Macrotermes* and *Odontotermes* spp.)

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Microbiota of termites is crucial for nitrogen cycle activities and degradation of recalcitrant components of plant biomass that influence soil structure and carbon mineralization in tropical and subtropical regions. The aim of this study was to isolate and characterize gut bacteria that may be potentially associated with nitrogen metabolism from two fungus-feeding termites (*Macrotermes* and *Odontotermes* spp.). Twenty termites from the intact colony of each termite species were aseptically degutted. Gut homogenate was inoculated and cultured on selective media for the isolation of pure bacteria. Pure bacterial isolates were characterized using their morphological, biochemical and molecular characters. DNA was extracted from the isolates, polymerase chain reaction (PCR) amplified and sequenced. The 16S rDNA gene sequences were blastn analyzed against the Genbank database and phylogenetic analysis was performed using MEGA 5 software. All forty-six isolates reacted positive for the ammonifying, nitrate, catalase and nitrogenase tests. Phylogenetic analysis grouped the isolates into three phyla: *Firmicutes, Proteobacteria* and *Actinobacteria*. Isolates were affiliated with the genera *Pseudomonas*, *Citrobacter, Enterobacter, Proteus, Klebssiella, Bacillus, Staphylococcus, Rhodococcus* and *Micrococcus*. The results confirm that termites harbor diverse gut bacterial groups that have different physiological/enzymatic activities and might have functional implications in the termite-microbe symbiotic association.

Key words: Termites, symbiosis, mutualistic interactions, termite gut bacterial diversity, nitrogen fixation

INTRODUCTION

Termites (Order: *Blattodea*, Family: *Termitidae*) are a large and diverse group of soil macrofauna (Ahmed et al., 2011). They are thought to have originated about 150 million years ago and have since successfully colonized the temperate and tropical ecological zones (Thorne et al., 2000). Nearly 3,000 species of termites have been described and they are conventionally classified into lower and higher termites (Zhu et al., 2012). They are

important decomposers due to their ability to biodegrade complex substances like cellulose and hemicellulose found in plant materials (Sugimoto et al., 2000).

Termites harbor diverse microbial populations most of which are unique to the termite gut ecosystem (Otani et al., 2014). The microbiota associated with termites are crucial for degradation of recalcitrant components of plant biomass (Brune and Ohkuma, 2011) and this has a major influence on soil structure and carbon mineralization (Ohkuma, 2003). Termites' gut microbiota are exchanged between colony members and transferred to the next generation through trophallaxis (Brune and Ohkuma, 2011). The isolation and cultivation of bacterial strains from termite guts has partially contributed to their classification as decomposers of lignocellulose, uric acid and/or other aromatic compounds, as nitrogen-fixers and/or as H_2/CO_2-acetogens (Breznak, 2000). Nonetheless, the majority of the microbial species (~99%) are difficult or currently uncultivable, thus limiting understanding of their function in the gut ecosystem (Breznak, 2000).

The macrotermitinae comprises of the economically important termite species (Ahmed et al., 2011) that have been comprehensively studied (Mathew et al., 2012; Makonde et al., 2015; Otani et al., 2014, 2015). Isolation and characterization of termite gut bacterial species faces challenges since majority of the bacterial species are difficulty to culture (Breznak, 2000). Considering the plant-biomass degradation and nitrogen metabolism in termites, the presence of diverse bacterial lineages in termites (Makonde et al., 2015; Otani et al., 2015;) and the evolutionarily diverse termite species, the termite gut microbial populations are of significant interest and need to be isolated and characterized in order to better understand the termite symbiotic systems. In this study, the authors isolated and partially characterized some termite gut bacterial species that have different physiological/enzymatic activities. The results indicate some functional roles of the termite gut bacterial isolates.

MATERIALS AND METHODS

Sample collection and processing

Termite samples were collected in July, 2012 at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) in Juja (latitude 10° 0' 5'' S, Longitude 37° 0' 00" E; at an altitude of 1525 m above the sea level), Kiambu County, Kenya. Termite mounds (approximately 1 to 3 km apart), which were colonized by *Odontotermes* and *Macrotermes* species were excavated to a depth of ≈1.0 m. Termite samples were collected and put into sterile plastic boxes. Worker-caste termites were used in the experiments due to their foraging activities during the establishment and maintenance of the fungus gardens. Twenty (20) worker termites (for each termite sample from different mounds) were degutted separately using sterile fine tipped forceps; subsequently, the guts were homogenized in 10 ml of sterile normal saline. The resultant gut homogenate was then serially diluted using sterile normal saline into 10^{-1}, 10^{-2} and 10^{-3} that were subsequently used for the isolation of the gut microbial symbionts.

Isolation and purification of gut bacteria

Isolation of the different groups of gut bacteria was performed using selective media. Nitrifying bacteria were isolated using a mineral medium for nitrifying bacteria with the following composition: Na_2HOP_4, 13.5 g; KH_2PO_4, 0.7 g; $MgSO_47H_2O$, 0.1 g; $NaHCO_3$, 0.5 g; $(NH_4)_2SO_4$, 2.5 g; $FeCl_3$ $6H_2O$, 14.4 mg, $CaCl_2$ $7H_2O$, 18.4 mg; agar, 15 g. All the components were mixed and brought up to a 1 L volume of distilled water at pH 8.0. The isolation of denitrifying bacteria was done using the recommended nitrate agar medium, while the ammonifying bacteria were isolated using peptone agar medium. The nitrogen fixing bacteria were isolated using nitrogen free media containing: K_2HPO_4, 1 g; $MgSO_4.7H_2O$, 0.2 g; $CaCO_3$, 1 g; NaCl, 0.2 g; $FeSO_4.7H_2O$, 5 mg; glucose, 10 g; $NaMoO_4$, 5 mg; agar, 15 g; distilled water, 1 L; pH 7.0. The spread plate technique was used as described by Holt et al. (1994). In each case, plates were incubated and monitored daily at 30°C for up to 72 h. Bacterial colonies that formed on the plates were subsequently subcultured on the corresponding media previously described until axenic cultures were obtained.

Morphological and physiological characterization of the isolates

Colony morphology of the pure isolates was described using standard microbiological criteria with special emphasis on pigmentation, shape, form, elevation and margin formation. Preliminary characterization by Gram staining of the isolates was done as described by Holt et al. (1994). Temperature and pH ranges and optima for growth were determined in LB broth. Growth of isolates was measured using the Shimadzu model UV240 spectrophotometer at 660 nm in cuvettes with a 1-cm light path. The ability to tolerate sodium chloride and ammonia was tested by inoculating the isolates in LB broth supplemented with different concentrations of sodium chloride (0, 3, 5, 7 and 10%) and ammonium sulphate (0, 5, 10, 15 and 20%) as described by Muller et al. (2006).

Biochemical and enzymatic characterization

Catalase test was conducted by growing bacteria in nutrient broth overnight at 37°C. Catalase activity was observed by adding few drops of 3% H_2O_2 to the broth cultures, kept on the glass slides. The production of effervescence due to catalase catalyzed breakdown of H_2O_2 to molecular oxygen indicated a positive reaction. The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined using urea brot media containing a phenol red indicator. Bacterial cultures were inoculated

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in the medium and incubated at 37°C for 48 h. A positive reaction was indicated by development of deep pink color. For nitrate reduction test, the isolates were incubated at 37°C overnight followed by addition of 0.5 ml each of sulphanilic acid (0.8% in 5 N acetic acid) and α-naphthylamine (0.5% in 5 N acetic acid). The appearance of red or pink color indicated the positive test for nitrate reduction. Hydrogen sulfide production test was performed using SIM agar (Peptone 30 g/l, beef extract 3 g/l, ferrous ammonium sulphate 0.20 g/l, sodium thiosulphate 0.025 g/l, agar 3 g/l (pH 6.0) mixtures) that was inoculated with actively growing cultures. Blackening along the line of inoculation shows a positive test after 3 - 5 days at 30°C.

For methyl red (MR) and Voges–Proskauer (VP) tests, the MR-VP broth was inoculated with actively growing bacterial cultures and incubated at 30 C for 48 h. An uninoculated tube with the medium served as a negative control. MR indicator (Barrit's reagent) was added and positive cultures for the MR test appeared red while positive cultures for the VP test gave a rose coloration. An Indole test detects the production of indole from the amino acid tryptophan. Tryptone broth was inoculated with the isolates and incubated at 30°C for 48 h. Kovac's reagent (3 drops) was added into the tubes. A red colored layer on the surface of the tube indicated a positive test for indole. The citrate utilization was performed by inoculating the bacterial cultures on Simmon's citrate agar plates and incubating at 30°C for 48 h. A blue colourization of the medium indicated a positive reaction. The ammonifying and nitrifying test were determined by inoculating bacterial cultures in test tubes containing 5 ml peptone broth and ammonium sulfate broth media, respectively. The cultures were incubated at 30°C for 5 days and the presence of ammonia, an indicative of ammonification was detected by a yellow color when 3 drops of Nessler's reagent were added to the test cultures. A positive reaction for the nitrifying test was indicated by the development of a blue-black color upon addition of a few drops of Trommsdorfs reagent and sulfuric acid to the test cultures.

Qualitative enzymatic screening

Qualitatively screening of the isolates for production of enzymes such as cellulases, xylanases, amylases, proteases, gelatinases, lipases and nitrogenases was performed. Screening for cellulases and xylanasese was performed according to the procedures described by Ruijassenaars and Hartmans (2001). Briefly, the freshly growing bacterial cultures were spot inoculated on nutrient agar plates supplemented with 0.2% carboxymethyl cellulose (CMC) and xylan substrates for cellulases and xylanases, respectively. The plates were incubated at 30°C for 5 days and were overlaid with Congo-red (1 g/ml) solution for 15 min. After washing the plate surface with 1 M NaCl, clear zone around colony indicated the enzyme activity. Gelatin hydrolysis was performed in nutrient gelatin medium, which was inoculated with a loopful of actively growing bacterial cultures and incubated for 3 days at 30°C. Control tubes solidified when placed in ice whereas medium in inoculated tubes remained unsolidified, showing positive gelatin hydrolysis test.

To detect amylase activity, nutrient agar plates supplemented with 0.3% soluble starch were aseptically inoculated with actively growing bacterial cultures and incubated for 3 days at 30°C. The plates were then flooded with Gram's iodine, a clear yellow zone around the inoculation spots indicated starch hydrolysis. The protease activity was detected using casein hydrolysis where skim milk agar (Skim milk powder 100 g/l, agar 15 g/l) plates were aseptically spot inoculated with actively growing cultures and incubated for 3 days at 30°C. Clear zones around the inoculation spots indicated casein hydrolysis. The production of lipases by the isolates was determined by culturing the isolates on basal media (1% KH_2PO4, 0.01% $MgSO_4.7H_2O$, 0.005% $CaCl_2.2H_2O$, 1% NaCl and 1% Na_2CO_2) supplemented with 1% olive oil (domestic grade) as the sole carbon source. The plated medium was then inoculated by spotting bacterial cultures and incubated for 3 days at 30°C. Lipase production was indicated by the precipitation of calcium crystals around the colonies. The nitrogenase activity of the bacterial cultures was estimated by conducting the acetylene reduction assay. The isolates were grown in 5 ml of nitrogen free semi-solid media in 15 ml serum bottles sealed by rubber stoppers. The air (10%) was removed and replaced with acetylene. The head space of the cultural tube was sampled (1 ml of air) to determine ethylene production after twelve hours on a Shimadzu Gas Chromatograph (GC-9A, Japan). A standard ethylene gas was used as a positive control. An un-inoculated tube was used as a negative control as described by Eckert et al. (2001).

Molecular characterization of the isolates

Genomic DNA (gDNA) extraction and PCR amplification

Pure isolates were separately inoculated into sterile falcon tubes containing 15 ml Luria Bertani (LB) medium and incubated for 48 h in a shaking incubator (at 150 rpm) at 37°C. The isolate cultures were then centrifuged at 6000 rpm for 10 min. The resultant pellets were separately re-suspended in sterile micro tubes containing 0.2 ml of tris-ethylenediaminetetraacetic acid (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The cell suspensions were used for gDNA extraction using the UltraClean® Mega soil DNA isolation kit (MO BIO Laboratories, Inc.) according to the manufacturer's protocol. Purified DNA was quantified photometrically (NanoDrop; Thermo Fisher Scientific, Germany) and used as a template for amplification of 16S rRNA genes using the universal bacterial primers (8F 5'-AG (A/G) GTTTGATCCTGGCT-3' forward primer and 1492R 5'-CGGCTACCTTGTTACGACTT-3' reverse primer) according to their position in relation to the *Escherichia coli* gene sequence (Lane, 1991). For each PCR, 1 µl (25 ng/ µl) of the template was mixed with TaKaRa Ex Taq™ HS (5 units/µl) according to the manufacturer's protocol. The PCR conditions were as described by Mackenzie et al. (2007) except the final extension was at 72°C for 8 min. PCR product size was checked using a 1% agarose gel stained with ethidium bromide. The amplicons were gel purified using Macherey-Nagel NucleoSpin extract II kit as per the manufacturer's protocol and eluted in 30 µl of TE Buffer (5 mM, pH 8.0).

DNA Sequencing and phylogenetic analysis

Sequencing of the purified PCR products was done using a commercial service provider (Macrogene, South Korea). Sequences of the isolates were manually edited in chromas and checked for presence of artifacts or chimeric structures using the Mallard software (Ashelford et al., 2006). A search for similar sequences using BLASTN program was performed, and sequence alignment was performed using the CLUSTAL Omega program (http://www.clustal.org). A neighbor-joining tree of the aligned sequences was constructed using MEGA V5.10 (Tamura, 2011). Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). To obtain support values for the branches, bootstrapping (Felsenstein, 1985) was conducted with 1000 replicates. All sites, including gaps in the sequence alignment, were excluded pairwise in the phylogenetic analysis. Using the resultant neighbor-joining tree, each isolate was assigned to the proper taxonomic group. The taxonomic

assignment was confirmed at a 90% confidence level using the naïve Bayesian rRNA classifier on the RDP website (Cole et al., 2005). All sequences were deposited in GenBank nucleotide database with the accession numbers KF872743 to KF872766.

RESULTS

Characterization of the isolates

A total of 46 bacterial isolates were isolated from guts of *Odontotermes* and *Macrotermes* using selective media. Morphologically (Table 2), the isolates exhibited diverse colony characteristics differing in their form, color, margin, cell arrangement and the Gram reaction. The cell shape was either rod or cocci with a positive or negative Gram reaction (Table 1). Biochemical tests revealed that all the isolates were positive for the ammonifying, nitrifying and catalase tests (Table 1 and Figure 1A). About 85% of the isolates were positive for the citrate test and this indicated their ability to use citrate as a source of carbon. Fifty two percent of the isolates were positive for the urease test that demonstrates the ability of the isolates to attack nitrogen and carbon bonds in amide compounds. Forty-one percent and thirty percent of the isolates were positive for the MR and VP tests (Table 1 and Figure 1A). For the nitrate reduction test, only seventeen isolates (35%) were positive. This showed that these isolates could reduce nitrates to nitrites or beyond. Only 15% of the isolates could produce indole from the amino acid tryptophan as revealed by the positive indole production test. Twenty-eight percent of the isolates had the ability to produce hydrogen sulfide from substrates such as sulfur containing amino acids and organic sulfur as they were positive for the hydrogen sulfide production test (Table 1 and Figure 1A).

The growth pH of the isolates ranged from pH 4.0 to 8.0. Isolates (Bacto15, 24, 25, 26, 28 and 41) grew well at pH 4 and 6 (Figure 1B), while isolate Bacto31 had an optimum growth at pH 6.0 and Bacto40 had the least growth at all the pH ranges. The isolates also exhibited growth at different temperatures; however the optimum growth temperatures ranged from 30 to 37°C. Isolates (Bacto31 and 40) had the least growth in all temperature ranges (Figure 1B). They also showed varied tolerance to different concentrations of sodium chloride up to 7% with a generally poor growth at 10% sodium chloride concentration. The isolates exhibited varied tolerance to different concentrations of ammonium sulfate up to 15% and a generally poor at 20% of ammonium sulfate demonstrating that the isolates could not tolerate very high levels of ammonia (Figure 1B). Isolates (Bacto21, 31 and 40) had the least growth in almost all ammonium sulfate concentrations. All the isolates tested positive for the acetylene reduction test (ARA) meaning they all produced the nitrogenase enzyme (Figure 1A). Forty-six percent of the isolates were positive for casein hydrolysis while 28% of the isolates were able to hydrolyze starch and gelatin (Table 1 and Figure 1A). 30% of the isolates were positive for cellulase activity while 20% of the isolates were positive for the xylanase activity. This showed that these isolates were able to degrade cellulose and xylan, respectively, suggesting their potential role in the degradation of plant biomass in the environment. Twenty-four percent of the isolates were positive for lipolytic activity (Table 1 and Figure 1A).

Affiliation of 16S rRNA gene sequences of the isolates

A total of 26 representative isolates from guts of *Macrotermes* and *Odontotermes* spp., were selected based on the morphological (Table 2) and biochemical characteristics (Gram, catalase, urease, methyl red and Voges Proskauer tests) for further characterization using molecular approach. The isolates (prefixed as Bacto with their accession numbers in parenthesis) in the inferred phylogenetic tree were phylogenetically diverse and affiliated with known members from different phyla including *Firmicutes, Proteobacteria* and *Actinobacteria* (Figure 2A and B). Comparison of the newly isolated 16S rRNA gene sequences to known sequences in the Genbank database using Blastn analysis indicated sequences similarities of >90% with known sequences (Table 2). Most of the isolates (27%) were closely affiliated with members of the genus *Enterobacter* with >95 sequence identity. Four isolates (Bacto19, 24, 26 and 35) had >98% sequence identities with known members of the genus *Pseudomonas* that together formed a single sub-cluster supported with a bootstrap value of 99% (Table 2 and Figure 2A). Bacto26 [KF872751] and Bacto35 [KF872760] were obtained from *Macrotermes* spp. and were phylogenetically identical and had 100% sequence affiliation with *Pseudomonas libanensis* [KC789764] and *Pseudomonas rhodesiae* [KF923822]. Bacto19 [KF872744] was also obtained from *Macrotermes* sp. and was 100% affiliated with *Pseudomonas monteilii* [KF475842] while Bacto24 [KF872749] was isolated from *Odontotermes* sp. and on the same branch together with other *Pseudomonas* species (Table 2; Figure 2A). This was supported by a bootstrap value of 99%. Four isolates (Bacto32, 34, 39 and 40) were affiliated with members of the genus *Serratia* [with 97–100% sequence identities] (Table 2). These isolates formed a single sub-cluster with several other members of the genus *Serratia* as indicated in the inferred phylogenetic tree (Figure 2A). Bacto32 and 34 were isolated from *Odontotermes* spp. while Bacto39 and 40 were from *Macrotermes* spp. Isolate Bacto37 [KF872762] was 100% affiliated with *Klebsiella pneumonia,* while Bacto41 [KF872766] was 100% related to *Citrobacter farmer* (JX393004) and clustered with other *Citrobacter* species. Bacto28 [KF872753] had 100%

Table 1. Biochemical and enzymatic properties of bacterial isolates obtained from the guts of *Odontotermes* and *Macrotermes* species collected from JKUAT in July 2012.

Isolate code	Gram test	Catalase test	Urease test	Nitrate reduction	Citrate utilization	HS production	Ammonifying test	MR Test	VP test	Nitrite test	Indole test	Casein hydrolysis	Starch/ gelatin hydrolysis	Cellulase	Xylanase	Lipase
Bacto1	+	+	-	-	+	-	+	-	-	+	-	-	+	-	-	-
Bacto2	+	+	-	+	-	-	+	-	-	+	-	+	-	-	-	+
Bacto3	+	+	-	-	+	-	+	-	-	+	-	-	-	-	-	+
Bacto4	+	+	-	-	+	-	+	-	-	+	-	-	+	-	-	+
Bacto5	-	+	+	-	+	-	+	-	-	+	-	+	+	-	+	+
Bacto6	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-
Bacto7	+	+	-	-	+	-	-	+	-	+	-	-	-	-	-	-
Bacto8	-	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-
Bacto9	-	+	+	-	-	+	+	-	-	+	-	-	-	-	-	+
Bacto10	-	+	+	-	+	+	+	+	-	+	-	-	-	-	-	+
Bacto11	+	+	-	+	+	+	-	-	-	+	+	+	-	-	-	+
Bacto12	+	+	-	-	+	+	+	-	-	+	+	+	-	-	-	-
Bacto13	-	+	+	-	+	+	+	-	+	+	-	+	+	-	-	-
Bacto14	+	+	-	+	+	+	+	-	-	+	+	+	+	-	-	-
Bacto15	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-
Bacto16	+	+	+	+	+	+	+	-	-	+	+	-	-	+	-	-
Bacto17	-	+	+	+	+	-	+	-	+	+	-	+	-	-	-	-
Bacto18	-	+	+	+	+	-	+	-	+	+	-	+	-	-	-	-
Bacto19	+	+	+	-	+	-	+	-	-	+	-	-	-	-	-	-
Bacto20	-	+	-	+	+	-	+	-	+	+	-	-	+	+	-	+
Bacto21	+	+	-	-	+	-	+	-	-	+	-	+	-	-	-	-
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Bacto 26	-	+	+	+	+	-	+	+	-	+	+	+	-	+	-	-
Bacto 27	+	+	+	-	+	-	+	+	+	+	-	+	-	+	-	-
Bacto 28	-	+	+	+	+	-	+	+	-	+	-	+	-	-	-	-
Bacto 29	-	+	-	-	+	-	+	+	+	+	-	-	-	-	-	-
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Bacto 34	-	+	-	+	-	-	+	-	-	+	-	-	-	-	+	+
Bacto 35	+	+	-	-	+	-	+	+	-	+	-	-	-	-	-	+
Bacto 36	-	+	-	-	+	-	+	+	+	+	-	-	+	-	-	-
Bacto 37	+	+	+	-	+	-	+	-	+	+	-	-	-	-	+	-
Bacto 38	+	+	-	-	-	-	+	-	+	+	-	-	-	+	-	+
Bacto 39	-	+	+	-	+	-	+	+	-	+	-	-	+	+	+	-
Bacto 40	-	+	+	-	+	-	+	-	-	+	-	+	-	+	+	-
Bacto 41	-	+	+	-	+	-	+	-	-	+	+	+	-	+	-	-
Bacto 42	+	+	-	-	+	-	+	-	-	+	-	+	-	+	-	-
Bacto 43	-	+	+	-	+	-	+	-	-	+	-	+	-	-	-	-
Bacto 44	+	+	-	-	+	-	+	+	-	+	-	+	-	-	+	-
Bacto 45	+	+	-	-	+	-	+	+	-	+	-	+	-	+	-	-
Bacto 46	+	+	-	-	+	-	+	-	-	+	-	+	-	-	-	-

+ Denotes a positive result and - denotes a negative result for the test. MR– Methyl Red, VP – Voges-Proskauer.

Table 2. Taxonomic affiliation and percentage sequence similarities of bacterial isolates with closest relatives from the Genbank database.

Sample ID	Accession No.	Representative isolates ID	Host/Termite sp.	Closest taxonomic affiliation	Isolation Source	ID (%)
Bacto27	KF872752	-	*Odontotermes* sp.	*Enterobacter cancerogenus* strain Lpq4 (HQ154134)	rice paddy soil	99
Bacto33	KF872752	-	*Macrotermes* sp.	*Enterobacter cancerogenus* strain Lpq4 (HQ154134)	rice paddy soil	99
Bacto29	KF872754	-	*Odontotermes/ Macrotermes* spp.	*Enterobacter* sp. (AY596467)	N/A	100
Bacto36	KF872761	-	*Odontotermes* sp.	*Enterobacter* sp. (AY596467)	*N/A*	100
Bacto38	KF872763	-	*Macrotermes* sp.	*Enterobacter* sp. LCd2 (KF411753)	Alfalfa root nodules	99
Bacto22	KF872747	Bacto20	*Macrotermes* sp.	*Enterobacter* sp. RsN-1 (AB673456)	*R. speretus*	98
Bacto31	KF872756	Bacto11, 12, 14	*Odontotermes/ Macrotermes* spp.	*Enterobacter cancerogenus* strain Lpq4 (HQ154134)	rice paddy soil	96
Bacto34	KF872759	-	*Odontotermes* sp.	*Serratia* sp. Cd22 (AB673460)	*C. domesticus*	97
Bacto32	KF872757	Bacto5	*Odontotermes* sp.	*Serratia marcescens* strain Z1085 (KC212068)	N/A	100
Bacto39	KF872764	-	*Macrotermes* sp.	*Serratia nematodiphila* strain Z65 (KC212076)	Milk	100
Bacto40	KF872765	Bacto43	*Macrotermes* sp.	*Serratia nematodiphila* strain Z65 (KC212076)	Milk	100
Bacto24	KF872749	-	*Odontotermes* sp.	*Pseudomonas aeruginosa* strain GH2T (KC864775)	Water	99
Bacto26	KF872751	Bacto18, 17	*Macrotermes* sp.	*Pseudomonas libanensis* strain BGR5 (KC789764)	Soil	99
Bacto35	KF872760	Bacto44, 45	*Odontotermes/ Macrotermes* spp.	*Pseudomonas libanensis* strain BGR5 (KC789764)	Soil	99
Bacto19	KF872744	Bacto6, 7	*Macrotermes* sp.	*Pseudomonas monteilii* strain IHB B 2329 (KF475842)	Rhizosphere soil	100
Bacto15	KF872745	Bacto16	*Odontotermes* sp.	*Bacillus licheniformis* strain DGB (KF840408)	Sugarcane bagasse	98
Bacto30	KF872755	-	*Odontotermes* sp.	*Bacillus cereus* (JX155762)	Rhizosphere soil	100
Bacto37	KF872762	-	*Macrotermes* sp.	*Klebsiella pneumonia* (JX390619)	Ethnomedicinal	100
Bacto25	KF872750	Bacto33	*Odontotermes* sp.	*Staphylococcus* sp. MEF7 (JN660060)	Fermented food	100
Bacto13	KF872743	-	*Marotermes* sp.	*Bacillus simplex* (KJ161416)	Sediment	99
Bacto41	KF872766	-	*Odontotermes* sp.	*Citrobacter farmeri* strain W17-1 (JX393004)	Frog gut	100
Bacto28	KF872753	-	*Odontotermes/ Macrotermes* spp.	*Proteus mirabilis* strain ZK1 (KF471515)	Waste water	100
Bacto23	KF872748	-	*Macrotermes* sp.	*Micrococcus terreus* strain DL20 (HQ009859)	River water	99
Bacto21	KF872746	Bacto2, 3, 4	*Macrotermes/ Odontotermes* spp.	*Rhodococcus equi* strain SW9 (KF873018)	Soil	99

N/A denotes not applicable; ID denotes identity

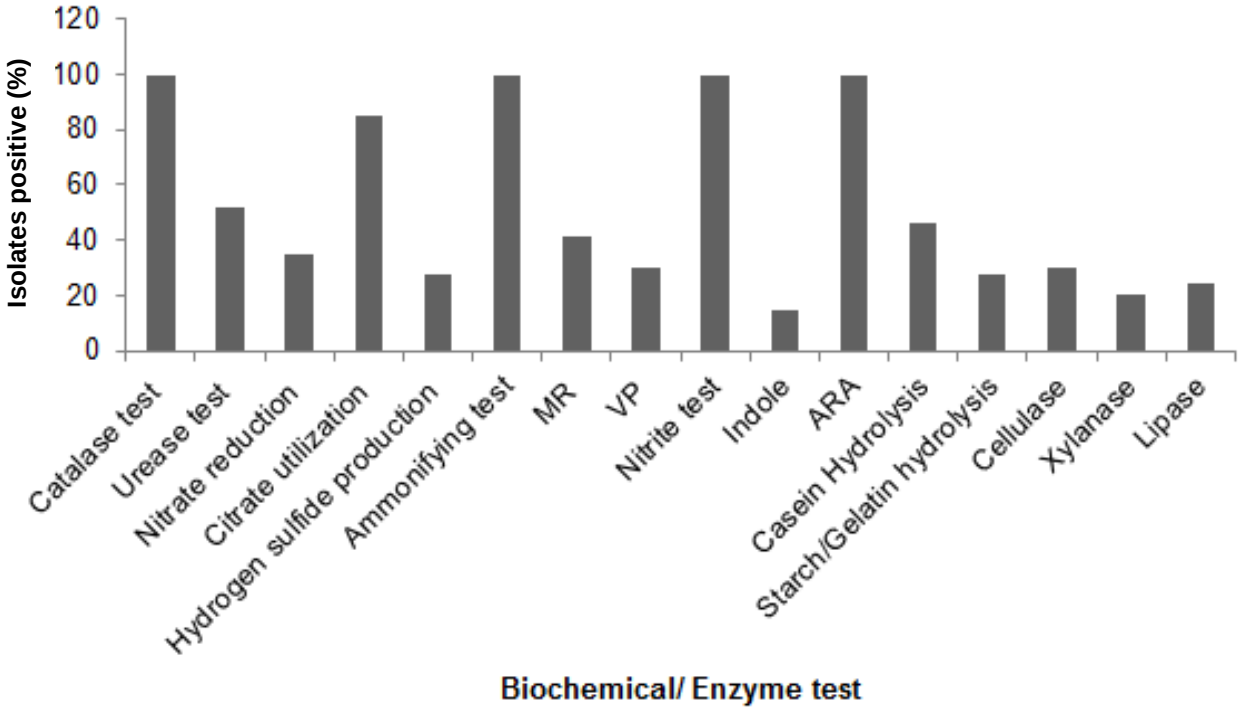

Figure 1A. Biochemical and enzymatic properties of bacterial isolates obtained from termite gut. MR 'denotes' Methyl Red; VP 'denotes' Voges–Proskauer; ARA 'denotes' acetylene reduction assay.

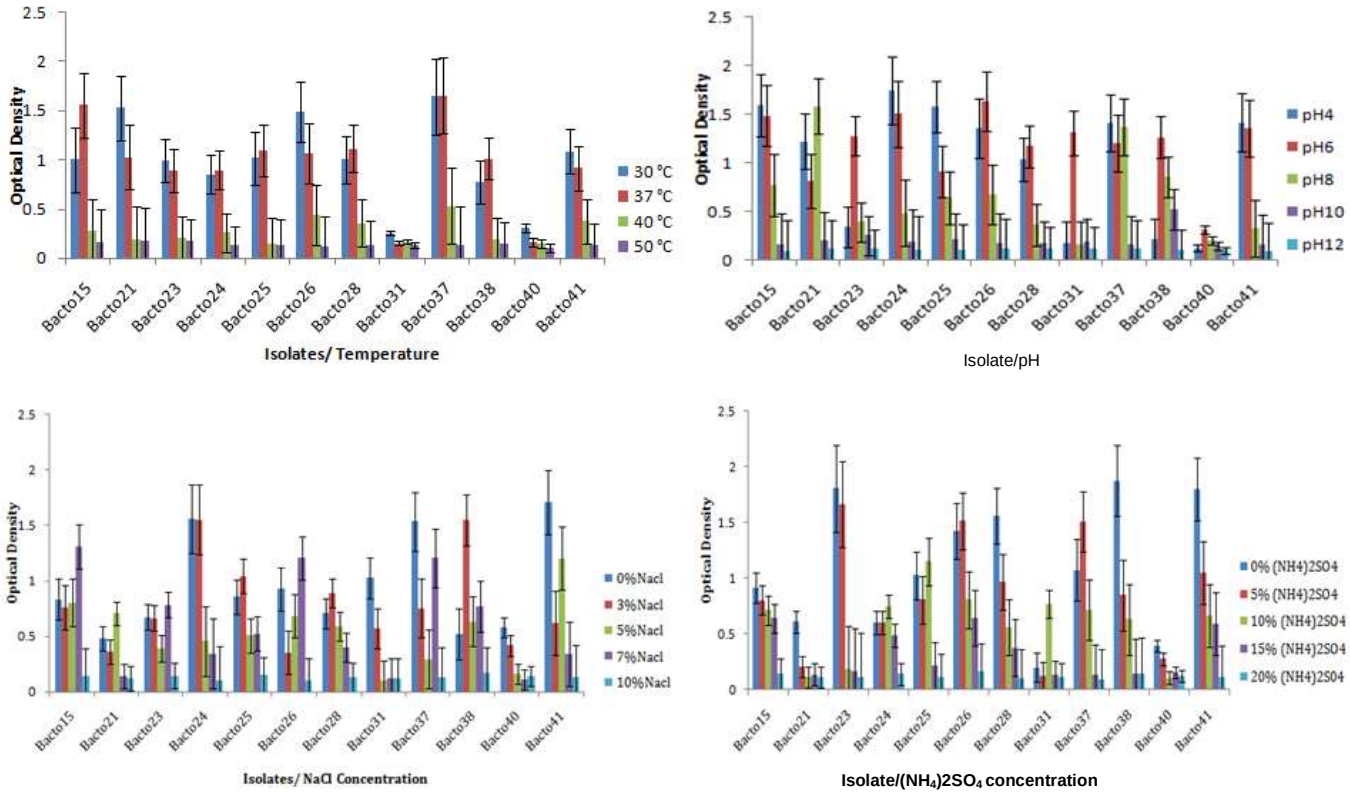

Figure 1B. Physiological properties of bacterial isolates.

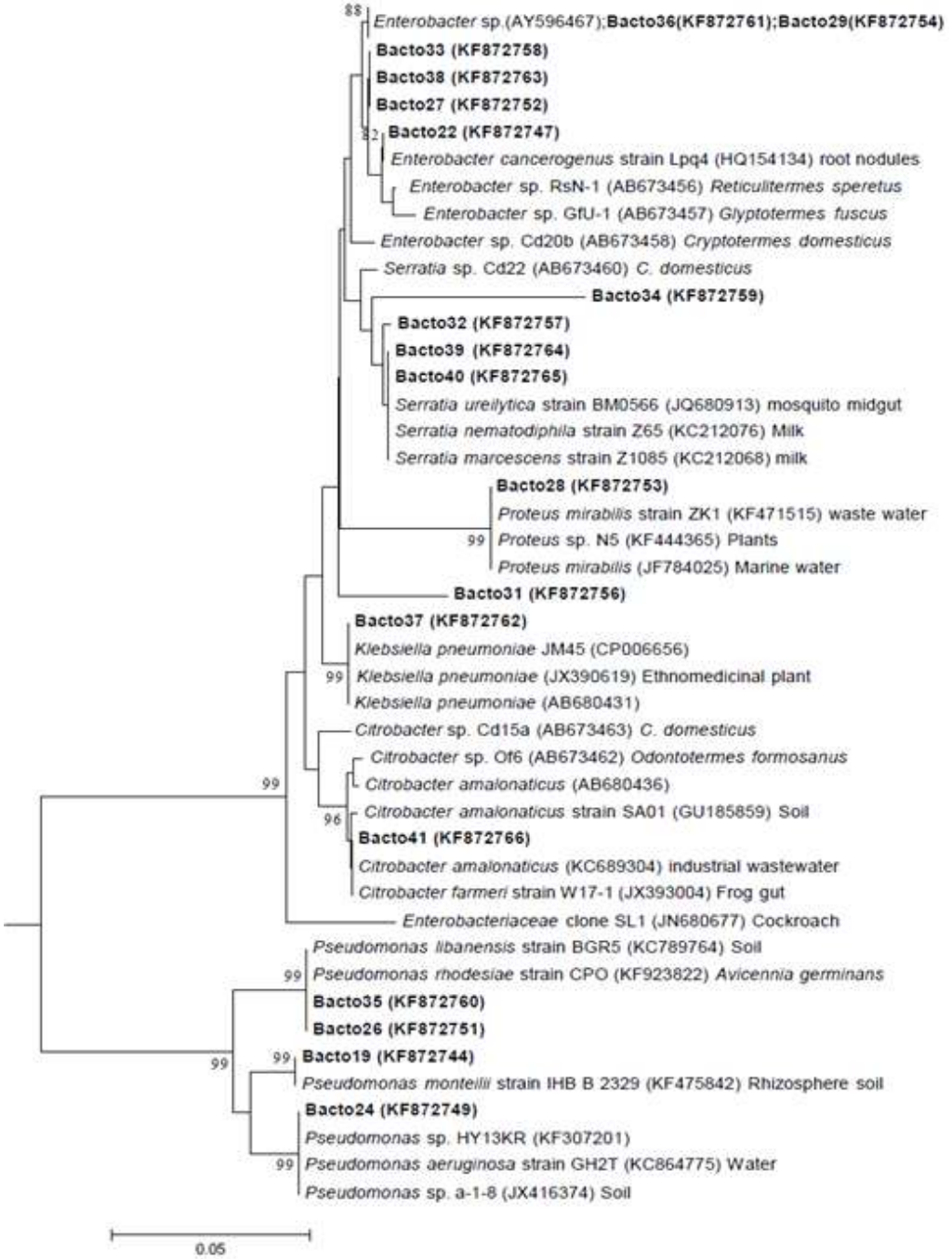

Figure 2A. Evolutionary relationships between partial 16S rRNA gene sequences of the isolates and some selected known bacterial species.

sequence similarity with other *Proteus* species that together formed a separate branch supported by a bootstrap value of 99% (Figure 2A). Isolates Bacto13, Bacto15 and Bacto30 were closely affiliated with members from the genus *Bacillus* (with between 98-100% sequence identities) and were from different termite species (Table 2). The three isolates formed a separate minor sub-cluster together with other known *Bacillus* species (Figure 2B). Bacto25 clustered with members of the genus *Staphylococcus*. Two isolates Bacto23 [KF872748] and Bacto21 [KF872746] formed a separate sub-cluster with some members belonging to the phylum *Actinobacteria.* Bacto23 had 99% sequence similarity with *Micrococcus terreus* (HQ009859) while

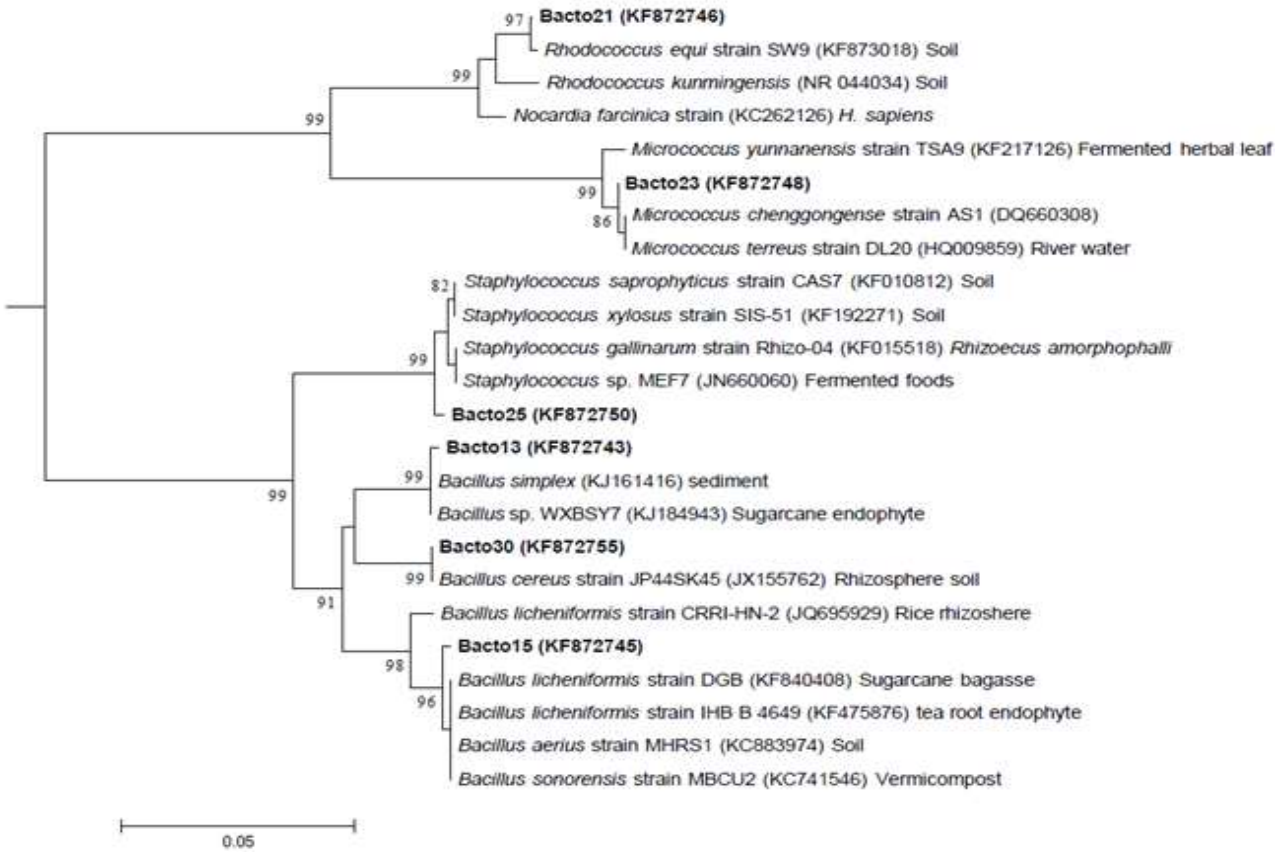

Figure 2B. Evolutionary relationships between partial 16S rRNA gene sequences of the isolates and some selected known bacterial species. The scale bar indicates approximately 5% sequence difference. Isolates are prefixed as Bacto and in bold font with accession numbers in parenthesis. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap re-samplings. The 16S rRNA sequence of *Methanoculleus thermophiles* (accession number, AB065297) was used to root the tree.

Bacto21 was 99% related to *Rhodococcus equ i* (KF873018) (Table 2 and Figure 2B). Four poorly resolved sequences (Bacto1, 5, 9 and 46) were excluded from the phylogenetic tree analysis.

DISCUSSION

In this study, the authors successfully isolated and partially identified termite gut bacterial microsymbionts that could be potentially involved in nitrogen cycle activities and degradation of plant biomass. The findings of this study support previous data that termites harbor gut bacterial symbionts, which aid in nitrogen fixation (Majeed et al., 2012) and lignocellulose digestion (Brune and Ohkuma, 2011). The isolates had the ability to degrade nitrogenous biopolymers and subsequently release ammonia, which is enzymatically converted to nitrites and nitrates as well as produce the enzyme catalase that converts hydrogen peroxide to water as demonstrated by the ammonifying, nitrifying and catalase tests, respectively. Majority of the isolates also had the ability to use citrate as the sole source of carbon. The abilities of some isolates to oxidize glucose and reduce nitrates to nitrites (Table 1 and Figure 1A) indicate their possible roles in the nitrogen metabolism activities (Lengeler et al., 1999). Notably, all the isolates demonstrated nitrogenase activity, which possible implicate them in nitrogen fixation activities. Brauman et al. (2015) demonstrated that termites' gut microbiota are responsible for nitrogen fixation that underscores nitrogen fixation activities within the guts of the fungus cultivating termites (Majeed et al., 2012).

Molecular characterization and phylogenetic analysis showed that all the isolates were affiliated with three bacterial phyla (*Proteobacteria*, *Actinobacteria* and *Firmicutes*) and that they belonged to ten different general (Figure 2A and B). The isolates Bacto22, 27, 29, 31, 33, 36, and 38 were obtained from different termite species (Table 2) phylogenetically related to the genus

Enterobacter (with >95% sequence similarity) and this was further supported by their ability to reduce nitrate to nitrite and being catalase positive. Members of *Enterobacter* that were previously isolated from termite guts were shown to play a role as nitrogen fixers (Adams and Boopathy, 2005). The detection of isolates (such as Bacto41, affiliated with the genus *Citrobacter*) demonstrates the presence of termite gut symbionts that can ferment glucose, reduce nitrates to nitrites and use citrate as a carbon source. Some members of this group have previously been isolated from the gut of termites (Adams and Boothy, 2005) and may play an important role in the nitrogen metabolism. The detection of gut isolates (Bacto19, 24, 26 and 35) belonging to genus *Pseudomonas* may suggest their contribution in assisting termites to degrade low-molecular-weight compounds derived from the breakdown of plant- and animal-biomass (Madigan and Martinko, 2005). Though Bacto35 and 26 formed a monophyletic sub-cluster, they exhibited different biochemical characters associated with members of the genus *Pseudomonas.* Bacto35 was Gram positive, MR positive, and Indole negative while Bacto26 had opposite reactions to these tests. Such contradiction in the biochemical tests may be due to the complications associated with this genus on its taxonomy that is better resolved by the analysis of the ribosomal RNA as depicted in the phylogenetic tree (Figure 2A).

The inferred phylogenetic tree positioned isolates Bacto37 and Bacto28 together with the genera *Klebsiella* and *Proteus,* respectively. Members of the genus *Klebsiella* are Gram negative, rod shaped and facultative anaerobic bacteria as observed from the morphological and biochemical results of isolate Bacto37. Some *Klebsiella* species including *Klebsiella pneumonia*, *Klebsiella oxytoca* and *Klebsiella planticola* are capable of fixing nitrogen and are classified as associative nitrogen fixers (Chelius and Triplett, 2000). Isolate Bacto23 was phylogenetically related to members of the genus *Micrococcus* with 99% sequence similarity.

Micrococcus has the ability to aerobically produce acid from glucose, glycerol and aesculin hydrolysis, major pigment production, motility, and conversion of nitrate to nitrite. It has been reported that members of this genus play a key role in the nitrogen cycle as denitrifying bacteria (Smith et al., 1999). Indeed, termites form a model system for studying host-symbiont relationship and bioreactor systems. The isolation of different bacterial strains from the guts of termites demonstrates that termites harbor diverse bacterial lineages, some of which perform different activities associated with plant biomass degradation and nitrogen metabolism.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Vibrio cholerae non-O1 in bivalve mollusks harvesting area in Bahia, Brazil

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The aim of this study was to characterize the antimicrobial resistance and pathogenicity potential of *Vibrio cholerae* isolates originated from water samples and bivalve mollusks. The strains were subjected to phenotypic identification and molecular confirmation using the species-specific initiator *(OmpW)*; minimum inhibitory concentration (MIC) was determined; and the production of metallo-β-lactamases (MβLs) and virulence potential of the strains by using the initiator *ctxAB* (cholera toxin), *tcp* (toxin co-regulator pilus), *rfb*O1 (serogroup O1) and *zot* (zonula occludens toxin) were investigated. Six isolates of the bacterium (three from water and three from bivalve mollusks) were confirmed through the biochemical and specific gene detection tests. The isolates presented a high susceptibility toward the tested antimicrobials (91%) (10/11). One of the strains from water showing resistance to imipenem (MIC 20 µg), and producing MβLs did not show any involvement of plasmids. The genes related to the virulence were not detected; and all of the *V. cholerae* isolates belonged to the non-O1 serotype. However, the presence of an imipenem-resistant and MβLs-producing *V. cholerae* in a river mouth aquatic environment, which is a natural aviary of bivalve mollusks, represents a risk to the health of the population and alarms the public health agencies.

Key words: Mollusc, public health, antibiotic resistance.

INTRODUCTION

Vibrio cholerae is the causative agent of cholera, which inhabits aquatic environments. Water has a significant role in its transmission and epidemiology of this disease leading to outbreaks at endemic, epidemic, and pandemic levels (Goel et al., 2010).

Based on somatic antigen (O antigen), *V. cholerae* is classified into serogroups or serovars, and a total of 206 serogroups of *V. cholerae* have been identified so far. The toxigenic serogroups O1 and O139 have been found to be directly associated with epidemic (O1 and O139) and pandemic (O1) cholera (Raychoudhuri et al., 2009). The O1 serogroup can be further classified into two biotypes, classical and El Tor. El Tor was disseminated around the world the etiological agent behind current

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pandemic (Espiñeira et al., 2010).

V. cholerae has frequently been isolated from environmental samples, which mainly consists of serogroups, non-O1/ non-O139. These serogroups may cause diarrheal diseases less severe than cholera and do not present epidemic potential (Wong et al., 2012). Although they normally do not produce a cholera toxin, they carry other virulent factors involved in the pathogenicity, including the production of hemolysins, proteases, hemagglutinins, and may have multiple drug resistance (Oufdou and Mezrioui, 2012). It is reported that in aquatic environment, one serogroup can be converted to another by homologous recombinations, or by mutation and/or rearrangement, as noticed in O139 lineage (Blokesch and Schoolnik, 2007).

Gram-negative bacteria may acquire, maintain and express new genetic information and become resistant to one or several antimicrobials (Walsh et al., 2002). The extensive and/or inappropriate use of antimicrobials in humans and animals, agriculture and aquaculture as well as improper disposal of antimicrobials contributes in the development of resistant bacteria in the environment (Manjusha and Sarita, 2013).

The study of resistance to antimicrobial agents in indigenous aquatic microorganisms is important since it indicates degree of modification of ecosystems by man. The release of antimicrobials in municipal sewage system, surface water, groundwater, soil sediments, and mud samples exerts a selective pressure on the environmental microorganisms, and thus contributes in the proliferation of resistant microbes (Baquero et al., 2008).

Metallo-β-lactamase, an enzyme produced by bacteria, hydrolyzes carbapenems, penicillin, and cephalosporin. The main members of MβL family include imipenemase (IMP) and Verona imipenemase (VIM). VIM is well known and commonly found in several bacterial species. It has eleven variants reported all over the world, especially in Europe and Asia (Villegas et al., 2006). In this study, *V. cholerae* strains were characterized according to the presence of genes causing virulence and resistance to antimicrobials. These strains were isolated from the river waters in Recôncavo da Bahia (Brazil), an area where bivalve mollusks are harvested.

MATERIALS AND METHODS

Isolation and identification

We analyzed six bacterial isolates suspects of *V. cholerae* from water samples and bivalve mollusks (*Crassostrea rhizophorae* and *Mytella guyanensis*) harvested from the river mouth of São Francisco do Conde, Bahia (S 12° 33' 52.4"/ W 038º 41' 40.5"). These samples belong to the microbial specimen collection of the Food and Environmental Microbiology Laboratory, at the Nucleus for Studies of Fishing and Aquaculture (NEPA), of the Federal University of Recôncavo da Bahia. The strains were stored in agar stock at 15°C.

Initially, the isolates were re-isolated and biochemically verified. The isolates were grown in BHI broth containing 1% NaCl (pH 8.5) at 37ºC for 24 h and then inoculated in Petri plates containing thiosulfate citrate bile salts sucrose (TCBS) agar. Biochemical identification was carried out with the help of the biochemical keys proposed by Noguerola and Blanch (2008). Molecular identification was performed with the help of PCR by using primers directed to *ompW* gene (external membrane protein) (304 bp); forward: 5' - CAC CAA GAA GGT GAC TTT ATT GTG- 3' and reverse: 5' - GGT TTG TCG AAT TAG CTT CAC C - 3' (Goel et al., 2007).

Total DNA extraction

The genomic DNA was extracted by using modified protocol proposed by Sambrook et al. (1989). Initially, 2 mL of culture cultivated in tryptone soy broth (TSB) containing 1% NaCl at pH 8.5 for 24 h was transferred to microtubes and frozen. After being frozen for 30 min, the material was defrosted and centrifuged at 5.0 rpm for 10 min. The supernatant was discarded; 1 mL of sterile water was added to the pellet and centrifuged again.

The pellet was re-suspended in 500 µL of extraction buffer (0.15 M NaCl, 50 mM Tris-HCL, 10 mM EDTA, 2% SDS, pH 8.0) supplemented with lysozyme and incubated in water bath at 65ºC for 1 h. Then 0.5 mL of chloroform: isoamyl alcohol (24:1) and 0.5 mL of potassium acetate (0.5 M) was added and centrifuged at 10.0 rpm for 15 min. The supernatant was collected and transferred to a new microtube and 1 volume of ice-cold isopropyl alcohol was added and centrifuged at 10,000 rpm for 15 min. The pellet was washed twice with cold 80% v/v ethanol, centrifuged and dried overnight. The DNA was re-suspended in 0.1 mL of TE buffer (10 mM Tris-HCl; 1 mM EDTA) and kept at -20°C.

Polymerase chain reaction (PCR) amplification

To confirm *V. cholerae* species and to detect virulence factors, the DNA was amplified using multiplex PCR technique with the help of specific primers. The specificity of the multiplex PCR was determined by using a standard *V. cholerae* strain (ATCC 19782). The PCR was performed in 25 µL reaction mixture containing target DNA. The amplification conditions used for detection of virulence factors were as follows: Initial denaturation at 94°C for 1 min, followed by 30 cycles of amplification, annealing at 59°C for 1 min, and extension at 72°C for 2 min. Before initiation of the first cycle, the reaction mixture was heated (10 min, 94°C) to complete denaturation of the template, after the last cycle, the reaction mixture was subjected at 72°C for 10 min for final extension (Goel et al., 2007).

The PCR amplified products were separated by using 1.5% agarose gel (at 150 V, 400 mA for 60 min) and visualized using transilluminator. The gels were photographed using digital documentation system Kodak EDAS290.

Detection of the virulence genes

For the detection of the virulence genes, the DNA was subjected to multiplex PCR using primers specifics for *ctxAB* (cholera toxin) (forward: 5' - GCC GGG TTG TGG GAA TGC TCC AAG - 3' and reverse: 5' - GCC ATA CTA ATT GCG GCA ATC GCA TG - 3'); *tcp* (toxin co-regulator pilus) (forward: 5' - CGT TGG CGG TCA GTC TTG - 3' and reverse: 5' - CGG GCT TTC TTC TTG TTC G - 3'); *rfbO1* (somatic antigen) (forward: 5' - TCT ATG TGC TGC GAT TGG TG - 3' and reverse: 5' - CCC CGA AAA CCT AAT GTG AG - 3'); *zot* (zonula occludens toxin) (forward: 5'- TCG CTT AAC GAT GGC GCG TTT T - 3' and reverse: 5' - AAC CCC GTT TCA CTT CTA CCC A - 3') (Goel et al., 2007).

Table 1. Occurence of *ompW* specific gene and *ctxAB, tcp, zot* and *rfbO1* virulence genes of *Vibrio cholerae* isolates tested by multiplex PCR amplification.

Isolates	Specific gene		Virulence profile		
	***ompW* (304 bp)**	***ctxAB* (536 bp)**	***Tcp* (805 bp)**	***Zot* (947 bp)**	***rfbO1* (638 bp)**
Vc5	+	-	-	-	-
Vc6	+	-	-	-	-
Vc7	+	-	-	-	-
Vc8	+	-	-	-	-
Vc9	+	-	-	-	-
Vc10	+	-	-	-	-
V. cholerae ATCC 19782	+	+	+	+	+

Antimicrobial susceptibility

The antimicrobial susceptibility was performed by disk diffusion method (CLSI, 2010) using commercially available antibiotic-containing disks (Laborclin, Brazil): nalidixic acid (30 µg), ampicillin (10 µg), gentamicin (10 µg), cefalotin (30 µg), ceftazidime (30 µg), ciprofloxacin (30 µg), chloramphenicol (30 µg), imipenem (10 µg), nitrofurantoin (300 µg), sulfamethoxazole + trimethoprim (25 µg), and tetracycline (30 µg). Bacterial strains *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used as reference controls.

The minimum inhibitory concentration (MIC) of antimicrobials was determined for the strains showing antibiotic resistance. We applied the method of increasing antimicrobial dilution in Mueller-Hinton broth (HiMedia) with the concentrations of 15, 20 and 25 µg, starting from the concentration immediately higher in the commercial discs (CLSI, 2010).

Detection of the resistance mediated by R-plasmids

The presence of R-plasmids was tested for the strains showing an antimicrobial resistant profile. We used acridine orange (AO) (Merck) at a concentration of 100 µg mL^{-1} as a curing agent. After culturing at 37°C for 24 h, the samples were aliquoted (200 µL) to the tubes containing Luria Bertani (LB) broth (control) and LB plus AO and incubated them at 37°C for 24 h. These cultures were submitted again to the antibiogram (Molina-Aja et al., 2002). We extracted plasmid DNA from the strains with antimicrobial resistance by using the Plasmid Mini Kit I (Omega/Bio-Tek®, GA) and separated it on 0.8% agarose gel. The gels were digitally photographed by an L-pix system (Locus Biotechnology).

Determination of the production of metallo-β-lactamases (MβLs)

The cultures having imipenem resistance were evaluated for the production of MβL enzymes by disk approximation test using 2-mercaptopropionic acid (Sigma-Aldrich, Buchs, United States) close to a ceftazidime disk, according to the protocols proposed by the Yong et al. (2002), Lee et al. (2003) and CLSI (2010). After standardization (10^8 CFU mL^{-1}), the inoculum was suspended in 17 mL of Mueller-Hinton agar. Then the discs containing imipenem (10 µg), ceftazidime (30 µg), 3 µg of a 2-MPA (1.2 g mL^{-1}), or 10 µL of 0.5 M EDTA (pH 8) were positioned on the plate. The discs of EDTA and MPA were placed 15 and 20 mm apart (center to center), respectively, to that of the antimicrobial containing disc. The plates were incubated at 37°C for 18 h. The presence of an inhibition zone or increasing in its size around the discs containing imipenem and/or ceftazidime located close to the disc containing MPA and/or EDTA was considered positive for the MβL production.

RESULTS AND DISCUSSION

The *V. cholerae* isolates were identified based on their phenotype and further confirmed by the presence of *ompW* (Table 1). This indicates the efficiency of the dichotomous key based on the morphology of the cells and biochemical evidence. The *ompW* specific gene is highly conserved among *V. cholerae* strains belonging to different serotypes and biotypes (Goel et al., 2007).

The state of Bahia is in the third position of largest domestic producer of marine extractive fishing in the country, it is the first producer of the region northeast (Brazil, 2010). In the Baia de Todos os Santos, Recôncavo region da Bahia, area of this study, the activity collect shellfish is intense, especially bivalve mollusks. The risk of consumption of these organisms is due to ingestion of raw oysters not debugged or had mild heat treatment. This places the oysters on top of foods responsible for foodborne illnesses.

Another problem for the state is the serious problems of sanitation and industrial impacts are increasing on an increasing scale, risk environmental quality. The presence of pollutants not only endangers the health of the population, as commits to fishing activity, that in most cases it is the only source of income for many fishermen and seafood restaurants in the region.

The absence in region of virulent strains of *V. cholerae* is satisfactory, because it is a region that has suffered from the action antrópica, moreover supplies seafood in region. The lack of real epidemiological data makes extraction of areas of bivalve mollusks need to be monitored periodically, whereas cases of cholera occur in isolation in some regions of the country.

In 2012, according to the World Health Organization, 245.393 cases of cholera were reported in 48 countries

Table 2. Resistance microbial, determination of minimum inhibitory concentration (MIC), enzyme detection metallo-β-lactamases (MβLs) and R-plasmids presence in *Vibrio cholerae* isolates from water and bivalve mollusks in the estuary of the river Subaé, São Francisco do Conde, Bahia, Brazil.

Antimicrobial	[µg]	*Vibrio cholerae* (n=6)								
		Water (3) (%)			Bm (3) (%)			MIC (µg/mL)	MβLs	R-plasmids
		S	I	R	S	I	R			
Gentamicin	10	100	0	0	100	0	0	-	-	-
Ampicillin	10	100	0	0	100	0	0	-	-	-
Cephalothin	30	100	0	0	100	0	0	-	-	-
Ceftazidime	30	100	0	0	100	0	0	-	-	-
Imipenem	10	67	0	33	100	0	0	20 µg	+	-
Chloraphenicol		100	0	0	100	0	0	-	-	-
Nitrofurantoin	300	100	0	0	100	0	0	-	-	-
Nalidixic acid	30	100	0	0	100	0	0	-	-	-
Ciprofloxacin	30	100	0	0	100	0	0	-	-	-
Sulfamethoxazol+trimethoprim	25	100	0	0	100	0	0	-	-	-
Tetracycline	30	100	0	0	100	0	0	-	-	-

S, Susceptible; I, intermediate; R, resistant; Bm, Bivalve mollusks; MβLs, Metallo-β-lactamases.

across the globe (WHO, 2009). The largest number of cases has been observed in African countries (WHO, 2013). In America, only six cases have been reported. In Brazil, there has been no registered case of indigenous cholera since 2005; despite of increased number of *V. cholerae* O1 has been isolated from environmental samples.

The 26 confirmed cases have been registered in Brazil during the outbreak between 2004 and 2005 in the state of Pernambuco. The Northeastern region had large number of cases due to its climate and poor sanitation (SVS, 2008).

Virulence profile

We did not detect the genes *ctxAB, tcp, rfbO1* and *zot* in the isolates of *V. cholerae* (Table 1). The pathogenesis of the infections caused by vibrios is complex due to a variety of virulence factors, such as cytotoxins, enterotoxins and lithic enzymes (Masini et al., 2007). This does not overrule the possibility of the strains causing gastroenteritis, given their virulence is associated to several mechanisms. Similar results were reported by Wong et al. (2012) for environmental samples.

In drinking ground water samples analyzed in India, was detected the presence of the gene *ompW* in all the isolates, but could not find any evidence of the genes *rfbO1*, *tcp*, *ctxAB* and *zot*. The majority environmental isolates belonging to serogroups non-O1 and non-O139 are not toxigenic in general (Tamrakar et al., 2009). The gene of cholera toxin (*ctx*) is essential to cause the disease cholerae, and present only in *V. cholerae* strains O1 and O139 (Goel et al., 2007).

However serogroups non-O1 and non-O139 of environmental origin can be considered strains without risk, it is known that the aquatic environment can serve as a reservoir for the emergence of pathogenic strains from populations not pathogenic, due acquisition of genetic mobile materials, thus creating new pandemic strains (Islam et al., 2013).

Profile of antimicrobial susceptibility

The *V. cholerae* strains showed 91% (10/11) susceptibility to the drugs commonly used by the Brazilian population (Table 2). The bacterial resistance profiles are closely related to the environmental conditions and pressures to which the strain has been exposed

The susceptibility of the *V. cholerae* to tetracycline is satisfactory considering it is one of the first drugs chosen to treat infections caused by vibrios (Han et al., 2007). Fluoroquinolones (ciprofloxacin) and aminoglycoside (gentamicin) are used to treat infections caused by *V. cholerae* O1 and O139 (Okuda et al., 2007). However, the appearance of antibiotic resistant *V. cholerae* strains has restricted its use in to patients with severe dehydration (Kitaoka et al., 2011).

In Iran, Raissy et al. (2012) observed resistance to gentamicin in 83.3% strains and tetracycline in 18.1% strains of *V. cholerae* isolated from fish. Zanetti et al. (2001) reported 88.9% of the isolates in a marine environment to be resistant to ampicillin (MIC > 64 mg) due to the production of β-lactamases.

In this study, antimicrobial resistance to imipenem with an MIC of 20 µg (water isolate) was observed (Table 2). This fact suggests that along the course of Subaé River

until its mouth, wastewater from hospitals may be discharged in the river, causing an antibiotic contamination. Imipenem is a broad spectrum antibiotic used in the treatment of infections caused by β-lactamase producing enterobacteria. The occurrence of enzymes capable of inactivating carbapenems has increased the microbial resistance, thus limiting the options available for treatment (Ikeda et al., 2012).

The presence of the *V. cholerae* strains resistant to carbapenems poses a risk for the propagation of the microbial resistance in the environmental bacteria, when the aquatic environment is efficient in the selection of the resistant bacterial populations via exchange of resistance genes carried on mobile genetic elements (Kitaoka et al., 2011).

The study of microbial resistance is relevant from the point of view of public health. In purview of this, it is important to monitor the extent to which ecosystems is modified by man, especially when antimicrobials are released in municipal sewage system with urine and feces (Baquero et al., 2008).

The Brazilian authorities, aiming to minimize the problem of the antimicrobial resistance in the country, have limited the use of antibiotics by prescription only. However, flaws in the treatment of the sewage stations besides implication of animal prophylaxis have also contributed to the discharge of antibiotic residues into water bodies.

While finding the genetic origin of imipenem resistance, an active involvement of plasmids could not be detected in the extraction of plasmid DNA (Table 2); one possibility for this observation is that plasmids might have been lost during the storage of the isolates. According to Smitt and Bidochka (1998), the growth conditions, storage, and environmental conditions may cause changes in the plasmid, and may influence its stability.

In a study in Kerala, India, the antimicrobial resistance mediated by plasmids in vibrios was reposted from seafood. However, in several studies, no strains with plasmids were reported; this indicates occurrence of potential chromosomal resistance (Manjusha and Sarita, 2013).

Detection of metallo-β-lactamases (MβLs)

The *V. cholerae* isolates showing resistance to imipenem also showed ability to produce MβLs enzymes (Table 2). These enzymes make the bacteria resistant to a large number of antimicrobials, especially carbapenems (Padhi, 2011). Carbapenems belonging to a class of antibiotics reserved to treat severe infections caused by organisms are already resistant to the latest penicillins and cephalosporins (Fritsche et al., 2005).

V. cholerae has developed an ability to overcome the antimicrobial effect due to the presence of efflux pumps that act on several classes of antimicrobials and produce enzymes that may hydrolyze complex antimicrobials. In addition, *V. cholerae* has a strong capacity to share resistant genes for antimicrobials through integrons and plasmids (Mandal et al., 2012).

Due to the capacity to inactivate several microbial agents, an increase in the prevalence of this microorganism would drastically compromise its ability to efficiently treat hospital infections or the ones acquired in the community mostly caused by Gram-negative bacillus. These enzymes, due to posing a global threat via their resistance mechanism, are among the greatest concerns for the medical community.

The presence of the enzyme New Delhi metallo-β-lactamase (NDM-1) in *V. cholerae* and *Shigella boydii* in the environment has been reported. The blaNDM-1 gene has very high ability of genetic exchange between the environmental bacterial species. The increase in *V. cholerae* strains resistant to the prescription or excessive use of antimicrobials, as well as the absence of adequate monitoring (Padhi, 2011).

The Antimicrobial Surveillance Program (SENTRY) has documented a world-wide raise in the index of occurrence and number of types of MβLs; this increase is a matter of concern, especially in Asia, Europe and Latin America (Fritsche et al., 2005). In Korea, approximately 10 to 50% of the resistance to imipenem in *Pseudomonas aeruginosa* and *Acinetobacter* spp. is due to the production of MβLs, which can spread very quickly to several species of Gram-negative bacillus via transferring genes for MβLs enzymes of the type *Verona imipenemase-2* and *Imipenemase-1* (Yong et al., 2002). In Brazil we do not have data *Vibrio cholerae* strains metallo-β-lactamase positive only was reported clinical cases of *Pseudomonas aeruginosa* and *Acinetobacter* spp. The chelating agents (EDTA/MPA) are efficient in inhibiting MβLs enzymes. The inhibition of these enzymes by EDTA is an important characteristic used to distinguish MβLs from other β-lactamases (Lee et al., 2003). According to the same authors, imipenem-ethylene diamine tetra acetic acid (IPM-EDTA) and ceftazidime-mercapto propionic acid (CAZ-MPA) are the simple methods to detect MBL-producing strains using simple disc for the diffusion of MβLs.

Conclusion

The strains of *V. cholerae* originating from São Francisco do Conde, Bahia, did not have genes related to the toxicity of these bacteria and were susceptible to most antimicrobials evaluated. However, considering the genetic plasticity of these bacteria in the aquatic environment, the presence of metallo-β-lactamase enzymes is an indicator of the risky situation to which the population is exposed. The monitoring of these environments may be an efficient strategy in predicting outbreaks and epidemics involving *V. cholerae* as

pathogenic agent.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

An evaluation of the use of probiotics and manure composting as strategies to reduce levels of Shiga toxin-producing *Escherichia coli* in sheep

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Healthy ruminants appear to be the main reservoir of Shiga toxin-producing *Escherichia coli* (STEC). Importantly, this pathogen is shed in faeces of sheep and can cause outbreaks of human illness ranging from diarrhea to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) caused by Shiga toxin-producing *E. coli* (STEC) have been reported worldwide. The manure of ruminants when used as agricultural fertilizer can serve as a vehicle for STEC contamination of fruits, vegetables, water and soil. The aim of the present study was to evaluate whether the use of probiotic strains of *Ruminobacter amylophilus, Ruminobacter succinogenes, Succinovibrio dextrinosolvens, Bacillus cereus sub toyoi, Lactobacillus acidophilus* and *Enterococcus faecium,* supplemented to the daily oral food ration provided to sheep, together with composting of their feces, may be used as a strategy to reduce STEC levels on a farm. The first stage of the present study was performed during a six-week period with a total of 160 sheep distributed among four groups comprised of 40 sheep each. Group A did not receive either STEC or probiotic, Group B received probiotic alone, Group C received STEC plus probiotic and Group D received STEC alone. After the sheep were inoculated, samples of their feces were collected and the number of STEC and *E. coli* were counted. In the second stage of the study, after the six-week period, all fecal material was composted into four separate heaps. A possible protective effect of the probiotic strains against colonization by STEC was observed. It was also observed that composting was very efficient at eliminating or decreasing the STEC population. Although the number of STEC isolates was effectively decreased in all compost heaps, the Group C derived compost heap was found to have a lower amount of STEC than the Group D derived compost heap. These findings suggest that the use of probiotics, such as lactic bacteria, together with composting manure may be an efficient strategy to decrease the STEC population on a farm.

Key words: Composting, *Escherichia coli*, probiotic, Shiga-like-toxin

INTRODUCTION

Healthy ruminants appear to be the main reservoir of Shiga toxin-producing *Escherichia coli* (STEC) (Sanderson et al., 1999). Importantly, STECs are zoonotic pathogens that can cause food-borne diseases in

humans, ranging from diarrhea to hemorrhagic colitis (HC) and severe cases such as hemolytic uremic syndrome (HUS) (World Health Organization, 1998). Worldwide, sheep have been shown to be a major reservoir for STEC, including countries such as Norway (Urdahl et al., 2001), Russia (Kudva et al., 1998), Germany (Beutin et al., 2004), Spain (Rey et al., 2003) and Brazil (Vettorato et al., 2003)

Probiotics may be used as an alternative for decreasing the number of pathogenic bacteria, thereby decreasing the spread of these strains on a farm (Lema et al., 2001; Chaucheyras-Durand et al., 2010). The mechanisms by which probiotics cause microbial interference in the gut include nutrient competition, generation of an unfavorable environment and competition for attachment or adhesion sites resulting in reduced colonization by pathogenic bacteria (Caplice and Fitzgerald, 1999).

Manures composted for agricultural use have been shown to possess reduced amounts of zoonotic pathogens (Erickson et al., 2009). However, when this process is inefficiently applied or managed, fecal contamination of agricultural soil presents a potential risk of infection to humans and animals (Wu et al., 2009). Composting has been defined as intense microbial activity leading to decomposition of most biodegradable materials (Adani et al., 1997). Composting also allows the complete or partial degradation of key chemical compounds (Whitney and Lynch, 1996).

The use of probiotic strains to supplement the ration of livestock (Lema et al., 2001; Chaucheyras-Durand et al., 2010) and the use of composting to decrease the population of pathogenic microbes (Pourcher et al., 2005; Murkherjee et al., 2006; Gonçalves and Marin, 2007) have both been reported as efficient alternatives to decrease the spread of pathogenic strains on a farm. However, these studies were done separately, and there is little information about the combined use of the two practices to decrease the STEC population present on a sheep farm. Therefore, the aim of the present study was to evaluate whether the use of probiotic strains supplemented to the daily oral ration provided to the sheep, together with the composting of their feces, may be used as a strategy to reduce STEC levels on a farm.

MATERIALS AND METHODS

Probiotics and STEC strain used

The probiotic bacteria used in this study were *Ruminobacter amylophilus, Ruminobacter succinogenes, Succinovibrio dextrinosolvens, Bacillus cereus* sp *toyoi, Lactobacillus acidophilus* and *Enterococcus faecium* isolated from bovine rumina and intestinal tracts following the recommendations of Hungate (1975). These bacteria have the following features: they are nonpathogenic, enzyme-producing and resistant to lactic acid and low pH (Rigobelo and Ávila, 2012). It was performed the inoculum count resulting in 3×10^8 colony forming units (CFU) per gram, and then, each strain was lyophilized and mixed all together. Each ration was supplemented with 0.2% probiotic inoculums, and the treated animals received 200 g of rations per animal per day. Water was supplied freely.

It was used a STEC strain previously isolated from the feces of healthy sheep, and the presence of virulence genes was detected by a multiplex PCR (Vidal et al., 2005) in the Laboratory of Bacteriology and Veterinary Pathology. This strain carries *stx1, stx2* and *eae* genes and belonging to O101 sorogroup.

Trial design

A total of 160 sheep were sourced from five rural properties of Dracena region (Sao Paulo, Brazil) and housed at the research facilities of Sao Paulo State University (UNESP) located in Dracena city. The sheep Santa Ines breed at the fattening stage. The animals were selected based on equivalence of body weight (41±2 kg) and age (9-12 months). All groups were fed a commercial diet (Rações Pioneira, Ribeirão do Pinhal, Brazil) of identical composition. The animals were separated into four different groups (A, B, C and D) with 40 sheep in each group and kept separated for the six weeks of the experimental work. The animals belonging to Group A, the control group, did not receive either STEC or probiotics. Group B received probiotics in the feed for six weeks. Group C received a single oral dose of an STEC strain with the same probiotics given to Group B (probiotic given in the feed for six weeks). Group D received a single oral dose of the same STEC as given to Group C. This study was conducted in accordance with the ethical guidelines for investigations involving laboratory animals and was approved by the Ethics in Animal Research Committee (EARC), protocol number is 21/2010 of UNESP- Univi Estadual Paulista, Sao Paulo, Brazil.

Inoculation of sheep with STEC

All sheep of Groups C and D received orally a 40 ml of solution containing 2×10^9 viable STEC carrying the *stx1*, *stx2* and *eae* genes in 0.9% saline solution, as previously described (Ávila et al., 1986). After inoculation, the animals were monitored daily for changes in rectal temperature and the development of diarrhea.

Identification of STEC from feces

During the six-week period that the animals were kept separate, feces were collected directly with a rectal swab from each animal from each group weekly. The feces were then transported to the laboratory aseptically in a bottle. In the laboratory, the feces were weighed, and 1 g was inoculated into a bottle containing 10 ml Brilliant Green Bile broth, and the bottles were incubated at 37ºC, for 12 h. Next, 0.1 ml of broth was collected and cultured on MacConkey agar at 37ºC for 12 h according to Wollum, (1982). Colonies suspect of *E. coli* were identified based on the colony characteristics, Gram staining and biochemical profile (Koneman et al., 1997). It was performed the DNA extraction of the samples and then analyzed by PCR for the presence of *stx1*, *stx2* and *eae* genes

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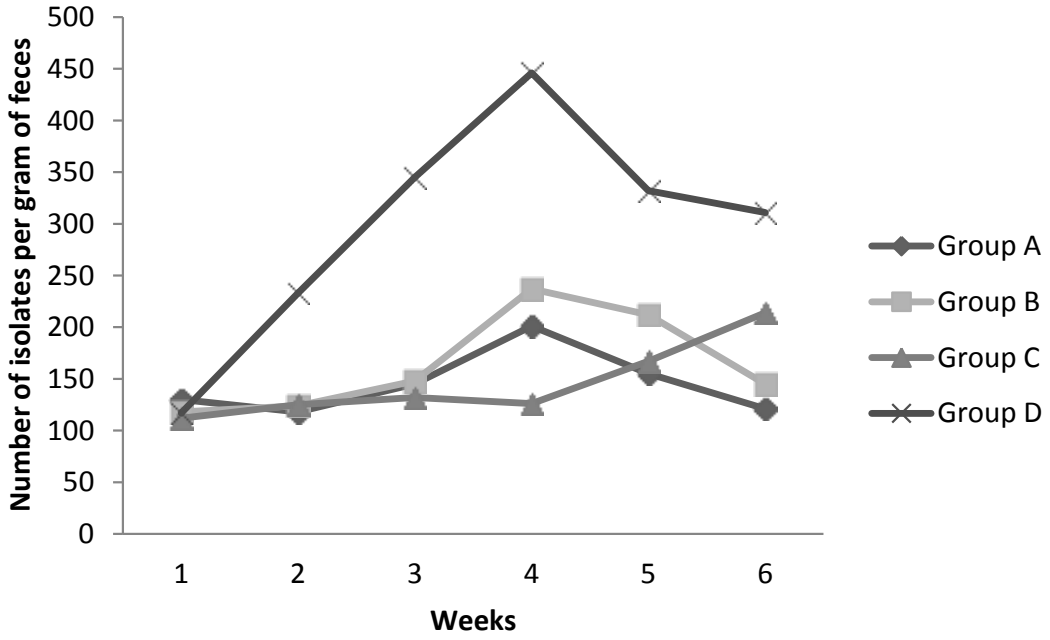

Figure 1. Number of STEC recovered from the feces of sheep in the different groups.

as previously described (Vidal et al., 2005). It was also performed the count of all samples carrying the *stx1*, *stx2* and *eae* genes, the same pattern as present in the STEC challenge strain.

Construction of compost heaps

For six weeks, the sheep's feces accumulated in the stalls of each group of sheep. During this period, the material of each group was not mixed and they were protected against birds, because these animals can transmit STEC. At six weeks post-inoculation, the material was collected separately for construction of compost heaps. The collected fecal material was heaped into four piles of 250 kg each, and the piles were used for the construction of separate compost heaps. Each heap was formed with the following dimensions: 0.5 m high, 0.5 m wide and 1.0 m long. Three parts of dry grass to one part of sheep's feces were added to each compost heap to equilibrate the ratio of nitrogen to carbon thus facilitating the decomposition of all material. Each day, the heaps were watered up to the saturation point, which was approximately 60% wet, and all material was turned every three days. Process control was based on temperature development. The study was performed at the Campus Experimental of Dracena from January to May, 2010 when the typical ambient temperatures average was 28ºC. Under the heaps, six equidistant points were marked, from which samples were collected weekly and were transported aseptically to the laboratory for analysis. The samples were collected for seven weeks and were analyzed for temperature, total number of bacteria and whether or not the bacteria carried the *stx1*, *stx2* and *eae* genes, through the methods cited below.

Isolation and identification of *E. coli* and STEC from compost heaps

During the composting period, the feaces in stalls was removed from housing pens to avoid the re-infection by STEC strains, and 25 g of compost were collected weekly from each sampling point and transported to the laboratory. In the laboratory, the samples were inoculated into 225 ml of Brilliant Green Bile broth, mixed and incubated overnight at 37ºC for 12 h. Next, 0.1 ml was collected and spread plate on MacConkey agar. The *E. coli* isolates were identified based on colony characteristics, Gram staining and biochemical profile (Koneman, et al., 1997). A loopful of colonies in the plate was collected, mixed and grown overnight in Luria Bertani (LB) broth at 37ºC. The DNA extraction was done according to Keskimaki et al. (2001) in which bacteria were pelleted from 1.5 ml of broth, suspended in 200 µl sterile distilled water and boiled for 10 min. Following centrifugation of the lysate, a 150 µl sample of the supernatant was stored at -20ºC as a template DNA stock. A multiplex PCR to detect *stx1*, *stx2* and *eae* genes was performed as described (Vidal et al., 2005). Strains testing negative for *stx1*, *stx2* and *eae* genes were considered as *E. coli* non-STEC.

Statistical analysis

A chi-square test was performed using the SAS software (SAS Institute 2001, technical report release 8.2, Cary, NC, USA) to determine the significance of the results.

RESULTS

Throughout the six-week period, the temperature range of all animals was 38.9 to 40.0ºC. No adverse effects were observed in the animals receiving the STEC strain or probiotics during this study.

The number of STEC strains re-isolated from the feces of animals in each group (A to D) for six weeks is shown in Figure 1. The group that yielded the highest number of STEC was Group D. The number of STEC isolates from Groups A, B and C did not differ statistically. The only group that presented a significant difference was Group

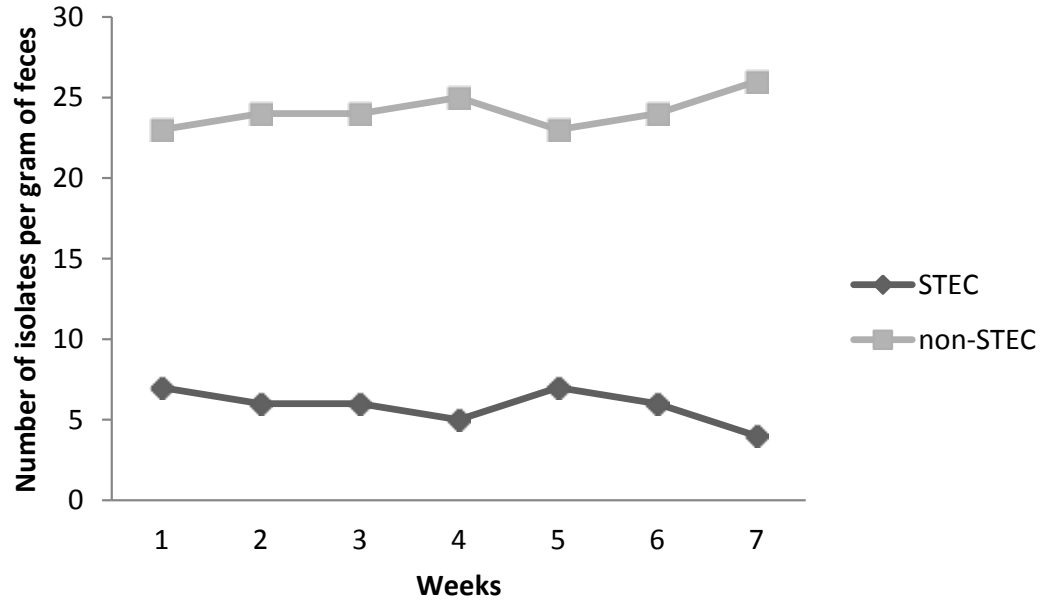

Figure 2. Number of STEC and *E. coli* non-STEC isolated from the compost heap for Group A.

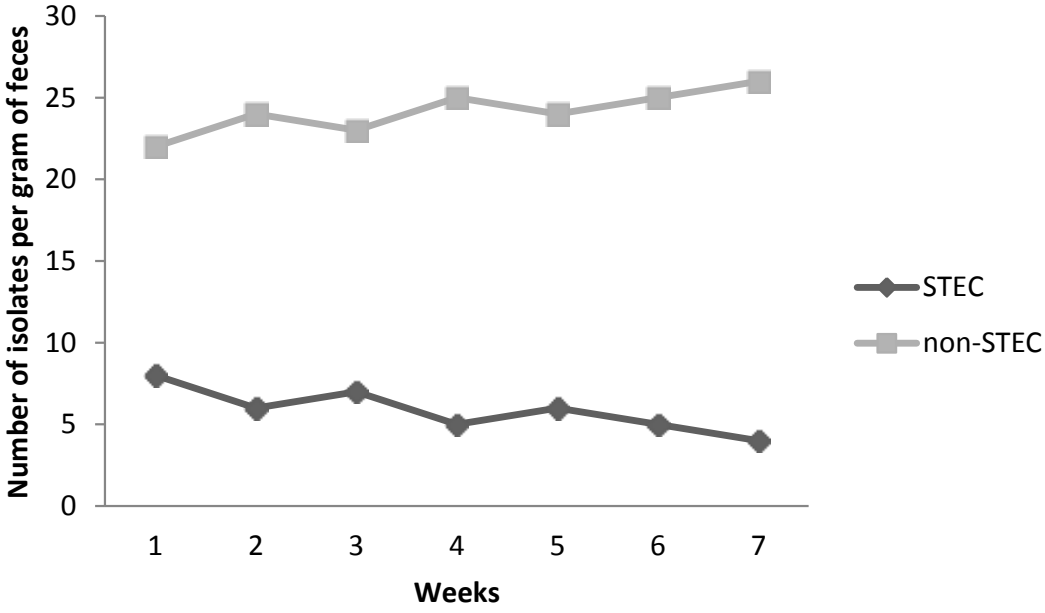

Figure 3. Number of STEC and *E. coli* non-STEC isolated from the compost heap for Group B.

D, which received one single dose of inoculum at a concentration of 2 x 10^9 cfu per ml of STEC.

During seven weeks, five *E. coli* strains were isolated from six equidistant points on the compost heaps, totaling 30 *E. coli* strains per heap per week. The number of STEC and *E. coli* non-STEC isolated weekly from all groups is shown in Figures 2 to 5. Both the control Group A and Group B were the groups from which the lowest number of STEC strains were isolated. Group C had the third lowest number of STEC strains. The group from which the largest number of STEC was isolated was Group D. However, the numbers of STEC isolates did not differ statistically between the groups. The compost heap that presented the largest initial number of *E. coli* strains was Group D. This fact was observed in spite of Group C having received the same concentration of STEC and the

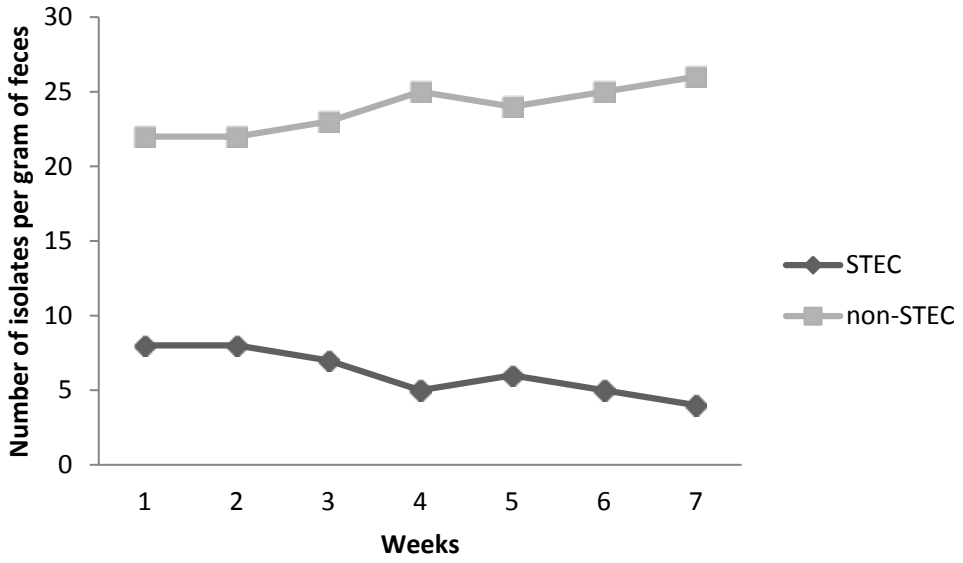

Figure 4. Number of STEC and *E. coli* non-STEC isolated from the compost heap for Group C

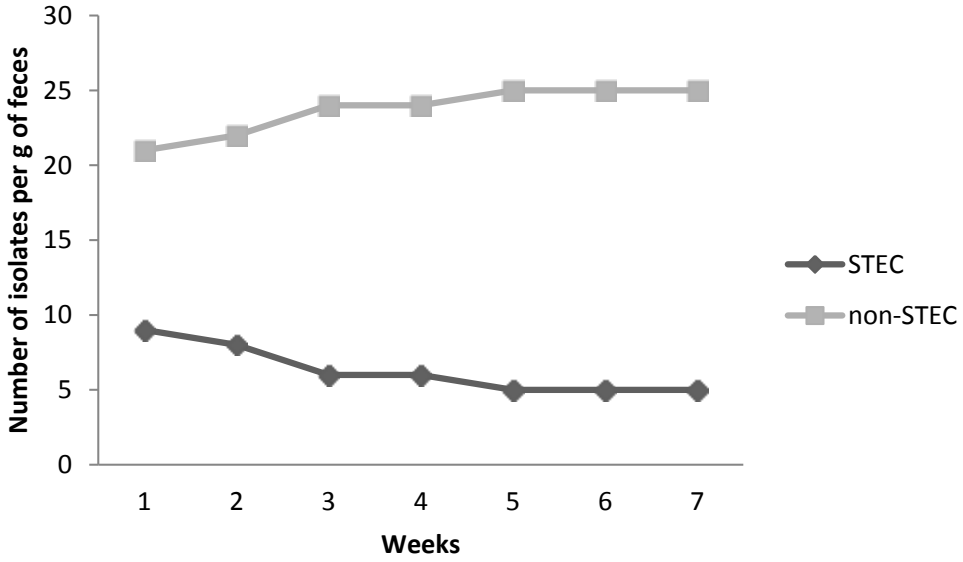

Figure 5. Number of STEC and *E. coli* non-STEC isolated from the compost heap for Group D.

probiotic strains. In this group, there was a lower number of strains spread into the feces, and these numbers were similar to those measured in Groups A and B.

There were no statistical differences between the numbers of STEC isolated from the compost heaps. All groups were efficient in the elimination of pathogenic strains. Group D had the largest initial number of STEC strains compared to the other groups. However, the composting process was efficient in the elimination high microbial concentration. The number of *E. coli* isolates began to decrease during the fourth week and had their lowest values during the sixth week. The composting process occurred normally among the compost heaps, that is, there was no difference in either temperature or the quality of materials composted. The average temperatures ranged from 12 to 65ºC for all treatments.

DISCUSSION

A range of enteric zoonotic pathogens, such as STEC, are present in animal manure when it is applied to agricultural land as a fertilizer, and these pathogens can be transmitted to both humans and animals (Borczyk et al., 1987; Pell, 1997). These fertilizers are provided by the livestock industry at large volumes annually (Haug,

1993; Pell, 1997; Sasaki et al., 2006; Mukherjee et al., 2006). STECs are zoonotic pathogens that can cause food-borne diseases in humans, ranging from diarrhea to HC and severe cases such as HUS (World Health Organization, 1998). Moreover, there are several studies showing the prevalence of STEC in sheep in Brazil, and because of it, the control of STEC has a great importance in public health (Martins et al., 2015; Vettorato et al., 2003).

The reasons leading to the colonization of the ruminant gut by STEC are unknown. Elucidating the relationships between ruminants and STEC may allow the development of interventions to prevent colonization by STEC, thereby eliminating STEC from the feces (Magnuson et al., 2000; Grauke et al., 2002). Moreover, the frequency, magnitude, duration and transmissibility of STEC strains in sheep are different in relation to other pathotypes, suggesting that the STEC strains are better adapted to persist in the alimentary tract of sheep (Cornick et al., 2000).

Rambaud et al. (1993) suggested that the protective effects caused by probiotics such as lactobacilli occur because the lactobacilli act as an adherence barrier to the surface of the gut. Probiotics may also produce their effects with viable as well as nonviable bacteria, suggesting that metabolic or secreted factors or structural cellular components may mediate their immune modulatory activities (Borches et al., 2009). The exact mechanism of balancing and interference of probiotic strains with the intestinal microbiota in sheep is unknown. More studies are necessary to investigate these relations. Lactic bacteria supplemented in the ration has been used as a strategy for decreasing the spread of *E. coli* in sheep (Lema et al., 2001; Guarner and Malagelada, 2003; Chaucheyras-Durant et al., 2010). In the present study, the group that received the probiotic had reduced colonization by STEC, resulting in a lower number of STEC being recovered from the feces of these animals, probably because of the lactic bacteria present at probiotic. This suggests that there was a protective effect against colonization by STEC. The protective effect of lactic bacteria occurs through a mechanism of competitive exclusion, including competition for nutrients and adhesion sites in the gut (Guarner and Malagelada, 2003; Millette et al., 2007). Several studies show that STEC was found in healthy ruminants, like sheep, cows and goats (Djordjevic et al., 2001; Zschöck et al., 2000), maybe because of it, the STEC inoculum used in the current study did not cause diarrhea. More studies are needed to explain whether this same effect would occur with a higher dose inoculum.

Together with the use of lactic bacteria to decrease the spread of pathogenic strains, it is possible to use of compost heaps as another means of decreasing the microbial population of the sheep manure. The composting process helps to ensure the hygiene of the final compost product (Pourcher et al., 2005; Murkherjee et al., 2006; Gonçalves and Marin, 2007). Previous studies have shown a long-term survival of more than 21 months for *E. coli* O157:H7 in manure held under a variety of environmental conditions (Kudva et al., 1998). In the current study, the survival of STEC strains was not greater than non-Shiga toxin producing *E. coli* as determined by comparing the number of isolates for each during the seven weeks. Gonçalves and Marin (2007) observed that the STEC strains seemed to be more sensitive to the action of temperature than the ordinary strains. All treatments that cause a decrease of the pathogen population of manure will certainly contribute to reduced dissemination of these pathogens on a farm as well as a reduced occurrence of outbreaks caused by these pathogens. Many studies have reported the efficiency of composting in decreasing the microbial population (Islam et al., 2004; Klein et al., 2010; Alexander et al., 2011). However, there is little information regarding the degree to which non-O157 STEC cells can survive in manure. Fukushima et al. (1999) reported the survival of STEC for up to 12 weeks when the temperature remained at 25ºC. Recently, Fremaux et al. (2007) reported the elimination of non-O157 STEC strains when submitted to composting in manure heaps after nine and 16 days at 35 and 56ºC, respectively. These results were similar to the present study in which the maximum temperature of the compost heaps was 65°C.

Although the number of STEC isolates was effectively reduced among all the compost heaps, the initial contamination of Group C by STEC during the first week that received probiotics together was lower than the Group D that received STEC alone. This high initial contamination by STEC in the feces might present a potential risk of contamination and spread of STEC on a farm, and the probiotics may contribute to decreasing this concern. Our findings suggest that the use of probiotics such as lactic bacteria, together with composting manure, may be an efficient strategy to decreasing the population of STEC on a farm.

Conflict of Interests

The authors have not declared any conflict of interest.

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