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Full Length Research Paper

Epidemiology, prevalence and antibiotic susceptibility profiles of methicillin-resistant *Staphylococcus aureus* in farm animals and farm workers in the Central Region of Ghana

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important microorganisms which has increasingly become resistant to most commonly used antimicrobials. This research investigated the epidemiology, nasal carriage prevalence and the antibiotic susceptibility profile of MRSA among farm animals and farm workers in the Central Region of Ghana. A total of 396 nasal swabs were collected from farm animals (94.9%) and farm workers (5.1%). Antibiotic susceptibility test was carried out on Mueller Hinton agar using Kirby-Bauer disc diffusion method. Epidemiological risk factors were assessed using pre-designed questionnaires. Results showed that the overall prevalence of MRSA in the Central Region of Ghana was 49.2% (195/396). Pigs recorded the highest nasal carriage prevalence of 50.2% (157/313) followed by sheep 45.1% (23/51), goats 50% (6/12) and humans 45% (9/20). MRSA isolates were 100% susceptible to both Vancomycin and Augmentin. Epidemiological risks factors for nasal colonisation of farm workers in this study were: direct contact with pigs (p=0.000), last period of antibiotic administration (p=0.020), and the type of apparels worn (nose mask (p=0.000), and gloves (p=0.020)). Epidemiological risk factors for nasal colonisation of farm animals in this study were: the type of antibiotic administered (p=0.000) and the last period for antibiotic administration (p=0.000). Phenotypic detection of MRSA and their resistance to the tested antibiotics should be a cause of alarm in the Central Region of Ghana.

Key words: Methicillin-resistant *Staphylococcus aureus*, prevalence, nasal carriage, epidemiology, farm animals, workers.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is of a major concern in clinical medicine due to the importance of β-lactams in the therapy of staphylococcal infections. In addition, morbidity and mortality for MRSA

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patients compared to patients infected with methicillin-susceptible *S. aureus* (MSSA) is on the rise (De Kraker et al., 2011). In Ghana, the children's block of the Korle-Bu Teaching Hospital (KBTH), was closed down in January, 2012 as a result of an MRSA outbreak in the ward (GNA, 2012). The epidemiology of this bacterium in animals has also gained global attention in recent years due to the spread of multidrug resistance and their increasingly evidenced zoonotic potential. In a study by Haenni et al. (2011) in France, MRSA isolates had identical characteristics to the human Geraldine clone (ST5, spa-type t002, and the same virulence genes, resistance pattern, and SCCmec cassette type I). These livestock-associated (LA)-MRSA are considered to be zoonotic and people with occupational contact with livestock, for example, farmers, veterinarians, and workers at abattoirs, are frequently exposed and colonised (Cuny et al., 2013). In 2007, a report on transmission of MRSA between dairy cows and milking personnel alerted people working with dairy cattle of the occupational health risks (Juhász-Kaszanyitzky et al., 2007).

Due to the interspecies transmission of MRSA and the health risks of MRSA colonisation, there is the need to research into the roles animals play in human MRSA infections. In Ghana, very little is known about the nasal carriage prevalence of MRSA among humans (Egyir et al., 2015, Odonkor et al., 2012). In addition, there are no published data on MRSA carriage in farm animals and farm workers. Hence, this study was designed to determine the epidemiology and antimicrobial susceptibility patterns of MRSA in apparently healthy farm animals and farm workers in the Central Region of Ghana.

MATERIALS AND METHODS

Study location

The study was conducted in 9 of the 15 administrative districts in the Central Region of Ghana. These were Komenda Edina Eguafo Abrem (KEEA), Cape Coast, Twifo Heman Lower Denkyira (THLD), Abura Asebu Kwamankese (AAK), Assin, Gomoa, Effutu, Mfantseman and Agona. These regions were selected to represent the North, South, East and Western zones of the region. The Central Region is located in the South-Western centre of Ghana. It is on latitude of 5° 29' 59.99 N and a longitude of -1 00' 0.00''. The Central Region shares boundaries with the Ashanti Region to the north, Greater Accra Region to the south-east and to the west by Western Region. It is bound to the south by the Gulf of Guinea. It has an annual temperature range of 24 and 34°C, annual rainfall pattern averaging 800 to 1500 mm and a relative humidity of between 50 and 85%. The region has six months each of rainy and dry seasons. The population of the Central Region has been projected to be 2,521,118 in 2018 (Ghana Statistical Service, 2013).

Sampling technique

A list of almost all farms in the districts of the region was obtained from the Ministry of Food and Agriculture. Out of this list, simple random sampling technique was used to select the farms for this study. The districts were selected based on convenience, that is, based on the proximity of the districts to the researcher. A total of 18 livestock farms (two from each district) that reared pigs, sheep and goats were randomly selected across all the nine districts of the study area. Because most of the farmers reared pigs with goats and sheep as a supplement, pigs were allotted 80% of the total sample size. In calculating the minimum sample size for this study, the formula described by Daniel (1999) was used.

$$n = Z^2 P(1-P) / d^2$$

Where, n = sample size, Z = z statistic for a level of confidence, P = expected prevalence or proportion (in proportion of one; if 20%, P = 0.2), and d = precision (in proportion of one, if 5%, d = 0.05).

For the level of confidence of 95%, which is conventional, Z value is 1.96. Also, since there was no know prevalence in the study area, the P value was perceived to be 50% (Daniel, 1999).

A total of 396 nasal samples were obtained from pigs, sheep, goats and farm workers in the study area.

Questionnaire survey and epidemiological data

A structured questionnaire, interviews and observations were used to elicit data on potential risk factors for the colonisation of MRSA in farm animals and the farm workers. From the farm workers, demographic data (gender, age, educational background, occupation), farm- and animal-related variables (exposure to pigs, handling antimicrobial drugs to animals, use of hygiene/protective measures, and occupational activities), life style determinants (eating preferences, exposure to raw meat, smoking, contact with pets), and medical history (exposure to health care facilities, antibiotic usage) were collected for each farmer at each sampling moment. For the animals, data such as age, sex, type of antibiotics used on them and the frequency of antibiotic use were collected.

Sample collection

From each farm, 22 nasal samples were collected from apparently healthy pigs, goats, sheep and workers. From each farm, at least one sample was taken from the farm workers. One nasal swab per individual was taken from the anterior nares of farmers and livestock using sterile swabs in liquid transport medium (ESwab, Copan, Brescia, Italy). Samples were stored at 4°C and transported to the laboratory.

Ethics approval and consent to participate

Ethical approval was acquired from the Institutional Review Board of the University of Cape Coast, Ghana. The nasal samples from the animals were collected with the help of veterinarian.

Isolation of MRSA

The swabs were streaked onto selective MRSA agar plates (ORSAB, OXOID, UK). These plates were incubated for 24 to 48 h at 37°C and examined for the blue, raised MRSA colonies.

Anti-microbial susceptibility testing

The MRSA isolates were subjected to antibiotic susceptibility test using disc diffusion method by Kirby-Bauer (1966). The following antibiotics were used: penicillin (15 µg), ampicillin (10 µg),

Table 1. Isolation rates of MRSA among different species of animals and districts in the Central Region of Ghana.

District	MRSA Isolates of species				
	Pigs [n/N (%)]	Sheep [n/N (%)]	Goats [n/N (%)]	Man [n/N (%)]	Total [n/N (%)]
KEEA	30/39 (76.9)	0/0 (0)	0/0 (0)	2/5 (40)	32/44 (72.4)
CC	19/28 (67.9)	6/15 (40)	0/0 (0)	0/1 (0)	25/44 (56.8)
THLD	15/38 (39.5)	0/0 (0)	1/2 (50)	2/4 (50)	18/44 (40.9)
AGONA	23/44 (52.3)	0/0 (0)	0/0 (0)	0/1 (0)	23/44 (52.3)
ASSIN	21/44 (47.7)	0/0 (0)	0/0 (0)	0/0 (0)	21/44 (47.7)
MFANTSEMAN	10/28 (35.7)	5/15 (33.3)	0/0 (0)	0/1 (0)	15/44 (34.1)
EFUTU	7/12 (58.3)	12/21 (57.1)	5/10 (50)	1/1 (100)	25/44 (56.8)
GOMOA	17/43 (39.5)	0/0 (0)	0/0 (0)	1/1 (100)	18/44 (40.9)
AAK	15/37 (40.5)	0/0 (0)	0/0 (0)	3/7 (42.9)	18/44 (40.9)
Total	157/313 (50.2)	23/51 (45.1)	6/12 (50)	9/20 (45)	195/396 (49.2)

Table 2. Distribution of MRSA isolates among various Age groups and sex of animals.

Parameter	MRSA isoaltes						Total No.	%
	Pig		Sheep		Goat			
	Freq./Sample size	%	Freq./Sample size	%	Freq./Sample size	%		
Age of animal								
<4 months	75/151	49.7	1/6	16.7	-	-	76	48.4
4-10 months	68/130	52.3	5/10	50	0/2	0	73	51.4
11-15months	8/16	50	7/12	58.3	2/4	50	17	53.1
>15months	6/16	37.5	10/23	43.5	4/6	66.7	20	44.4
Sex of animal								
Male	86/166	51.8	5/9	55.6	1/4	25	92	51.4
Female	71/147	48.3	18/42	42.9	5/8	62.5	94	47.7

tetracycline (30 µg), Cotrimoxazole (25 µg), Erythromycin (5 µg), Gentamicin (10 µg), Vancomycin (30 µg), Cloxacillin (5 µg), Cefuroxime (10 µg), Augmentin (30 µg), Meropenem (10 µg), and Ciprofloxacin (5 µg). Approximately, between 2 and 5 isolated colonies from a pure culture were emulsified in sterile nutrient broth and the turbidity of the inoculum was compared with 0.5 McFarland Standard. Mueller-Hinton Agar (Lab M Limited, Lancashire, UK) plates were prepared and a loopful of the inoculum was seeded on the surface of the media. Subsequently, a sterile cotton swab was used to spread the organism evenly on the Mueller-Hinton agar. The antibiotic discs were placed on the agar plates using sterilised forceps. The plates were read after 24 h of incubation at 37°C under aerobic condition. *S. aureus* ATCC 25923 was used as a quality control strain. The sensitivity of the isolates to various antibiotics were classified in accordance with the guidelines of the National Committee for Clinical Laboratory Standards (CLSI, 2016) as susceptible, intermediate or resistance for each antibiotic tested by measuring the zone of inhibition around the antibiotic disc.

RESULTS

Nasal carriage prevalence of MRSA in livestock and farmers

A total of 396 samples were collected from farm animals and farm workers. This comprised 313, 51, 12 and 20 nasal swabs from live pigs, sheep, goats and related farm workers, respectively from the 9 districts. In summary, 50.2% (157/313), 45.1% (23/51), 50% (6/12) and 45% (9/20) MRSA were isolated from pigs, sheep, goats and farm workers, respectively representing an overall prevalence of 49.2%. From Table 2, a total of 195 MRSA were isolated of which most were isolated from livestock between the ages of 15 months and below. The overall isolation rate of MRSA was higher among female animals. Among the various districts, KEEA district recorded the highest MRSA isolates (72.7%) with Mfantseman district recording the lowest MRSA isolates (34.1%) from both animals and farm workers (Table 1).

Epidemiological risk factors for MRSA colonisation

From Table 4, it can be seen that, respondents for the potential risk factors were 5/20, 0/20, 3/20, 3/20 and 9/20 for age groups < 18 years, 18-24 years, 25-34 years, 35-44 years and > or = 45 years, respectively. Among these respondents, no MRSA isolates were isolated from age

groups 18-24 and 35-44 years. Individuals below the age of 18 years recorded the highest MRSA isolates of 4 out of 5 respondents representing 80% followed by age group 45 and above (4/9, 44.4%) and age group 25-34 (1/3, 33.3%). MRSA nasal carriage was not significantly associated with age of the respondents (p= 0.368).

From Table 4, all 20 respondents were male and 9 were MRSA carriers representing 45%. Although few, people with no educational background recorded the highest percentage of MRSA isolate (66.7%) but statistically, educational background was not significantly associated with MRSA colonisation (p= 0.189). It could be statistically deduced that, MRSA colonisation is not related to the purchase of pork, beef, chevon, mutton and chicken in this study (Table 4).

Exposure to raw meat is not significantly associated with MRSA nasal colonization (p= 0.717). Statistically, smoking was not a major risk factor for MRSA colonisation in this study (p= 0.739). Having direct contact with pigs (p= 0.000) and the last period of taking antibiotic (p= 0.020) were statistically, major risk factors for MRSA nasal colonization in farm workers.

From Table 4, it can be seen that two major factors may predispose farm animals to MRSA nasal colonisation. Statistically, it can be seen that the type of antimicrobial to treat microbial infections (p= 0.010) and the period of antibiotic administration to animals (p= 0.000) are highly associated with MRSA nasal colonisation in farm animals.

Table 3. Phenotypic Antibiotic susceptibility patterns of MRSA isolated from both animals and man in Central Region of Ghana.

Antibiotics		MRSA = 195		P-value
		No.	%	
Penicillin	Susceptible	-	-	0.000
	Intermediate	-	-	
	Resistant	195	100	
Ampicillin	Susceptible	-	-	0.000
	Intermediate	-	-	
	Resistant	195	100	
Cloxacillin	Susceptible	-	-	0.000
	Intermediate	-	-	
	Resistant	195	100	
Erythromycin	Susceptible	-	-	0.000
	Intermediate	48	24.6	
	Resistant	147	75.4	
Tetracycline	Susceptible	-	-	0.073
	Intermediate	85	43.6	
	Resistant	110	56.4	
Vancomycin	Susceptible	195	100	0.000
	Intermediate	-	-	
	Resistant	-	-	
Cotrimoxazole	Susceptible	13	6.7	0.000
	Intermediate	70	35.7	
	Resistant	112	57.4	
Cefuroxime	Susceptible	5	2.7	0.000
	Intermediate	13	6.7	
	Resistant	177	90.8	
Gentamycin	Susceptible	30	15.5	0.000
	Intermediate	33	16.9	
	Resistant	132	67.7	
Ciprofloxacin	Susceptible	155	79.5	0.000
	Intermediate	32	16.4	
	Resistant	8	4.1	
Augmentin	Susceptible	195	100	0.000
	Intermediate	-	-	
	Resistant	-	-	
Meropenem	Susceptible	1	0.51	0.000
	Intermediate	25	12.8	
	Resistant	169	86.7	

Antibiotic susceptibility pattern of MRSA isolates

A total of 195 MRSA isolates of this study were subjected to antibiotic susceptibility test against 12 antimicrobial drugs. From Table 3, most of the MRSA isolates were sensitive to Vancomycin (100% sensitivity), Augmentin (100% sensitivity) and Ciprofloxacin (79.5% sensitivity). However, no isolate was sensitive to Penicillin or β-lactam associated antibiotics. Accordingly, the highest resistance was observed for Penicillin (100%, 195), Cloxacillin (100%, 195), and Ampicillin (100%, 195). The resistance of MRSA to other antibiotics is as follows: Cefuroxime (90.8%, 177), Meropenem (86.7%, 169), Erythromycin (75.4%, 147), Gentamycin (67.7%, 132), Cotrimoxazole (57.4, 112) and Tetracycline (56.4%, 110) (Table 3). Except for Tetracycline (p=0.073), all other susceptibility test results of antibiotics against MRSA isolates were statistically significant (p<0.05).

DISCUSSION

This is the first known study to identify MRSA colonisation in farm animals and farm workers in Ghana. The presence of MRSA in animals and humans in the study area is of veterinary and public health concern.

Nasal carriage has an important role in the epidemiology and pathogenesis of MRSA infection in humans and animals. The overall prevalence rate of MRSA in the

Table 4. Epidemiologic risk factors for MRSA nasal carriage among farm workers and farm animals in the central region of Ghana.

Associated factor		Freq./Sample size	MRSA Carriers		P-Value
			No.	%	
Age group	< 18 years	5/20	4/5	80	0.368
	18-24 years	0/20	-	-	
	25-34 years	3/20	1/3	33.3	
	35-44 years	3/20	-	-	
	> or = 45	9/20	4/9	44.4	
Sex	Male	20/20	9/20	45	
	Female	0/20	-	-	
Educational background	None	3/20	2/3	66.7	0.189
	Junior high	10/20	5/10	50	
	Senior high	4/20	1/4	25	
	Tertiary	3/20	1/3	33.3	
Meat Normally Purchased	**Pork**				0.096
	Yes	14/20	7/14	50	
	No	6/20	2/6	33.3	
	Beef				0.000
	Yes	4/20	-	-	
	No	16/20	9/16	56.3	
	Mutton				0.020
	Yes	2/20	1/2	50	
	No	18/20	8/18	44.4	
	Chevon				0.000
	Yes	0/20	-	-	
	No	20/20	9/20	45	
	Chicken				0.096
	Yes	17/20	7/17	41.2	
	No	3/20	2/3	66.7	
Exposure to Raw Meat	Frequently	3/20	2/3	66.7	0.717
	Occasionally	8/20	3/8	37.5	
	Seldom	9/20	4/9	44.4	
Smoking of Cigarette	Yes	5/20	4/5	80	0.739
	No	15/20	5/15	33.3	
Date of Hospitalisation	< 3 months ago	8/20	2/8	25	0.096
	> or = 3 months ago	12/20	7/12	58.3	
Last Time of Taking Antibiotic	< 3 months ago	3/20	1/3	33.3	0.020
	> or = 3 months ago	17/20	8/17	47.1	
Direct Contact with Pigs	Yes	18/20	9/18	50	0.000
	No	2/20	0/2	-	

Table 4. Contd.

Bruises during Direct Contact	Yes	6/20	4/6	66.7	0.739
	No	14/20	5/14	35.7	
Hours/Week in Direct Contact	1-20 hours	10/20	4/10	40	0.717
	21-40 hours	3/20	3/3	100	
	> 40 hours	7/20	2/7	28.6	
Collected Blood/Urine From Livestock?	Yes	14/20	6/14	42.9	0.317
	No	6/20	3/6	50	
Apparels Worn During Contact	**Boats**				
	Yes	20/20	9/20	100	0.000
	No	0/20	-	-	
	Long coats				
	Yes	6/20	4/6	66.7	0.739
	No	14/20	5/14	35.7	
	Gloves				
	Yes	0/20	-	-	0.000
	No	20/20	9/20	100	
	Nose mask			-	
	Yes	0/20	-	100	0.000
	No	20/20	9/20		
Antimicrobials to Treat Livestock Infection	Tetracycline	285/396	110/285	38.6	0.010
	Penicillin	111/396	75/111	67.6	
Period of Antibiotic Administration to animals	< 3 months ago	236/396	148/236	62.7	0.000
	> or = 3 months ago	160/396	47/160	29.4	

Central Region of Ghana was 49.2%. The prevalence of colonisation both at the farm level and the individual animal level on most farms, where MRSA was present, was striking. Most of the tested animals on many farms of this study, especially pigs, were nasally colonised by MRSA, though on some farms, only a small number of animals were nasally colonized with MRSA. It is unclear whether differences in management on farms were associated with this variation. It would be interesting to re-test low prevalence farms to determine whether the prevalence of MRSA colonisation has increased, as it is possible that the low prevalence could indicate recent introduction of MRSA.

Results from the present study showed that the carriage rate of MRSA was higher than that for Asian countries such as China, which recorded carriage rate in pigs as 58/509, 11.4% (Cui et al., 2009); Korea 21/657, 3.2% (Lim et al., 2012); Malaysia 1.4% (Neela et al., 2009); Japan 0.9% (Baba et al., 2010) and Hong Kong 16 to 21.3% (Guardabassi et al., 2009). Also, the prevalence of MRSA found in pigs (50.2%) in this study was different in comparison to other European studies. For example, in Belgium, an estimated 44% were carriers (Crombé et al., 2012); in Germany, a prevalence of 52% was reported for fattening farms (Alt et al., 2011), and there was 56% prevalence in pig holding companies in the Netherlands (Broens et al., 2011). Moreover, in La Rioja (Northern Spain), Gómez-Sanz et al. (2010), described a prevalence of 21 and 49% in fattening and suckling pigs, respectively, at the slaughterhouse level. These differences can be attributed to variations in microbiological methods (sampling technique, culture and method of MRSA identification), local infection control standards, local prevalence of MRSA and husbandry methods.

This carriage prevalence in farm animals in Ghana could be attributed to the higher stocking density which was observed throughout the visit. Previous studies

indicated that higher density increased the risk of MRSA colonisation (Van Duijkeren et al., 2007; Battisti et al., 2010). *S. aureus* is not typically regarded as a pathogen in pigs, sheep and goat and even with a high prevalence of MRSA colonisation in these animals, clinical infections have not been widely reported in this species. However, a report by Van Duijkeren et al. (2007), implicating MRSA in exudative dermatitis in pigs raises potential pig health concerns. Although the percentage of MRSA nasal carriage in sheep was remarkably lower than those observed in pigs and goats, the reason for this is not clear but it is suggested that variation could partly be due to differences in nasal physiology and self-care behaviors of these animals. Efforts should be made to characterise possible reservoirs in order to reduce the spread of MRSA among farm animals especially pigs.

Although definite conclusions cannot be made because of small sample size, observations of this study on farm workers are consistent with numerous studies demonstrating that people working in close contact with animals colonised with MRSA have a high risk of culture positive nasal swabs (Smith et al., 2009; Weese, 2010; Wulf et al., 2008). Further confirmation of this phenomenon is unlikely to provide new insight unless accompanied by efforts to understand its biological nature and implications for occupational health.

Furthermore, although very high prevalence has been described in this study and in other cross-sectional studies of farm workers and farm animals, the routine use of multiple enrichment methods to culture samples is likely to result in detection of samples with low numbers. Quantification of MRSA from culture positive farm workers may provide a more meaningful context for evaluating contamination versus colonisation events and informing assessment of associated health risks. Transmission of methicillin resistant variant between animals and humans has been frequently reported via direct contact (Deiters et al., 2015) or indirect routes such as the environment and food chain (Peterson et al., 2012).

Although direct comparison cannot be made because a control group was not used in the assessment of human colonisation, the prevalence of colonisation in farm workers (45%) was quite high and is much greater than has been reported in some research work in the general population in Accra. For example, a study by Odonkor et al. (2012) in the Greater Accra Region of Ghana estimated a population colonisation of 84 MRSA out of the 250 *S. aureus* isolates, giving a prevalence rate of 33.6% of the samples collected from microbiological samples from hospital in Accra-Ghana. Further, a study on MRSA prevalence on people who visited the University of Ghana Hospital was only one (9.1%) out of 11 *S. aureus* positive isolates (Pesewu et al., 2014). Therefore, it is likely that personnel working with farm animals specifically pigs as seen in this study are at higher risk for MRSA colonisation compared to the general Ghanaian population.

There were also some differences in MRSA nasal carriage rate among various districts in the Central Region, with KEEA district recording the highest and Mfantseman district recording the lowest. Reasons for these differences cannot be understood but may be attributed partly to the variation in geographical locations, the differences in the proportions of animals sampled and the extent of risky environmental exposures. Egyir et al. (2014) reported variations in the distribution of MRSA in the Northern Region of Ghana and attributed it apparently to geographical variations.

Antimicrobial resistance profiles of the isolates from farm animals and farmers

The antimicrobial resistance profiles shown in the present study are comparable to many other studies within and outside Ghana reiterating the fact that pathogens are likely to develop resistance to most commonly used drugs (Penicillin, Ampicillin and Erythromycin) as opposed to less commonly used drugs Ciprofloxacin and Vancomycin (Verkade et al., 2014; Odonkor et al., 2012; Moyo et al., 2014). Unlike other previous studies, all the MRSA isolates were somehow resistant to all the classes of antibiotics used in the study except for Vancomycin, Augmentin and Ciprofloxacin. This study agrees with Sapkota et al. (2006), who reported that MRSA was susceptible to Ciprofloxacin and Vancomycin. The present study showed the resistance of MRSA to Penicillin (100%), Ampicillin (100%) and Tetracycline (54.6%). These findings agree with the findings of Shibabaw et al. (2013), who among others reported resistance of MRSA to Penicillin (94%) and Tetracycline (73.8%) around Addis Ababa. This confirms the previous finings that MRSA strains have been recognised to be resistant to almost all β-lactam antibiotics (Lowy, 2003). The observed resistance patterns to some of the conventional antibiotics, which are usually frequently prescribed in the study area, are alarming because of the high resistance rates among nonclinical isolates such as those obtained in this study. This further reaffirms the critical role of commensals in public health. The observed high level of resistance (65 to 100%) to almost all of the antibiotics might be due to consumption of antimicrobials (Moulin, 2001) as growth promoters (Perrier-Gros-Claude et al., 1998), used extensively in livestock husbandry in the study area.

The present study has demonstrated the existence of alarming levels of resistance of MRSA to commonly used antimicrobials (Penicillin and Ampicillin) in the farms of this study. The results were in accordance with reports from earlier studies in other countries (El-Jakee et al., 2008; Gentilini et al., 2002) suggesting a possible development of resistance from prolonged and indiscriminate usage of some antimicrobials. Vancomycin

used to be the last antibiotic for treating infections caused by such resistant isolates (Boucher et al., 2010; Fitzgerald et al., 2001; Bhalakia et al., 2006). This study reaffirms this fact as all MRSA isolates were sensitive to Vancomycin.

Meanwhile, the wide range of multiple antibiotic resistance showed a divergence between the static-use (in situation where a fixed antibiotic regimen is used) and the adaptive-use (in using varieties or wide range of antibiotics due to observed low performance of the earlier one(s)), which may imply consistent use of various antibiotics in these farms on the animal, to achieve a non-chemotherapeutic advantage (Laxminarayan et al., 2011). This implies that the organisms might have developed resistance over a period of exposure without medical prescription. Therefore, this exposure of the animal bacterial flora to antibiotics appears to be encouraging emergence of resistance across a wide range of antibiotics (Barbosa, 2000). It is, therefore, important to control the misuse or any other non-therapeutic use of antibiotics. This study is, therefore, important to the agricultural sector in Ghana as far as animal health is concerned. It is also important to the human health sector due to the possibility of zoonotic infections, even if the organism in the animals had its original source from previous human contact. Farm animal workers, especially piggery workers, should be diligently hygienic as the animal is a consistent source of MRSA. The relatively high prevalence of MRSA observed among conventional herds in the study confirms that routine antimicrobial use in farm animal especially pigs is a sufficient cause for emergence of LA-MRSA.

Epidemiologic risk factors for MRSA colonisation among farm workers and farm animals

In this study, factors that could be associated with lower socioeconomic status were positively associated with MRSA carriage, educational status (less than high school or general educational developmental degree versus higher levels (Schinasi et al., 2013). Similarly, other studies have reported positive associations between lower socioeconomic status and MRSA (Casey et al., 2013). A recent population-based study in Pennsylvania found that community-acquired MRSA infection was associated with community economic deprivation (Casey et al., 2013). Why lower levels of educational achievement would be a risk factor, is also difficult to speculate, unless this is a surrogate for lower socioeconomic status and, therefore, crowded living conditions. Epidemiologic risk factors that have significant association with MRSA nasal carriage among farm workers and farm animals in this study were: direct contact with pigs (p=0.000), the last period of taking antibiotic (p= 0.020), type of antimicrobial to treat microbial infections (p=0.10) and the period of antibiotic administration to animals (p=0.000) and whether or not one wear apparels such as nose mask (p=0.000) and gloves (p=0.000). Similar studies showed that the rate of nasal colonisation of MRSA is high when there are predisposing risk factors like antibiotic usage in previous 4 weeks (Aiello et al., 2006; Farley et al., 2008). All respondents who wore boots were MRSA carriers. This means that wearing boots is not a sole channel or factor for assessing risk factor for MRSA nasal carriage.

It was observed that younger individuals both farm workers and farm animals had high MRSA nasal carriage. Why younger individuals and females would be at higher risk for MRSA colonisation could not be determined in this study but can be partly due to their weaker immune response. Statistically, sex was not an associated risk factor for carrying MRSA in the current study, which was in agreement to others (Graham et al., 2006; van Cleef et al., 2011). The protective measures taken by some of the farmers did not prevent them from becoming colonised with MRSA. This could be a result of breaches in adherence to these measures, e.g., poor hand hygiene after removal of gloves or the reuse of contaminated apparels, or because of contamination outside pig farms.

Although not determined, dust may also be a potential risk factor for acquiring MRSA for individuals who tested positive for MRSA and who had no direct contact with livestock. In a rural region in Lower Saxony, Germany, local residents who visited farms, e.g. to buy meat, had a 3.2-times higher risk (95% CI: 1.4-7.4) of colonisation with MRSA than did people without occupational livestock contact (Bisdorff et al., 2012).

A correlation between exposure time on farm and human colonisation has been shown elsewhere (Graveland et al., 2011), which is reaffirmed by the present results. However, a larger number of participants would be needed for more reliable results.

Conclusions

The data from this study showed that MRSA is present in Central Region of Ghana and farm animals especially pigs can serve as reservoir of this multi-drug resistant organism. The carriage rate of MRSA is highest among pigs than goats, sheep and humans respectively. Antimicrobial resistance is a clear and present danger. MRSA isolates were all resistant to all the antibiotics except Vancomycin, Ciprofloxacin and Augmentin. Some epidemiological risks factors observed in this study were; direct contact with pigs, last period of antibiotic administration, type of antibiotic administered and not wearing apparels like nose mask and gloves. Based on the results, it is recommended that all MRSA isolates identified with the phenotypic method in this study should be confirmed using molecular tools. Molecular characterisation should be done to determine the genetic relatedness between MRSA strains of farm animals and

MRSA strains of farm workers in the Central Region of Ghana. In future, surveillance studies using large sample size, should be conducted so as to make the findings robust.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

MRSA, Methicillin resistant *Staphylococcus aureus*; **MSSA,** methicillin susceptible *Staphylococcus aureus*; C.I., confidence intervals.

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Full Length Research Paper

Root symbioses in two legume-grass consortia inoculated with soils obtained from degraded coal mining areas in reclamation

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The intense exploitation of coal deposits in Southern Brazil has caused severe degradation problems in extensive areas. In order to recover such areas, the use of revegetation with species adapted to disturbed environments and with the capacity to establish mutual relationships with microorganisms have been of great value. The objective of this study is to characterize plant-root symbioses in two grass-legume (*Calopogonium mucunoides* with *Brachiaria decumbens* and *Vicia sativa* with *Brachiaria decumbens*) consortia inoculated with soil of the Carboniferous Basin in the state of Santa Catarina. Areas evaluated were at different stages of land reclamation and the influence of those consortia on the occurrence of rhizobia, arbuscular mycorrhizal fungi, and in the community of endophytic bacteria were evaluated. The study was done in two independent experiments in a completely randomized design, with five replications; done under greenhouse condition. There were seven treatments - five inoculated (with soil obtained from areas of 2, 4, 6 and 12 years of recovery, and a reference area) and two control treatments without inoculation (with low and high concentrations of mineral nitrogen). After 50 days of implantation, soil and plant material were collected to characterize root symbioses by nodule counting, nitrogen fixing bacteria isolation, mycorrhizal occurrence (%), and characterization of root endophytic bacterial communities. Only the calopogonium-brachiaria consortium was able to nodulate with rhizobia from the recovering coal mining areas. Arbuscular mycorrhizal fungi (AMF) and endophytic bacteria occur in the vetch-brachiaria and calopogonium-brachiaria consortia regardless of the time of recovery. The microbial communities present in soils with different stages of recovery are more efficient in promoting plant growth in the calopogonium-brachiaria consortium and this behavior may be associated with the calopogonium's ability to associate with autochthonous rhizobia.

Key words: Environmental recovery, revegetation, plant-growth-promoting, rhizobia, *arbuscular mycorrhiza.*

INTRODUCTION

The Carboniferous Basin in the state of Santa Catarina (CBSC) is the second most important in Brazil, and for a long time the deposition of tailings, following coal extraction, was done with no control or soil preservation (Lopes et al., 2009). In the last decades, many areas were abandoned after open-pit mining, which entailed the removal of large strips of vegetation, erosive processes, the release of toxic gases into the atmosphere, and the loss of soil organic matter. The sites lost or significantly decreased their self-healing capacity, requiring active intervention to reestablish a non-degraded condition (Rocha-Nicoleite et al., 2013). Faced with such facts, a civil action was proposed, which obliges the coal industry, the State and the Federal Government to carry out projects aimed at recovering those areas. Revegetation is a rehabilitation alternative that can aid in this process, since it promotes the control of erosive processes, resulting in the recovery of soil properties (Siqueira et al., 2008).

Plant species used in the revegetation of such areas must have adaptive capacity to degraded environments. Works involving revegetation with tree legumes have been developed in the region in the last decade. However, the use of these plants presents limitations due the deep root systems that may affect the structure of soil built which is preformed in tailings areas with the purpose of being a barrier for confinement the residues. Hence, the use of other legumes, mainly herbaceous species, may pose an alternative. Leguminous plants such as *Calopogonium mucunoides* Desv. (calopo) and *Vicia sativa* L. (vetch) consorted with grasses such as *Brachiaria decumbens* Stapf. (brachiaria) are used for the revegetation programs in the region, as they increase the organic material deposited in the soil surface, favoring biological activity, and accelerating the recovery process (Rocha-Nicoleite et al., 2013). Other important characteristics of these plants are good adaptation to acid soils of low natural fertility containing high levels of aluminum. These plants are able to establish mutualistic relationships with symbiotic microorganisms such as rhizobia (in the case of leguminous plants) and arbuscular mycorrhizal fungi (AMF) (leguminous plants and brachiaria), which provide nutrients (such as phosphorous) to the plant (Ampomah and Huss-Danell, 2016; Ferreira et al., 2016; González et al., 2018). Besides rhizobial and mycorrhizal symbionts there is a great diversity of other microorganisms occupying the interior of the plant tissues, also known as endophytes. Those may present the capacity to promote plant growth through several mechanisms, including production of phytohormones and siderophores, solubilization of phosphates, among others (Timmusk et al., 2011; Brígido and Glick, 2015; Santoyo et al., 2016). Altogether, these microorganisms interacting with plants can play a crucial role for the establishment and development of plants in degraded environments, such as those commonly found in coal mining areas.

Despite calopo and vetch being able to form a symbiotic relationship with rhizobia, not much is understood about the development of the symbiosis under stressful conditions, which greatly affect the outcome of the symbiotic process. Similarly, little is known about the impact of the stressful conditions imposed by the coal-mining degraded soil in the mycorrhizal and root endophytic communities of brachiaria, calopo and vetch. Moreover, in order to maximize the revegetation procedure, future inoculants adapted to the harsh environmental conditions present in the coal-mining recovery areas need to be developed. Therefore, the objective of the present work is to characterize plant-root symbioses in calopo+brachiaria and vetch+brachiaria consortia inoculated with soils from mining areas at different stages of land reclamation, and determine the influence of those consortia on the occurrence of rhizobia, AMF, and in the community of endophytic bacteria.

MATERIALS AND METHODS

Data collection

The collection of data was done in June 2015. Different areas were chosen according to the recovery time after mining, therein designated: two years (A2), four years (A4), six years (A6) and 12 years (A12) under a revegetation regime. The pH for the soils of those areas was 4.66, 4.53, 3.80 and 4.91, respectively. A2 and A4 are located in the municipality of Lauro Muller and have the following coordinates: 28º19'08.97''S 49º26'20.93"W and 28º33'26.62"S 49º27'56.19"W respectively. A6 is located in Treviso 28º26'10.78"S 49º23'36.04"W and A12 in Siderópolis 28º35'09.30"S 49º25'25.93"W. Soil samples were also collected in a reference area (RA) with no mining history in Lauro Muller-SC (28°22'32.1"S 49°20'31.9"W), and with a typical vegetation cover of dense ombrophilous forest. The chemical characterization of the soils and the plant species present in these areas was summarized in Silva (2016). For the collection, five random sites were chosen within each area, located 100 to 200 m apart from each other (depending on the size of the area). At each sampling site a central point was chosen and at a 4 m radius 4 soil samples were collected (at each cardinal point), at a depth of 0-20 cm, forming together 400 g of soil per sample, which was used as source of inoculum.

Plant growth assay

The experiment was conducted under greenhouse conditions using

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two consortia: one of *Calopogonium mucunoides* and *Brachiaria decumbens* Stapf. (calopo+brachiaria), and another with *Vicia sativa* and brachiaria (vetch+brachiaria). Experiments were done in 280 cm³ pots containing an autoclaved mixture of sand and vermiculite (1:1; v/v) inoculated with 50 g of soil-inoculum. For this purpose, 15 mL of brachiaria seeds and two legume seeds, previously disinfected with 2% sodium hypochlorite for two minutes and washed six times in sterile distilled water, were sown. For each consortium, a completely randomized design (five replications) was used, consisting of five treatments with the inoculum source (corresponding to soils obtained from areas A2, A4, A6, A12 and RA), and two control treatments without inoculation: low (5.25 mg N) (C-N) and high concentration of mineral nitrogen (52.535 mg N) (C+N), totaling 35 experimental units for each consortium. Weekly, 50 ml of a half-strength Hoagland and Arnon (1950) nutrient solution were added to the pots. The experiment was conducted for 50 days, when the soil and plant material were collected for evaluation. The aerial part of the plants was placed in a drier with air circulation at 65°C until constant weight, to determine the shoot dry biomass (SDB). The nitrogen content in the shoots was determined by the Kjedahl semi-micro method according to Tedesco et al. (1995). Accumulated nitrogen was calculated by multiplying the nitrogen content with the respective SDB content.

Rizhobia and mycorrhizal occurrence

Nodules were detached and counted from the roots of the leguminous plants. Then, ten nodules were selected per pot to isolate the rhizobia. For this, nodules were disinfected with alcohol (95.5%) for 60 seconds, sodium hypochlorite (2.5%) for 2 minutes, and washed six times in sterile distilled water. Nodules were then macerated and inoculated into Petri dishes containing yeast mannitol agar medium- YMA extract (Vincent, 1970). Subsequently, plates were incubated for a period of 14 days at 28 °C. Then, isolates were characterized morphologically after 10 days of incubation via bromothymol blue in YMA medium and congo red in YMA medium. The following morphological characteristics were evaluated: growth time, pH change, color, shape, surface and border of the colony, absorption of the indicator, and mucus production. Strains of *Rhizobium leguminosarum* (SEMIA 384), *R. tropici* (CIAT899), *Bradyrhizobium japonicum* (BR 1602), and *Bradyrhizobium* sp. (SEMIA 6144) were used as reference.

Root samples from each treatment were separated, washed and stained according to Koske and Gemma (1989), and the percentage of colonization estimated (Giovannetti and Mosse, 1980). The soil spore density was obtained from samples of 50 g of soil collected in each treatment. Spores were obtained by wet sieving (Gerdemann and Nicolson, 1963), followed by sucrose gradient centrifugation. After extraction, spore counting was performed using a stereomicroscope (16X).

Root endophytic bacteria community determination

Samples of 0.5 g of consortium roots were disinfected following Da Silva et al. (2016). To verify the effectiveness of the disinfestation process, an aliquot of the last wash water was used for DNA amplification. Root samples were macerated in liquid nitrogen for DNA extraction using the 2% CTAB method (Doyle and Doyle, 1990). Amplification was done for the V3 region of the bacterial 16S rDNA gene using primers BAC338FGC and UN518R (Ovreås et al., 1997). Amplification was performed using 10 µmol L^{-1} of the primers and the PCR products analyzed by denaturing gradient gel electrophoresis (DGGE) following Da Silva et al. (2016). Acquisition of gel images was done on a Gel Logic 2200 Pro Photo Documentator (Carestream Health, New York, USA). The fragment (band) patterns were analysed with the program BIONUMERICS 7.10 (BioSystematica, Wales, UK).

Statistical analysis

Normality test (Shapiro-Wilk) and the homogeneity of variances (Cochran) were performed for the variables measured. The number of spores was transformed with the $\log_{10}$ function. The data were compared using analysis of variance and the means submitted to the SNK test ($p<0.05$) (ASSISTAT 7.7). The phenotypic attributes of the rhizobia were evaluated by a hierarchical clustering analysis using the software SYSTAT 11. The clusters obtained from the band profiles of the PCR-DGGE were analyzed using the Jaccard index and the UPGMA clustering model.

RESULTS

Occurrence of autochthonous rhizobia and mycorrhiza

The visual inspection of roots revealed the presence of nodules in the calopo+brachiaria consortium in all inoculated treatments (Table 1). No nodules were observed in the vetch+brachiaria consortium.

In the calopo+brachiaria consortium plants inoculated with soil from Areas A2, A4 and A12 exhibited on average 55 nodules per pot, 243% higher than those for area A6 (16 per pot). Fifty rhizobia isolates were obtained and their morphological and cultural features characterized. Two thirds of the isolates evaluated did not alter the pH of the culture medium. Most isolates of rhizobia (76%) exhibited intermediate or slow growth, and 60% of them had scant or low mucus production. These characteristics pointed to the low representativeness of the genus *Rhizobium*, which is fast growing, reduces the pH of the medium, and presents abundant mucus production.

Unlike the rhizobia, AMF were verified in both consortia. The percentage of mycorrhizal colonization ranged from 14 to 51% in the calopo+brachiaria consortium, and from 19 to 47% in the vetch+brachiaria consortium (Table 1). Plants inoculated with soil from Areas A2 and A6 showed the highest mycorrhizal colonization in both consortia (on average 47%). For the calopo+brachiaria consortium, the number of spores in areas in the initial stages of recovery (A2 and A4) was a range of 5- 6 times greater than in RA. On the other hand, in the vetch+brachiaria consortium AMF spores were detected in all treatments with the lowest values in areas A12 and RA. In area A6, about four times more spores were found in the vetch+brachiaria than in the corresponding calopo+brachiaria consortium.

Characterization of the community of endophytic bacteria

The results of the hierarchical cluster analysis for

Table 1. Mycorrhizal colonization (%) and number of AMF spores present in the calopo-brachiaria and vetch-brachiaria consortia inoculated with coal mined soils at different stages of recovery in the state of Santa Catarina.

Consortia	Treatments	Nodule number	Mycorrhizal colonization (%)	#spores 50 mL soil^{-1}
Calopo+brachiaria	A2*	62^{a}	46^{a}**	377^{b}
	A4	51^{b}	21^{b}	722^{a}
	A6	16^{c}	51^{a}	188^{d}
	A12	52^{b}	21^{b}	282^{c}
	RA	66^{a}	14^{b}	76^{e}
Vetch+brachiaria	A2	0	46^{a}	626^{a}
	A4	0	19^{b}	464^{a}
	A6	0	47^{a}	707^{a}
	A12	0	24^{b}	271^{b}
	RA	0	27^{b}	118^{c}

*A2, 2 years of recovery, A4: 4 years of recovery; A6, 6 years of recovery; A12, 12 years of recovery; RA, reference area. **Values followed by different letters in the same column for each consortium are statistically different according to the Scott-Knott test ($p<0.05$).

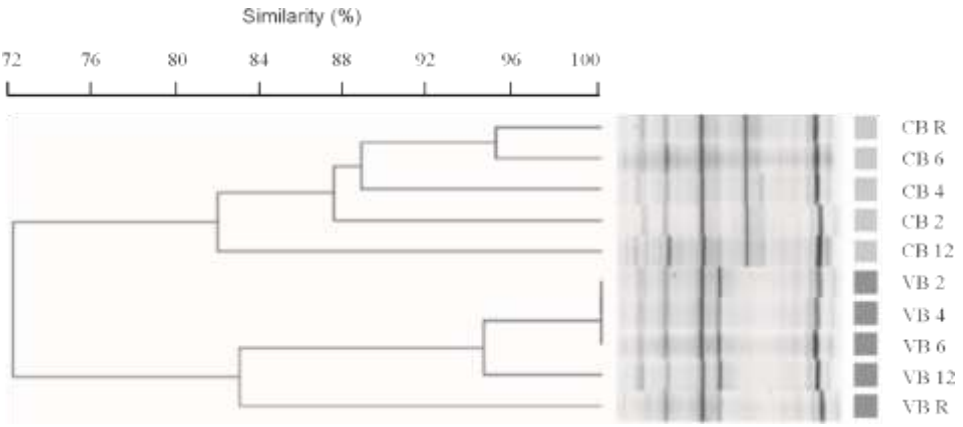

Figure 1. Hierarchical grouping of the community structure of endophytic bacteria. CB = calopo+brachiaria, VB= vetch+brachiaria, R = reference soil without mining tailings. 2, 4, 6, 12 = different recovery times for soils with mining tailings.

bacteria communities in the consortia can be observed in Figure 1. It can be seen that the structure of the endophytic community presents 72% similarity between consortia. There is also a formation of two groups with 82 and 83% similarity, made by samples from the vetch+calopo consortium, respectively. Thus, the determining factor of clustering was the type of legume present in the consortium and not the area used as inoculum source.

In the calopo+brachiaria consortium, the grouping presented some heterogeneity: AR was closer to areas with the lowest recovery times, whereas A12 had communities of more differentiated endophytic bacteria. In the grouping obtained for the vetch+brachiaria consortium, the grouping presented lower heterogeneity. Moreover, there was a separation between the RA and the areas under recovery. There were no marked differences between the microbial groups evaluated in soils with different recovery times in this consortium.

Effect of soil inoculation on plant growth and nitrogen accumulation

Shoot dry matter (SDM) and nitrogen accumulation of the calopo+brachiaria consortium were significantly influenced by the treatments. The highest SDM was accumulated by plants of the C+N treatment, followed by those inoculated with soil-inoculum of areas A2, A4, and A12 (Figure 2A). Treatments A6 and RA presented a biomass 33% lower than the C-N. Nitrogen accumulation was highlighted for the treatments with the soil-inoculum from areas A2, A4 and A12, which did not differ from C+N (Figure 2C). For those treatments, the average increase was 57% higher in relation to the C-N treatment. In the RA, the accumulation of nitrogen presented an intermediate value, and the A6 area again presented the lowest levels. On the other hand, in the vetch+brachiaria consortium there were increases in plant growth only in the C+N treatment. The inoculation did not influence

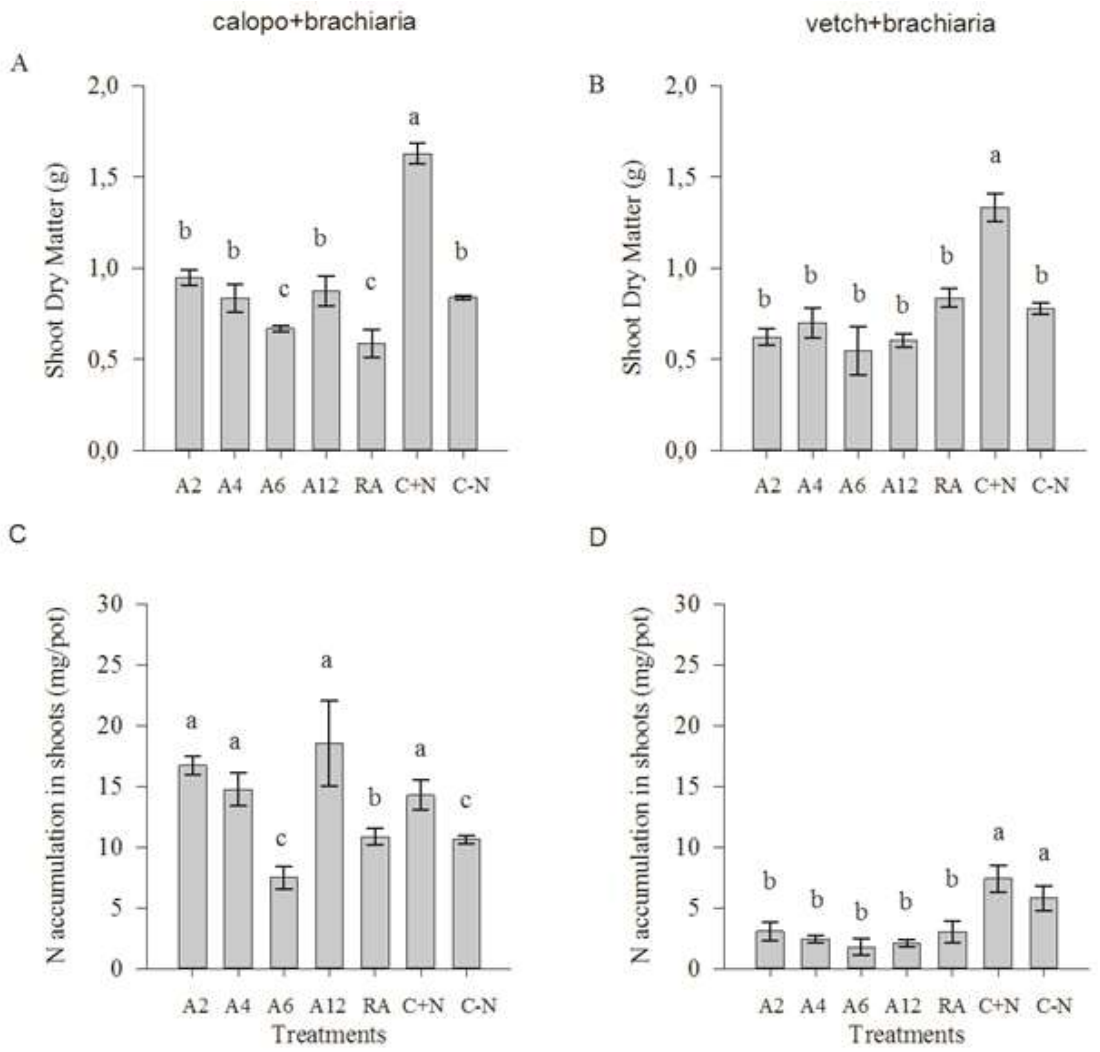

Figure 2. Effect of soil inoculation from coal mining areas under different times of recovery, on attributes related to growth and nutrition of plants grown in consortia: shoot dry matter for the calopo+brachiaria (A) and vetch+brachiaria (B) consortia, nitrogen accumulation in calopo+brachiaria (C) and vetch+brachiaria (D) consortia; RA = reference area without mining tailings. 2, 4, 6, 12 = years of recovery; C+N = non-inoculated control with high N after 50 days of growth; C-N = non-inoculated control with low N after 50 days of growth. Means followed by the same letter do not differ statistically by the Scott-Knott test ($p<0.05$). Vertical bars represent the standard error of the mean (n = 5).

accumulation of nitrogen since the inoculated treatments were lower when compared to the controls without inoculation (Figure 2B and 2D).

DISCUSSION

Symbiotic potential of calopo vs. vetch in different soils

Rhizobia

After water, nitrogen is the second limiting factor for plant growth. The Biological Nitrogen Fixation (BNF) process for nitrogen addition appears as a very environmentally friendly alternative since the nitrogenous industrial fertilizer inputs are reduced. Currently, it is accepted as a consensus that both the production and the use of nitrogen fertilizers result in a serious threat to the environment, since in both situations considerable amounts of nitrous oxide (N_2O) are generated, being one of the more powerful greenhouse gases there is (Crews and Peoples, 2004; Jensen et al., 2012). In this sense, the use of nitrogen fixing bacteria as an important way to make this nutrient available after the establishment of symbiosis with legumes has been intensively studied. Numerous studies have shown that symbiosis increases N accumulation for many legumes (Calheiros et al., 2013;

Moura et al., 2016).

In the present study, calapo was found to have a better response to inoculation than vetch, with mean increases in N accumulation four times higher in most inoculated treatments. In that respect, the selection of strains with high efficiency has become a constant search. Nevertheless, the choice should be for autochthonous strains, which have much higher survival rates in relation to non-autochtonous strains (Geetha and Joshi, 2013). In the present work, 40 indigenous rhizobia from recovered mining areas were obtained. They all share a common trait, which is the fact that they are all adapted to the local conditions and have competitive capacity with the microbial community for their establishment and permanence in coal mining degraded areas. These isolates were compatible with calopo but not with vetch.

The rhizobium-legume symbiosis is a highly specific interaction (Lopes et al., 2016). A previous study developed by our group described that autochthonous rhizobia from coal mining areas show low symbiotic compatibility with vetch, since nodulation was confirmed in only 12.5% of the isolates evaluated (Hernández et al., 2017). Furthermore, Spaink et al. (1991) showed that vetch is a very restricted plant that establishes interaction with only the genus *Rhizobium* that has receptors for a single type of nodulation factor. Recently, Ampomah and Huss-Danell (2016) studied the genetic diversity and phylogeny of bacteria isolated from nodules of six species of *Vicia* in Norway. In that study, 25 isolates were obtained, all of are which classified as *Rhizobium leguminosarum* sv. *viciae*. These descriptions suggest that isolates compatible with vetch are uncommon, a fact that matches the results presented in the current study.

AMF

AMFs are highly tolerant to abiotic stresses; plants colonized with AMF can mobilize more nutrients, tolerate water shortages, and reduce the impact of trace elements, among other characteristics that increase plant survivability in the initial stages of establishment and development (Siqueira et al., 2008). In this study, mycorrhizal colonization was similar in both consortia. With the exception of area A6, it was verified that in the vertch+brachiaria consortium there was a reduction in the number of AMF spores with the increase of recovery time. Several authors have described that in areas in the early stages of succession, mycorrhizal colonization is greater than for areas in more advanced stages (Zangaro et al., 2012; Sousa et al., 2014). In addition, plants inoculated with the A12 and RA soils present the lowest values of mycorrhizal colonization and number of spores, probably because the mycorrhizal symbiosis, as explained previously, presents a smaller competitive advantage in stable ecosystems. There are, however, variations in these patterns, as observed in area A6, which may be related to the inoculum potential of AMF present in the soils of the loan areas. In the process of revegetation, soils of different places, called loan areas, are added and provide topographic remodeling aside from supporting the vegetation to be introduced. The quality of this soil determines the number of propagules and the initial microbial diversity in each area to be recovered, so the starting point for the different areas in recovery is not necessarily the same.

Endophytic bacterial communities

In relation to the endophytic bacterial community structure, a high similarity between the consortia was expected, due to the presence of brachiaria in both treatments. However, the formation of two distinct groups was verified, and can be attributed to the genotype of the legume present in the consortium. These results are in agreement with those described in other studies in which it was shown that the genotype of the host, its stage of development, and the type of organism studied determine the community of endophytic microorganisms (Ottesen et al., 2013; Bodenhausen et al., 2013; Hardoim et al., 2015).

Moreover, in the vetch+brachiaria consortium, in the inoculated treatments, regardless of time, a very specific bacterial community structure can be observed, with similarity varying between 95-100% (Figure 1). In this work, restricted associations can be observed not only in the bacterial endophyte community structure, but also with rhizobia. This symbiosis was absent in vetch and may justify the different community structure of the consortium, being these microorganisms absent in the banding pattern or even influencing the bacterial community associated to the species.

Effect of soil inoculation on plant growth and nitrogen accumulation

The increases in N accumulation verified for treatments A2, A4 and A12 showed that in the calopo+brachiaria consortium the inoculation may substitute completely the addition of nitrogen fertilizers since the values were not significantly different between those treatments and the C+N treatment. The contribution of N in the calopo+brachiaria consortium is positively related to the presence of rhizobia ($r = 0.5^{**}$). On the other hand, in the vetch+brachiaria consortium the inoculation may contribute negatively for the accumulation of N, as shown in Figure 2.

In general, calopo was more responsive than vetch when inoculated with soil from coal mining areas, presenting increased ability to form symbiotic relationships. Calopo seems to be a better alternative to be used in future recovery programs since 80% of the

evaluated soils presented a microbiota that in symbiosis has the capacity to promote the growth of this legume. This fact is very positive considering that this is a legume native to South America adapted to low fertility soils and low pH, meanwhile vetch is a native legume from the temperate regions of the world (Ferreira et al., 2016; Camargos and Sodek, 2010). In the present study, there was no symbiotic association between vetch and any native rhizobia. However, the vetch+brachiaria consortium could be used as a prior crop once it promotes abundant AMF propagules that can establish symbioses later with the plants inserted by the recovery programs. Rhizobia isolates and AMF spores obtained in this study will be tested in symbiotic efficiency assays in calopo in order to obtain microbial inoculants appropriate to areas degraded by mining.

Conclusion

The calopogonio-brachiaria and vetch-brachiaria consortia are similar in the presence of root symbioses involving arbuscular mycorrhizal fungi and endophytic bacteria, regardless of the recovery time of the coal mining areas. The microbial communities present in soils with different stages of recovery are more efficient in promoting plant growth in the calopogonium-brachiaria consortium, and this behavior may be associated with the calopogonium's ability to associate with autochthonous rhizobia.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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