

Comparative Fatty Acid Profiling of *Klebsiella pneumoniae* and *Rhodococcus rhodochrous* Isolated from Spoilt Paints by Gas Chromatography

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Abstract

The use of fatty acids to study the differences in un-related microbes is limited. This study analyzes the fatty acids produced by two unrelated microorganisms: *Klebsiella pneumoniae* (Gram-negative, aerobic, non-endospore forming, usually encapsulated rod-shaped bacteria of the family *Enterobacteriaceae*) and *Rhodococcus rhodochrous* (metabolically versatile, non-spore-forming, non-motile actinomycete) isolated from spoilt paints. Fatty acids produced by the organisms were analyzed using an efficient MIDI-Sherlock gas chromatography method. *K. pneumoniae* was characterized by a high content of straight chain, branched chain, hydroxyl and cyclo-fatty acids made up of C12: 0, C13:0, C14:0 iso, C14:0, C15:0 iso, C15:0 anteiso, C15:1 ω 8c, C15:0, C16:0 iso, C16:1 ω 5c, C16:0, C15:03OH, C17:1 ω 8c, C17:0 cyclo, C17:0, C18:1 ω 5c and C18:0. *R. rhodochrous* was dominated by straight chain, monounsaturated and 10-methyl fatty acids. The inability to synthesize branched, cyclo- and hydroxyl- fatty acids, was observed in *R. rhodochrous* which composed mainly of C14: 0, C15: 1 ω 5c, C15:0, C16:1 ω 9c, C16:0, C17:1 ω 8c, C17:0, C17:0 10-methyl, C18: 1 ω 9c, C18.0, 10 methyl-C_{18:0} TBSA, C20:1 ω 9c, and C20:0. Descriptive statistics reveal a mean of 2.53, 15.10 and 15.15 for retention time (RT), equivalent chain length (ECL) and Peak name, respectively. Possible implications of the variations in fatty acid distribution may include differences in their abilities to produce various secondary metabolites and potentials to degrade a variety of xenobiotics.

Keywords: Fatty acids, paints, *Rhodococcus rhodochrous*, *Klebsiella pneumoniae*

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Introduction

An important component of cellular materials in many living organisms is the fatty acid, which also helps in identification and characterization of microbial community structure. Fatty acids are the basic building blocks of more complex lipids (Jenkins *et al.*, 2015). Therefore, their composition in different bacterial species can be used as a means for comparisons. The qualitative nature of gas chromatographic data allows for the authentic microbial biomass composition differences in a diverse community. Essentially,

by virtue of biosynthetic relationships, fatty acids can be categorized into two groups comprising the straight chain fatty acids (SCFAs) and the branched-chain fatty acids (BCFAs). Branched-chain fatty acids (isobutyric acid, 2-methylbutyric acid, and isovaleric acid) are primarily saturated fatty acids, both even-carbon- and odd-carbon-numbered, with one or more methyl branches, accordingly called mono- or multi-methyl BCFAs, respectively (Maheshwari *et al.*, 2020). They are mainly derived from the catabolism of branched-chain amino acids such as valine, leucine, and isoleucine (He *et al.*,

2019). SCFAs (formic acid, acetic acid, propionic acid, butyric acid, and valeric acid) are predominant end products of fermentation of dietary fibers by the anaerobic intestinal microbiota (He *et al.*, 2019). Previous studies have shown that bacteria with the straight-chain membrane system usually need unsaturated fatty acids for their growth. On the contrary, these fatty acids are not necessarily required for growth by bacteria with the branched-chain membrane system such as the iso-, anteiso-, and ω -alicyclic fatty acids (Cronan, 2006). *Klebsiella pneumoniae*, a human saprophyte in the nasopharynx and in the intestinal tract, has been shown to be the most medically important species of the genus *Klebsiella* known to cause community-acquired bacterial pneumonia, occurring particularly in chronic alcoholics (Cillóniz *et al.*, 2019). Previous studies also show that *Klebsiella*, an ubiquitous pathogen (Struve and Krogfelt, 2004) second only to *Escherichia coli* accounts for 13.1 % of nosocomial urinary tract infections (UTI) with especially higher occurrence in immunocompromised patients such as patients with neuropathic bladders or with diabetes mellitus (Fernando *et al.*, 2017). Interestingly, *K. pneumoniae* has also been most commonly isolated from environmental and aquatic samples. Incidentally, the environmental isolates have been described as being indistinguishable from human clinical isolates with respect to their biochemical reactions and virulence (Podschun *et al.*, 2001). The *K. pneumoniae* membrane is characterized by a fatty acid profile dominated to a large extent (50%) by the straight chain fatty acids, with the major ones being C12:0, C13:0, C14:0, C15:0, C17:0, C18:0 and C19:0 as well as -iso branched fatty acids and hydroxylated fatty acids (Vasyurenko *et al.*, 2001). Members of the actinomycetes have been widely studied because of their ability to produce various secondary metabolites and extremely high potentials to degrade a variety of xenobiotics, including toxic aromatic compounds as pyridine (Yoon *et al.*, 2000). *Rhodococcus* species have been found to contain C16:0, C18:1 cis9, 10-methyl-C-18:0 (TBSA) as major fatty acids (Yoon *et al.*, 2000). In the present study, variations in fatty acid profiles were investigated using the MIDI Sherlock system to analyze model spoilage organisms previously isolated from paints (Obidi *et al.*, 2017) based on pattern recognition

software and the database of the Sherlock data file. The study is important because the composition of fatty acids in different bacterial species can be used as a means for comparison as well as predict spoilage potentials in paint microbes.

Materials and Methods

Microorganisms

Klebsiella pneumoniae and *Rhodococcus rhodochrous* were already isolated in our earlier work (Obidi *et al.*, 2017). The isolates were maintained as stock cultures on Nutrient Agar (Difco) slants. Fresh (24 h) cultures were each streaked on a 4-quadrant pattern on two tryptic soy agar (TSA) plates and incubated at 28°C. They were harvested from the third quadrant with a sterile spatula into the bottom of a glass screw-cap test tube (MIDI, 2001).

Extraction

Extraction of the fatty acids from the bacterial cells was carried out using a reagent made up of 15% NaOH in methanol and water (1:1). Subsequently, saponification was carried out by adding 0.1ml of reagent 1 (methanolic base) into the culture tubes and covered tightly. The tubes were then vortexed for 5-10 seconds in three successions and subsequently placed in a rack and incubated in a 100°C water bath for 5 min, cooled briefly for about 60 secs and reincubated in the water bath for additional 25 min. The methylation process which ensures the conversion of the sodium salts of the fatty acids to fatty acids methyl esters was carried out for 10 mins at 80°C following an initial vortexing for 5-10 secs. Two (2.0) ml of the methylation reagent (6 N HCl-methanol solution, 3.25:2.75) was added into the tubes and covered tightly. The tubes were subsequently cooled in a tray of cold water. The fatty acid methyl esters were extracted from the acidic aqueous phase and transferred to an organic phase with a liquid-liquid extraction process with the addition of 1.0 ml of an extraction solvent (1:1 mixture of hexane and methyl *tert*-butyl alcohol). The tubes were tightly capped, placed in the hemocytometer and mixed end-over-end for 10 mins. Subsequently, a clean Pasteur pipette was used to remove and discard the aqueous (lower) phase as described by MIDI. Flame ionization

detection gas chromatography was performed using the MIDI system (Newark, Del.) and the various peaks were established. Retention time data was obtained from injection of the calibration mixture and is converted to Equivalent Chain Length (ECL) data for naming of the bacterial fatty acids. The ECL value for each fatty acid was therefore derived as a function of its elution time in relation to the elution times of a known series of fatty acids. The individual fatty acids were characterized by a pattern recognition software based on comparison to commercially available databases (MIDI, Inc.). Descriptive statistics involving minimum, maximum, mean, standard deviation and variance were computed for RT, ECL and Peak Name.

Results

The fatty acids isolated from *K. pneumoniae* and *R. rhodochrous* were characterized by gas chromatographic analysis and compared with the fatty acid profiles of many known bacterial species using pattern recognition software. The fatty acid chromatograms generated were studied and contrasted. Branched, hydroxyl and cyclo fatty acids were observed in *K. pneumoniae* (Table 1). Table 1 shows the identification of the various cellular fatty acids of *K. pneumoniae* as well as the differences in retention time (RT) and equivalent chain length (ECL) of the various fatty acids observed. In *K. pneumoniae* RT ranged from 1.6302 to 3.4279. In contrast, certain fatty acids observed in *K. pneumoniae* were not detected in *R. rhodochrous*, which instead possessed distinctive methyl fatty acids (Table 2). Table 2 displays the identification of types of cellular fatty acids of *R. rhodochrous* as well as differences in retention time (RT) and equivalent chain length (ECL) of the various fatty acids observed. In *R. rhodochrous*, RT ranged from 2.178 to 4.0321. However, some similarities were observed between the two organisms. Straight chain fatty acids predominate (41% and 46%) in each case and a good proportion (23.5% and 38%) of monounsaturated fatty acids were detected in both organisms. *K. pneumoniae* produced seven major straight chain fatty acids (C12:0, C13:0, C14:0, C15:0, C16:0, C17:0 and C18:0), two branched chain fatty acids (anteiso-C15 and iso-C16), one hydroxyl fatty acid (C15:03OH), three monounsaturated fatty acids (C15:1 ω 8c, C16:1 ω 5c and C17:1 ω 8c) and

one cyclo-fatty acid (C17:0 cyclo) (Table 1). *R. rhodochrous*, on the other hand, produced thirteen fatty acids comprising six straight chain fatty acids (C14:0, C15:0, C16:0, C17:0, C18:0 and C20:0); five monounsaturated fatty acids (C15:1 ω 5c, C16:1 ω 9c, C17:1 ω 8c, C18:1 ω 9c and C20:1 ω 9c) (Table 2), and in addition to these, *R. rhodochrous* was found to produce two methyl fatty acids (C17:0 10 methyl and C18:0 10 methyl, TBSA). Figures 1 and 2 show the gas chromatograms of the bacterial fatty acids isolated from the two organisms. The straight chain fatty acids (C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C19:0 and C20:0) were the most abundant fatty acids in both organisms. The monounsaturated fatty acids (C15:1 ω 5c, C15:1 ω 8c, C16:1 ω 9c, C17:1 ω 8c, C18:1 ω 5c, C18:1 ω 9c and C20:1 ω 9c) generally, were next in order of abundance. The hydroxylated fatty acids (C15:03OH) and cyclo propane fatty acids (C17:0 cyclo) were of low abundance and occurred only in *K. pneumoniae*. The results of the study show obvious differences in the fatty acid distribution patterns of the two organisms investigated. An obvious inability to synthesize the cyclo, hydroxyl and branched fatty acid was seen in *R. rhodochrous*. *Klebsiella pneumoniae* had 41 % of straight chain fatty acids compared to *R. rhodochrous* which had 46%. *K. pneumoniae* was observed to possess 23.5% of monounsaturated fatty acids, 6% of hydroxyl fatty acids, 6% of cyclo-fatty acids and 23.5% of branched-chain fatty acids. *R. rhodochrous* on the other hand had 38% of monounsaturated fatty acids and 15.3% methyl fatty acids. The differences in fatty acid profiles thus, constitutes the major distinguishing features of *R. rhodochrous* and *K. pneumoniae* in addition to some established distinctiveness in their phenotypic, phylogenetic and genetic characteristics.

Figures 3 and 4 show each variable in the dataset. The upper tail indicates the maximum value, while the lower tail represents the minimum value. The middle line across the boxplot represents the median (2nd quartile) of each variable in the dataset. For *K. pneumoniae*, the mean is 2.53, 15.10, 15.15; SD is 0.45, 1.50 and 1.47; while variance is 0.206, 2.251 and 2.163 for RT, ECL and Peak Name respectively. In the case of *R. rhodochrous*, mean is 3.03, 16.74, 16.8; SD is 0.52, 1.67, 1.67 while variance is 0.269, 2.774 and 2.816 for RT, ECL and Peak name

respectively. The result shows that there is a slight difference between the two major variables

(*K. pneumoniae*, *R. rhodochrous*) based on the number of observations analyzed.

Table 1: Identification of Cellular Fatty Acids of *K. pneumoniae*

RT	ECL	Peak Name	Percent
1.6302	12.0004	12:0	0.42
1.8917	12.9999	13:0	0.16
2.0733	13.6288	14:0 iso	0.12
2.1806	13.9998	14:0	12.24
2.3730	14.6309	15:0 iso	0.06
2.4014	14.7242	15:0 anteiso	0.45
2.4296	14.8168	15:1 ω 8c	0.08
2.4859	15.0009	15:0	-
2.6843	15.6321	16:0 iso	0.17
2.7760	15.9240	16:1 ω 5c	0.29
2.8009	16.0033	16:0	31.85
2.9687	16.5358	15:0 3OH	0.08
3.0567	16.8149	17:1 ω 8c	0.38
3.0875	16.9127	17:0 cyclo	1.14
3.1149	16.9998	17:0	0.40
3.4068	17.9319	18:1 ω 5c	0.07
3.4279	17.9994	18:0	0.17

RT, retention time; ECL, equivalent chain length

Table 2: Identification of Cellular Fatty Acids of *R. rhodochrous*

RT	ECL	Fatty Acid	Percent
2.1780	13.9999	14:0	1.46
2.4589	14.9217	15:1 ω 5c	0.36
2.4833	15.0017	15:0	--
2.7339	15.7989	16:1 ω 9c	--
2.7971	15.9998	16:0	0.43
3.0543	16.8155	17:1 ω 8c	22.13
3.1128	17.0013	17:0	7.11
3.2419	17.4130	17:0 10-methyl	2.93
3.3606	17.7918	18:1 ω 9c	1.16
3.4255	17.9988	18:0	32.83
3.5465	18.3944	18:010-methyl, TBSA	1.62
3.9684	19.7884	20:1 ω 9c	6.13
4.0321	20.0002	20:0	0.63
			0.35

RT, retention time; ECL, equivalent chain length

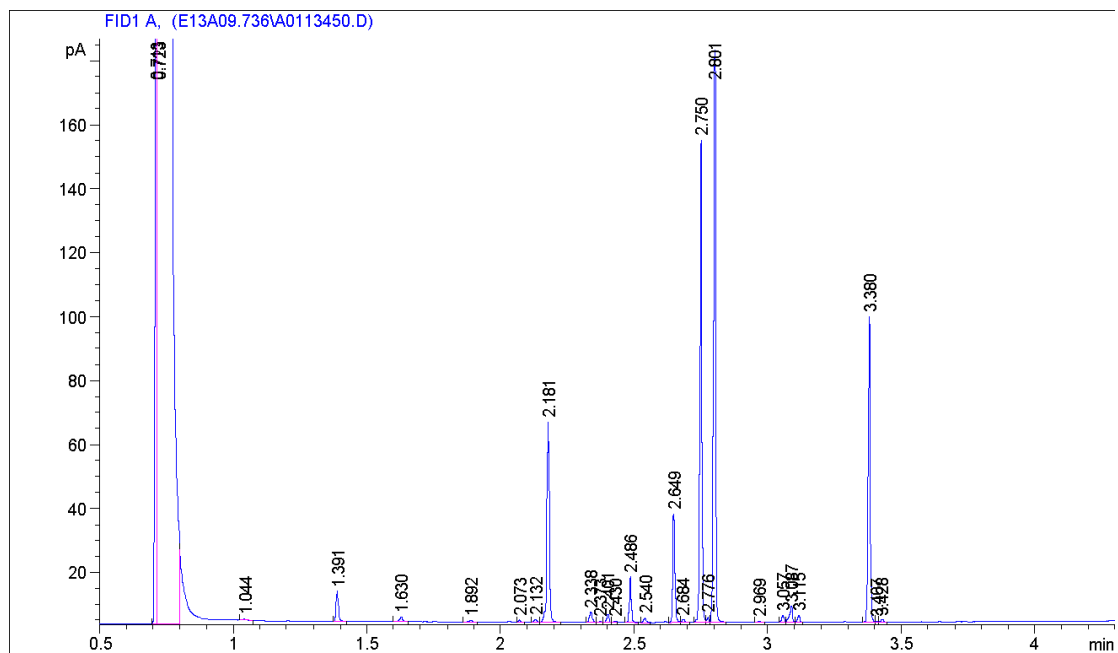


Fig. 1: Chromatogram of fatty acids of *Klebsiella pneumoniae*

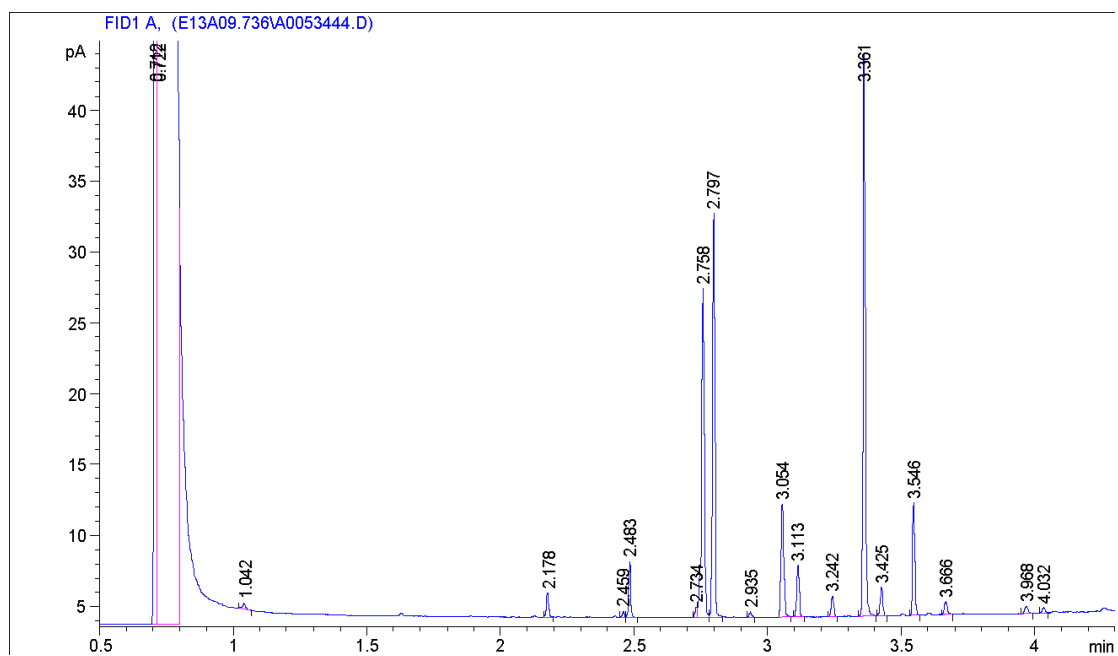


Fig. 2: Chromatogram of fatty acids of *Rhodococcus rhodochrous*

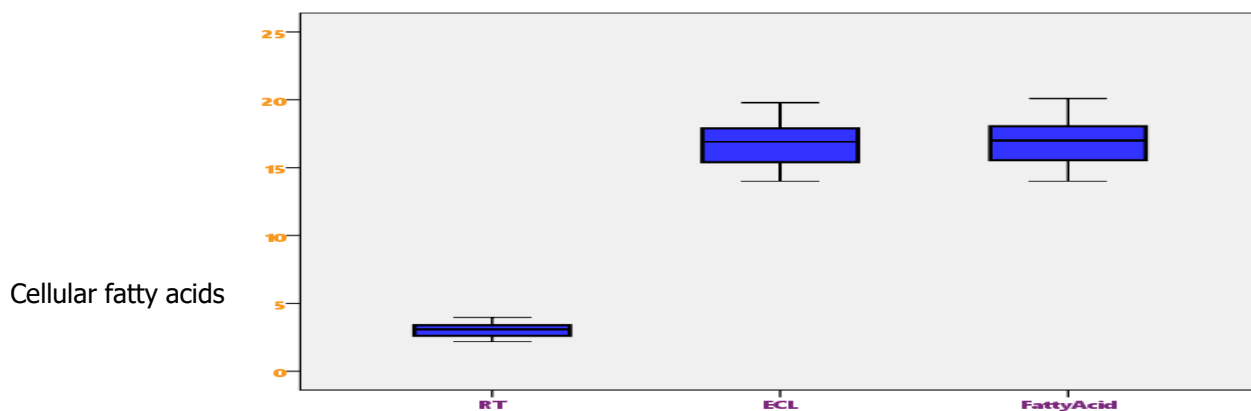


Fig. 3: Box Plot of *K. pneumoniae* Chromatogram parameters

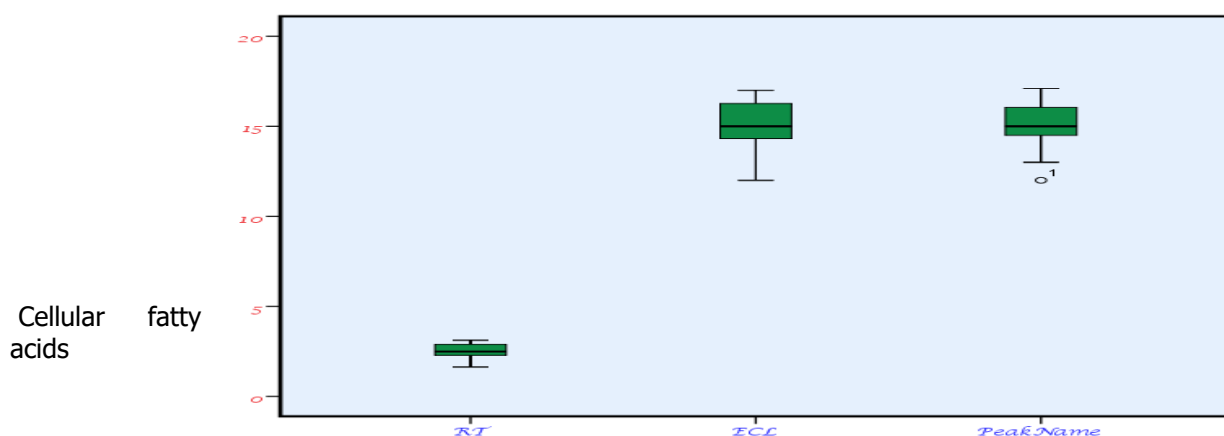


Fig. 4: Box Plot of *R. rhodochrous* Chromatogram parameters

Discussion

In the present study, fatty acid methyl ester (FAME) analysis was used to evaluate and compare the fatty acid profiles of *K. pneumoniae* and *R. rhodococcus*. The disparities in the fatty acid composition found in *K. pneumoniae* and *R. rhodochrous* are not unusual, being physiologically, biochemically, metabolically and taxonomically different organisms. *Klebsiella pneumoniae* was observed to possess 41 % of straight chain fatty acids and 11.8% branched fatty acids compared to *R. rhodochrous* which had 46% of straight chain fatty acids and no branched fatty acids. This is in agreement with a previous report by Lingfa *et al.*, (2020) which shows that straight chain fatty acids occur most commonly in bacteria and usually constitute about 10% of the total cellular fatty acids in bacteria which contain mostly branched-chain

fatty acids. All the saturated fatty acids, including the branched chain ones, are synthesized from acetyl coenzyme A (acetyl-CoA) as the primer and malonyl-CoA as the chain extender, followed, in some cases, by a modification of the fatty acid products. It is therefore believed that the enzyme system of the organism in question is responsible for these differences. Although branched chain fatty acids and straight chain fatty acids both increase membrane fluidity, they do not yield cells with identical morphologies or fitness for tolerating cold stress. Fatty acids having chain lengths shorter than 14 carbon atoms were observed in *K. pneumoniae* in contrast to *R. rodochrous*. This is in reasonable agreement with the report of (Wu *et al.*, 2020) who observed shorter chain lengths of fatty acids in *K. pneumoniae* compared to other isolates. A sensible reason may be that the cofactors involved in the synthesis are different and favor

the synthesis of shorter chain fatty acids in *K. pneumoniae*. Generally, the fatty acid compositions of bacteria are usually different from those of higher organisms in the sense that they have no polyunsaturated fatty acids (fatty acids with more than one unsaturation) (Tan *et al.*, 2012; Torres *et al.*, 2014; Bazinet and Laye, 2014). Substantial amounts (38%) of unsaturated and branched chain fatty acids were observed in *R. rhodochrous*. This might also contribute to having a cell envelope with greater flexibility. Similar results were obtained by Yoon *et al.*, (2000) who reported the occurrence of saturated, unsaturated and 10-methyl-branched fatty acids (10-methyl C18:0 TBSA) and major amounts of C16:0, C18:0 in several species of *Rhodococcus* tested. Branched chain fatty acids also have been found to enhance growth and virulence in bacteria and might give a cell envelope greater flexibility (Kaiser *et al.*, 2016). Ante-iso and iso branching (that is on the antepenultimate and penultimate carbons) have been reported to modify the thermotropic behavior and enhance the fluidity of lipid bilayers. Previous studies found that methyl branching reduces lipid condensation, decreases the bilayer thickness, and lowers chain ordering (Poger *et al.*, 2014). In addition, branching also results in the formation of kinks at the branching point, thereby enhancing the fluidity of lipid bilayers (Poger *et al.*, 2014). In this study, *K. pneumoniae*, an important pathogen, had branched chain fatty acids. Previous study has demonstrated the connection between pathogenicity and possession of branched-chain amino acids which are precursors for branched chain fatty acids (Kaiser *et al.*, 2018). Branched-chain amino acids represent important nutrients in bacterial physiology. They do not only support proliferation during infection, but also the evasion of host defenses (Kaiser and Heinrichs, 2018). The branched-chain amino acids are vital to both growth and virulence (Kaiser *et al.*, 2016). This probably explains why *Klebsiella* is highly virulent and highly pathogenic. The predominance of straight chain fatty acids in both organisms have been documented (Yoon *et al.*, 2000). So far, the branching of fatty acids have been described as being widespread in prokaryotic membranes. These branched-chain fatty acids are however, less common than the straight-chain fatty acids (Poger *et al.*, 2014). The fatty acid profile observed in the *K.*

pneumoniae is similar to those previously reported (Ringelberg *et al.*, 2008). An interesting occurrence of cyclo and hydroxylated fatty acids is particularly notable. The hydroxylated fatty acids (C15:03OH) and cyclo propane fatty acids (C17:0 cyclo) were of low abundance and occurred only in *K. pneumoiae*. This corroborates the report of Sohlenkamp and Gieger, (2016) who reported the occurrence of 3-Hydroxy straight-chain fatty acids in Gram-negative bacteria as the major acyl component of lipopolysaccharides in cell walls. Some researchers have indicated that a higher amount of Cyclo 17:0 indicates probable increase in certain groups of Gram-negative bacteria (Ibekwe and Kennedy, 1999). To further corroborate this fact, Ringelberg *et al.*, (2008) reported that n16:1w7c, n18:1w9c and n18:1w7c are bio-markers of fatty acids for Gram-negative bacteria. The reduced proportion of monounsaturated fatty acids (23.5%) in *K. pneumoniae* is usual among Gram-negative organisms. *R. rhodococcus* has been shown to possess physiological properties that are distinguishable from those of *K. pneumoniae*. It was observed by Yoon *et al.*, (2000) that in particular, the ability of *Rhodococcus* species to degrade pyridine was uncommon. Meanwhile, the ability to degrade pyridine has not been reported in *K. pneumoniae*. However, *K. pneumoniae* has been observed to tolerate and degrade tannic acid in very high concentrations. It is noteworthy that tannins, which serve as immune compounds in plants and protect them from ruminant and microbial attacks (Huang *et al.*, 2018) due to their toxicity due to microbial enzyme inhibition and substrate deprivation action on microbial membranes, were degraded by *K. pneumoniae*. The adaptation of *K. pneumoniae* to the toxicity of tannic acids has been linked to an increase in the degree of saturation of membrane lipid fatty acids (Pepi *et al.*, 2013).

The occurrence and prevalence of unsaturated fatty acids which are common to both organisms have been reported to play only a minor role in stress adaptation (de Carvalho and Caramujo, 2018) and are agents specific to aerobic bacteria (Erdogan *et al.*, 2013), and therefore may not have contributed to the degradation potentials observed in both organisms.

Conclusion

The study clearly showed that a fatty acid pattern consisting of a large proportion of straight chain, branched chain, hydroxyl fatty acids and a substantial amount of cyclo fatty acids is characteristic of *K. pneumoniae* (a typical Gram-negative organism) and may contribute to its degradative and physiological potentials. The absence of cyclo, hydroxyl and branched fatty acids was also confirmed to be typical in *Rhodococcus* species.

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