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Full Length Research Paper

Molecular detection of pathogenic *Leptospira* from cattle in peri-urban areas of Addis Ababa, Ethiopia

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Leptospirosis is a bacterial, zoonotic disease caused by pathogenic *Leptospira*. Rodents are known to carry pathogenic *Leptospira,* but livestock are also important hosts. The disease is economically important in cattle, causing abortion, decreased fertility and decline in milk yield. Pathogenic *Leptospira* are shed in cattle urine and can survive in the environment. Only a few studies have been performed in Ethiopia to investigate the presence of *Leptospira*. The aim of this study was to determine the prevalence of pathogenic *Leptospira* in cattle in peri-urban areas of Addis Ababa. A cross-sectional study was undertaken in peri-urban areas of Addis Ababa. Urine was collected from cattle. DNA was extracted and real-time PCR with melting curve analysis was performed to detect pathogenic *Leptospira*. Knowledge, attitudes and practices of the cattle-keeping households were assessed by a questionnaire and household level risk factors investigated using logistic regression. In total, 168 urine samples were collected from 168 cattle in 70 households. Pathogenic *Leptospira* were found in 3 of the 168 (1.8%) urine samples. Although potential exposure pathways were widely present in the households, no significant risk factors were detected in regression analysis. This study has shown that pathogenic *Leptospira* are present in cattle in peri-urban areas of Addis Ababa, which could be a potential threat for humans. These findings emphasize the need for large-scale studies concerning pathogenic *Leptospira* in Ethiopia, especially in communities with high human-animal interaction.

Key words: Pathogenic leptospira, PCR, Ethiopia, cattle, leptospirosis.

INTRODUCTION

Leptospirosis is a globally important zoonotic disease caused by bacteria of the genus *Leptospira*, which are thin, tightly coiled, spiral-shaped spirochetes (WHO, 2003; Picardeau, 2017). Pathogenic, intermediate and saprophytic *Leptospira* have been described. Saprophytic *Leptospira* are present in the environment and usually do not cause disease, while intermediate and pathogenic *Leptospira* species can cause disease in both humans and animals. Rodents are considered the main reservoir of leptospirosis, but a wide variety of wild and domestic

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animals including cattle, sheep,goats, horses and pigs can be infected. Once infected, animals can develop long term kidney colonization and shed *Leptospira* in urine. Transmission to other animals and humans occurs when *Leptospira* in urine-contaminated soil or water enters the body through mucous membranes, small cuts and abrasions (Levett, 2001; WHO, 2003; Allan et al., 2015).

Leptospirosis is found worldwide, but warm and humid tropical regions favour the survival and perpetuation of the spirochetes (Evangelista and Coburn, 2010; Hartskeerl et al., 2011). Globally, leptospirosis is estimated to cause 1 million clinical infections and 60,000 deaths each year in humans (Costa et al., 2015). However, information from the African continent is very sparse (De Vries et al., 2014; Costa et al., 2015) and limits a more accurate estimation of the global burden of leptospirosis. In humans, infections are often asymptomatic or a mild “flu-like” illness, while some patients develop severe illness with hemorrhage, hepatic and renal failure, called Weil’s disease (WHO, 2003). Detection of *Leptospira* is challenging, as direct detection with dark-field microscopy is not reliable and culture is too slow (weeks-months) (Vijayachari et al., 2001; Musso and La Scola, 2013; Karpagam and Ganesh, 2020). The gold standard test, namely the microscopic-agglutination-test (MAT), is labour-intensive, needs a panel of live leptospires and cannot differentiate well between current and past infections, but is able to differentiate serovars (Musso and La Scola, 2013; Karpagam and Ganesh, 2020). Serological tests, such as ELISA, and molecular assays are more practical. Importantly, molecular testing of urine provides a non-invasive option for diagnosis during early and late stages of infection and gives information on genotypic *Leptospira* species, which has largely replaced the traditionally-used serological classification (Musso and La Scola, 2013; Esteves et al., 2018; Vincent et al., 2019; Karpagam and Ganesh, 2020; Di Azevedo and Lilenbaum, 2021). Early detection and start of treatment affect the outcome of the disease in a positive way (WHO, 2003; Levett, 2001).

Leptospirosis is also an economically important disease of cattle. In adult animals, infection is often sub-clinical and the development of clinical signs depends on the infecting species. Cattle are maintenance host of *Leptospira borgpetersenii*, serovar Hardjo (Hardjobovis), which is associated with infertility, abortions, stillbirths, weak offspring and drop in milk production, but gives a more subtle clinical picture than infection with non-hardjobovis leptospires (WHO, 2009; Lilenbaum and Martins, 2014; Ellis, 2015). Acute leptospirosis can also occur in calves and is associated with fever, anorexia, diarrhea, icterohaemorrhagic syndrome and conjunctivitis (WHO, 2009; Ellis, 2015; Yadeta et al., 2016). Cattle are an important source of infection for humans because they can shed large numbers of *Leptospira* in urine over several months, although survival in the environment varies with species (Hairgrove, 2004; Barragan et al., 2017; Rocha et al., 2017; Hamond et al., 2022; Monti et al., 2023). In addition to urine, *Leptospira* can also be found in aborted fetuses, birth products and uterine discharges of infected animals, which can contribute to environmental contamination (Yadeta et al., 2016).

Ethiopia has amongst the largest livestock populations in Africa with many zoonotic diseases being endemic and has a high dependency on agriculture with many households having direct contact with animals (Grace et al., 2012; Shapiro et al., 2017; Management Entity, 2021). There is a paucity of research on leptospirosis in Ethiopia with a few studies performed in animals and only one small study in humans, all suggesting *Leptospira* being prevalent in the country (Moch et al., 1975; Yimer et al., 2004; De Vries et al., 2014; Tsegay et al., 2016; Desa et al., 2021; Marami et al., 2021). One recent study on leptospirosis in cattle in South-West Ethiopia has been published, which found a 24.5% seroprevalence of hardjo-specific antibodies using indirect ELISA (Desa et al., 2021). Other recent serological investigations in horses (44%) (Tsegay et al., 2016) and dogs (15%) (Marami et al., 2021) also reflected high levels of lifetime exposure in animals. There is a need to better understand the epidemiology of leptospirosis in cattle and species circulating in Ethiopia, because Ethiopia has the highest cattle population in Africa with a high livestock density in and around urban areas and the (peri-)urban dairy sector is targeted for development to meet the growing demand for milk and milk products (Management Entity, 2021; Shapiro et al., 2015; FAO, 2020). Therefore, the aim of this study was to investigate the prevalence of pathogenic *Leptospira* in cattle in peri-urban areas of Addis Ababa and to assess the knowledge, attitudes, practices and household level risk factors of cattle-keeping households regarding leptospirosis.

MATERIALS AND METHODS

Study design and study population

A cross-sectional study was conducted from February to October 2019 in peri-urban areas of Addis Ababa. Cattle-keeping households in four peri-urban sub-cities of Addis Ababa were eligible to participate. The four sub-city administrations recommended certain livestock-keeping woredas (districts of Ethiopia) within their sub-city. Animal health assistants of these woredas directed us to the households based on their lists of cattle-keeping households. We aimed to sample every adult animal in a household, with a maximum of 10 samples per farm. In practice, this was not always possible, as not all cattle were able to produce urine during the time of the visit. The minimum sample size for estimation of prevalence was determined using the single population proportion formula according to Thrusfield (2005): $n = (Z\alpha/2)^2 \times P\ (1\text{-}P) / d^2$.

As there were no studies published from Ethiopia investigating leptospirosis in cattle using molecular methods, other recent studies in East Africa using a PCR assay and performed among cattle were considered in the sample size determination (Dreyfus et al., 2017; Allan et al., 2018; Alinaitwe et al., 2019). The highest prevalence, 8.8%, was found among cattle in the capital city of Uganda

(Alinaitwe et al., 2019). Using this expected prevalence (P) of 8.8%, 5% precision, and a 95% confidence interval, a minimum sample size of 123 cattle was calculated. Taking into consideration field logistics, such as transport of the samples and availability of cattle in the households, a final sample size of 168 cattle was achieved.

Field data collection

A semi-structured questionnaire was used to collect data on household socio-demographics, livestock husbandry and water sources as well as knowledge, attitudes and practices (KAP) of the households in relation to leptospirosis and zoonoses more generally ("S1 Appendix"). Respondents were the household head or other adult aged >18 in the household. All interviews were conducted verbally in the local languages (Amharic and Oromo) by the lead investigator and with the assistance from veterinarians/ veterinary students or animal health assistants.

At least 15 mL of urine was collected from each animal (cow/heifer/bull/calf) in the household. A mid-stream urine sample was obtained during spontaneous urination or by gentle perineal massage performed by the investigator and animal health assistant, and collected in sterile bottles. The urine was neutralized immediately after collection with phosphate buffered saline 10x (Lucchesi et al., 2004). Urine samples were transported on the day of collection from the households to the laboratory, with a maximum transport time of 3 h.

Laboratory analysis

DNA was extracted from 140 µl of urine on the day of collection and stored at -20°C. DNA from the pellet was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, 2020). The DNA extraction method was tested by the main investigators on 140 µl of urine of known leptospirosis patients. Extracted DNA was subjected to quantitative real-time PCR using *Leptospira* specific lipL32 and lfb1 PCR detection assays (Bourhy et al., 2011). Lfb1 PCR involves an Evagreen real-time PCR assay, in which a lfb1 PCR product is revealed by a specific melting curve with a Tm of more than 80°C, also allowing species identification. The lipL32 PCR detection involves a TaqMan probe hydrolysis assay that specifically detects the real-time formation of a lipL32 PCR product. LFB1 F/R primers were used to amplify the *lfb1* gene while lipL32-47Fd and lipL32-301Rd primers were used to amplify the *lipL32* gene (Bourhy et al., 2011). The 25 µl PCR reactions contained 19.7 µl of master mix, 0.3 µL of Salsa polymerase and 5 µl of extracted bacterial DNA. Amplification was performed on a CFX96 real-time PCR system (BIO-RAD) with initial denaturation at 95°C for 1 min, followed by 45 cycles of 95°C for 10 s, annealing at 58°C for 30 s, and extension at 72°C for 10 s. These conditions were used for both primer sets ("S2 Appendix"). After PCR, the samples were heated from 40 to 95°C with continuous data acquisition. Purified leptospiral DNA control samples were provided by the Expertise Centre for Reference and Research on Leptospirosis/OIE Reference Laboratory for Leptospirosis (AMC, Department of Medical Microbiology and Infection Prevention, the Netherlands), and included the following species: *Leptospira interrogans*, *L. borgpetersenii* and *Leptospira santarosai*. For quality control purposes, extracted DNA was analysed in both the MRC-ET Advanced laboratory in Addis Ababa and the MRC-Holland laboratory in the Netherlands under identical conditions. The extracted DNA was transported frozen to the Netherlands.

Data management and analysis

Prevalence of pathogenic *Leptospira* was described as the number of animals with positive PCR detection for pathogenic *Leptospira* divided by the total number of animals tested. Melting curve plots were generated and analysed using CFX Manager Software v3.0.1 (BIO-RAD) to determine the average melting temperature for each positive sample in reference to control DNA, and thus suggest the species of positive samples. Data from questionnaires was entered into spreadsheets and Epi Info™ 7 statistical software. Demographic characteristics and knowledge, attitudes and practices were described using frequency counts and proportions. The association between household-level *Leptospira* status (outcome) and KAP questionnaire responses (9 explanatory variables) was explored using univariable logistic regression analysis, using Epi Info™ 7 statistical software. A p-value < 0.05 was considered as statistically significant. Given the small number of positive *Leptospira* detections and non-significant findings in univariable models further multivariable analysis was not pursued.

Ethical considerations

Ethical approval was obtained from the Department Research Ethical Review Committee (DRERC) of the Department of Microbiology, Immunology and Parasitology, College of Health Sciences of Addis Ababa University. Permission to conduct the study was also provided by the Addis Ababa City Administration Health Bureau and the Addis Ababa City Livestock and Agriculture Bureau. Permission for transport of DNA extracts to the Netherlands was obtained in 2020 from the Addis Ababa University, College of Health Sciences, Institutional Review Board (CHS/RTTD/257/2020).

Household respondents were asked to provide oral consent after they were informed (in the local language) about the purpose of the study, voluntary participation, right to withdraw at any time and that the data obtained would be treated as confidential.

RESULTS

Study population

Of the 76 households contacted, 70 households agreed to participate in the study. Reasons for non-participation were: unwillingness to provide urine samples (n=4) or cattle being absent at the time of visit (n=2). Demographic characteristics of respondents and household characteristics are shown in Table 1. The median household size of the studied households was 6 persons (ranging from 1-17 persons), with a mean of 6.6 persons ± 3.3 standard deviation (SD). Most of the respondents were either primarily farmers (40.0%) or unemployed (35.7%), while almost one-fourth (24.3%) were involved in private or governmental work. All households had access to piped water for drinking purpose, food preparation, hand washing, and cleaning. The majority of households faced frequent shortages of water; only a few (14.3%) households reported never or rarely having interruptions to their water supply. Of the 70 studied households, 60.0% also owned other domestic animals such as sheep, goats or horses. The number of cattle owned by households ranged from 1 to 60.

Prevalence of leptospirosis in cattle

Urine samples were collected from 168 cattle across the

Table 1. Demographic characteristics of the investigated peri-urban households in Addis Ababa and their respondents (N=70), 2019.

Demographic characteristics	Category	Number	Percentage
Gender	Male	42	60.0
	Female	28	40.0
Age	Below 30 years old	15	21.4
	30-60 years old	37	52.9
	More than 60 years old	18	25.7
Marital status	Married	43	61.4
	Single	16	22.9
	Divorced/Widowed	11	15.7
Education	No formal schooling	14	20.0
	Elementary school	29	41.4
	High school	14	20.0
	College	8	11.4
	University	5	7.2
Occupation	Primarily farmer	28	40.0
	Unemployed / Retired / Student	25	35.7
	Private / Government job	17	24.3
Animal husbandry	Only cattle	28	40.0
	Two or more livestock species	42	60.0
	Sheep	22	31.4
	Goat	8	11.4
	Equine	22	31.4
	Pigs	0	0
Available facilities	Electricity	70	100
	Telephone	69	98.6
	Radio or Television	69	98.6
	Piped water	70	100
	Toilet		
	Pit latrine with cement slab	54	77.1
	Pit latrine without cement slab	7	10.0
	Flush	3	4.3
	No toilet	6	8.6

70 households. Of these, three were positive for pathogenic *Leptospira* by real-time PCR (1.8%) (Figure 1). The three positive samples were all found in Yeka sub-city. Two positive samples came from different cattle within the same household, but the samples were taken on a different day. The positive samples were considered to be *L. borgpetersenii*, based on the melting curve, when compared with the positive controls (Figure 2), although it is difficult to distinguish species solely on the basis of the *lfb1* gene amplification product (Bourhy et al., 2011).

Knowledge, attitudes and practices

The majority (97.1%) of respondents had never heard about leptospirosis. Nevertheless, 85.9% of the households knew that animals could be a source of human infection and were able to mention how: by direct contact with animals, eating raw milk and meat, touching urine or birth products of cattle, or by rats. More than 60% of the households knew that urine from cattle could contain pathogens which can affect humans. Similarly,

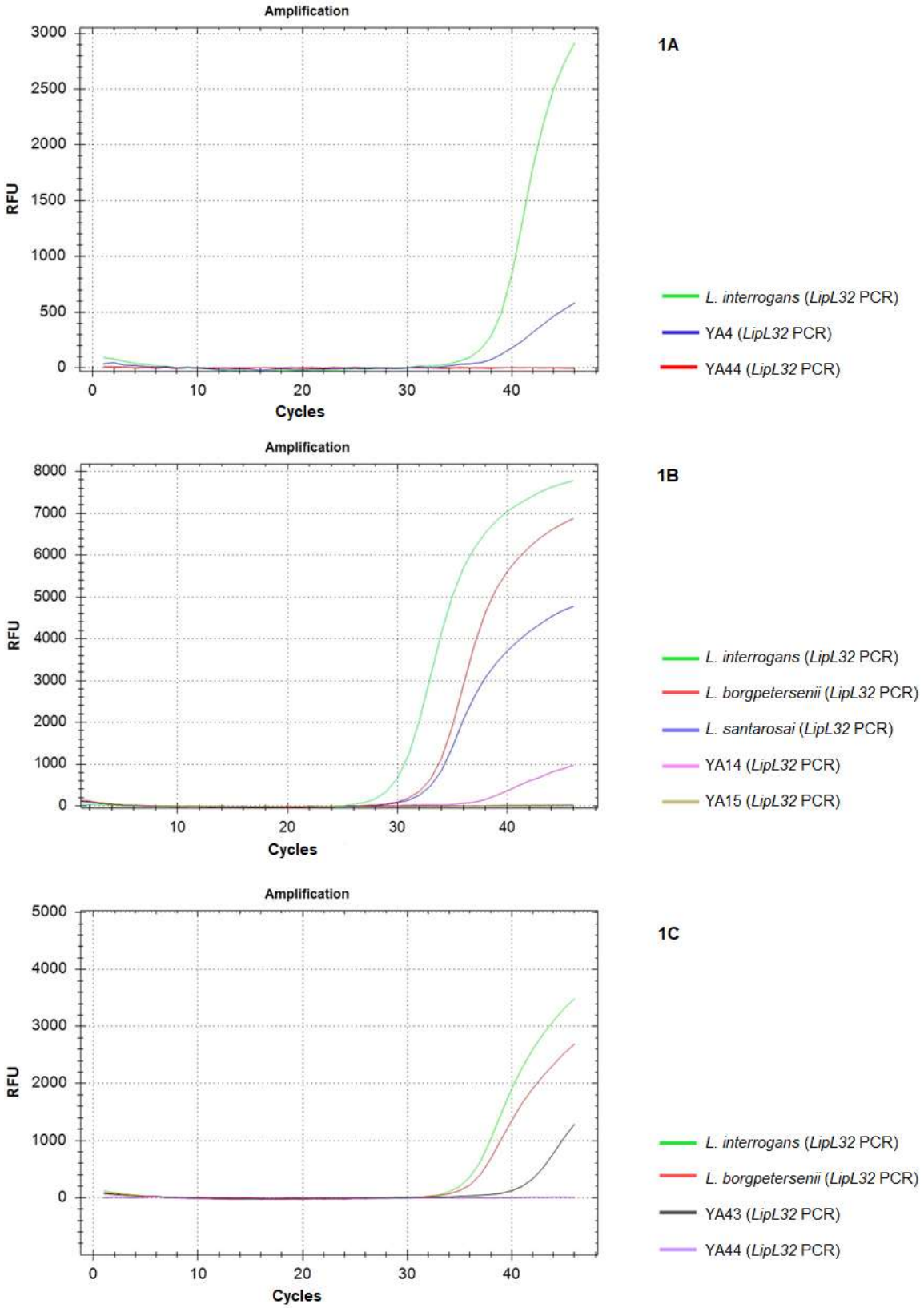

Figure 1. Leptospiral DNA detected in urine from 3 cows in peri-urban areas of Addis Ababa, Ethiopia, after being subjected to the lipL32 PCR reaction with TaqMan probe. Hydrolysis of the lipL32 specific TaqMan probe confirms the presence of *Leptospira* species in sample YA4 (1A), YA14 (1B) and YA43 (1C). RFU = Relative Fluorescence Unit.

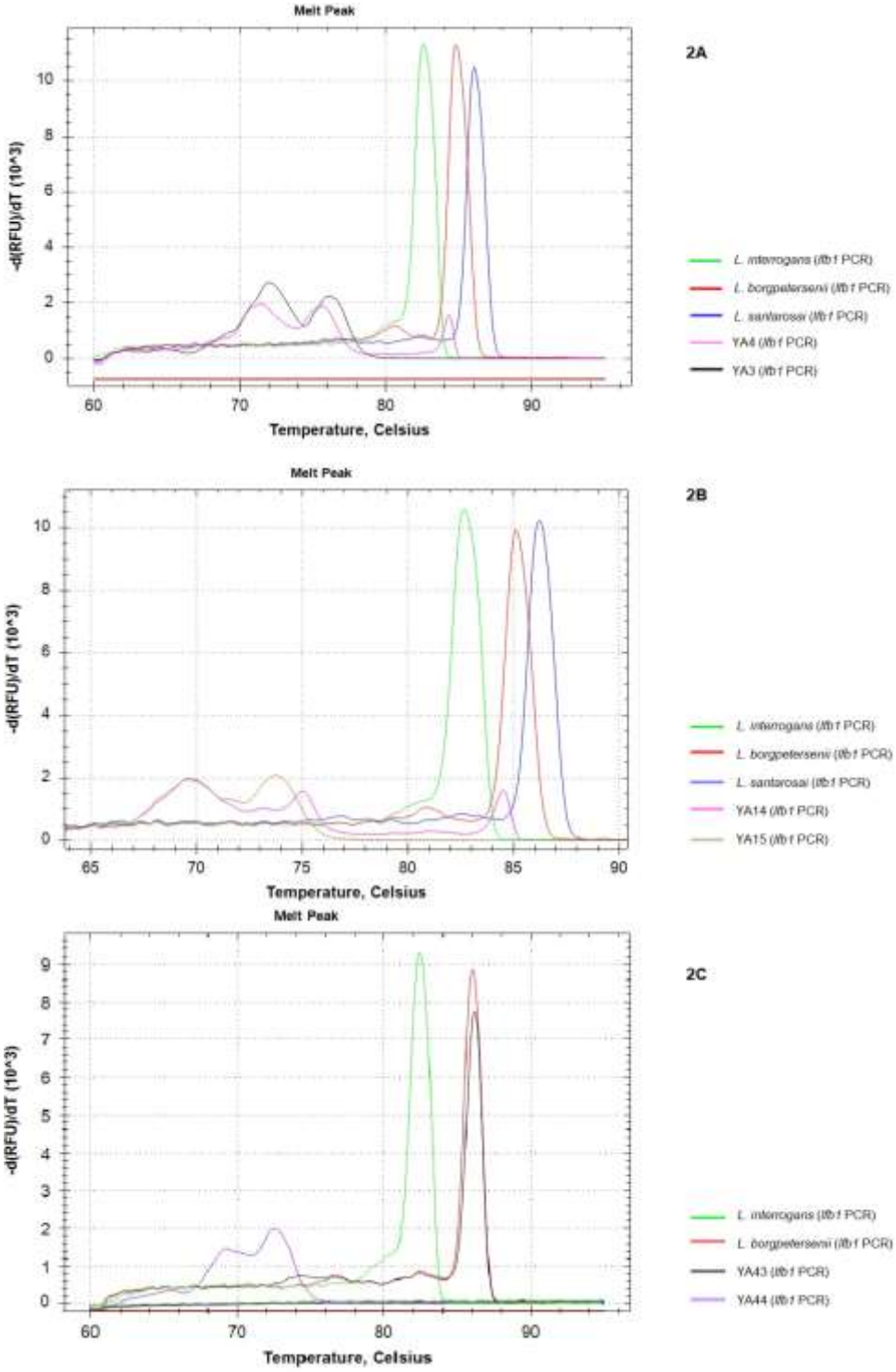

Figure 2. Melting curve analysis of DNA detected in urine from 3 cows sampled in peri-urban areas of Addis Ababa, Ethiopia, after being subjected to the previously described PCR reaction, using lfb1 primers in the presence of Evagreen. The melting curves of the *Leptospira interrogans*, *Leptospira borgpetersenii* and *Leptospira santarosai* positive control DNA samples are shown. The positive urine DNA samples YA4 (2A), YA14 (2B) and YA43 (2C) have a similar melting temperature with the *Leptospira borgpetersenii* control DNA sample. The fact that the melting peaks of urine DNA sample YA4 (2A) and YA14 (2B) are lower than the *Leptospira borgpetersenii* control DNA sample can be explained by the lower concentration of *Leptospira* in sample YA4 and YA14 than in the positive DNA control sample. RFU = Relative Fluorescence Unit.

Table 2. Univariate logistic regression analysis of explanatory variables for *Leptospira* positivity in peri-urban households of Addis Ababa, 2019.

Explanatory variable	N = 70 households		p-value
	Positive (%)	Negative (%)	
Knew that animals can be source of diseases	2 (100)	53 (77.9)	0.998
Knew about diseases transmitted by rats' urine	1 (50)	42 (61.8)	0.718
Knew about diseases transmitted by cattle's urine	0 (0)	42 (61.8)	0.998
Wet areas around the house	1 (50)	43 (63.2)	0.701
Walking through the water with animals	0 (0)	16 (23.5)	0.998
Two or more livestock species	2 (100)	40 (58.8)	0.998
Rats inside the house	1 (50)	46 (67.6)	0.586
Using rat traps or poison	0 (0)	30 (44.1)	0.998
Protective measures after dealing with diseased animals	2 (100)	55 (80.9)	0.998
Washing hands after dealing with animal excretions	1 (50)	44 (64.7)	0.535

rat's urine as a source of infection for humans was recognized by 62.3%. Nearly 70% of households reported that they frequently saw rats inside their houses and rat poison/traps were used by almost half (43.5%) of the households. Because leptospirosis is known to be transmitted by standing water contaminated by urine from domestic animals and rats, households were also asked about wet areas around their houses. More than half (61.4%) of the households stated that they had areas around the house that were wet, with more households (76.8%) reporting this during the rainy season. More than half (52.2%) of the respondents walked frequently in the wet areas around the house without shoes or with open-toed shoes. Walking with the animals through water happened in almost a quarter (23.2%) of the households. Water as a potential source of diseases was recognized by 82.9% of the households. Households were asked for symptoms that could be attributed to leptospirosis: 26.9% had seen fever in one of the household members during the last month, while kidney diseases, jaundice and bleedings were seen less often (9.0, 1.5 and 4.4%, respectively).

Finally, participants were asked how they managed sick animals. Animal health issues were resolved with veterinary assistance (92.4%) and/or by the households themselves (37.7%). Washing hands after dealing with animal excretions was practiced by around two third of the households (70.3%). Protective measures such as gloves or hand washing after dealing with diseased animals were reportedly practiced by 89.1% of the households.

Household-level risk factors for leptospirosis

Table 2 summarizes variables included in the univariable analysis. None of the 10 selected explanatory variables were significantly associated with cattle *Leptospira* positivity (p-value > 0.05).

DISCUSSION

This study is the first of its kind to detect pathogenic *Leptospira* by molecular methods in cattle in Ethiopia. The study confirms the presence of pathogenic *Leptospira* in peri-urban areas of Addis Ababa. Considering the large cattle population and high human-animal interaction in Ethiopia, this study provides important information on a potential threat for public health, as presence of pathogenic *Leptospira* in urine implies spread into the environment. Environmental contamination and exposure to animal excretions is the cause of leptospirosis infections in both animals and humans (WHO, 2003; Hartskeerl et al., 2011). The presumable identification of *L. borgpetersenii* is consistent with *Leptospira* species identified in cattle in Africa (Allan et al., 2015; Dreyfus et al., 2017). This study also confirms that a molecular assay targeting the *lipL32* and *lfb1* gene can be used to detect the presence of *Leptospira* in the urine of cattle.

The prevalence of leptospirosis in cattle in this study was 1.8% (3 positive samples out of 168 cattle tested). Comparing this study with previously published studies on *Leptospira* in Ethiopia is difficult because these studies used serological tests (Tsegay et al., 2016; Desa et al., 2021, Marami et al., 2021). The present study detected *Leptospira* DNA directly which reflects current or recent infection rather than cumulative exposure. Cattle may shed *Leptospira* intermittently in urine for up to 18 weeks (Rocha et al., 2017; Hamond et al., 2022) and thus PCR of urine is considered a useful, non-invasive diagnostic modality, particularly when understanding environmental contamination is of interest. One of the largest recent studies on urinary shedding of pathogenic *Leptospira* in cattle was done in New Zealand, where the urine of 4000 cattle was tested by real-time PCR and found a prevalence (2.4%) similar to ours, although majority of their cattle population was vaccinated and both environmental and cattle characteristics differ from the Ethiopian or African context (Yupiana et al., 2020).

The few published studies using molecular assays in cattle in African countries have shown similar or slightly higher prevalences than ours. In Egypt, leptospiral DNA was detected in 1.1% of 625 cows (urine or blood) (Samir et al., 2015). In Eastern Africa, *Leptospira* prevalences of 8.8% (kidney and/or urine) and 5.8% (only urine) were observed in Uganda (Alinaitwe et al., 2019) and 7.1% (kidney tissue) in northern Tanzania (Allan et al., 2018). A substantial higher prevalence was detected in South Africa, where *Leptospira* DNA was detected in kidney tissue samples of 26.9% (slaughtered) cattle (Dogonyaro et al., 2023), which could be related to the testing of kidney tissue instead of urine and to environmental factors.

Risk factors for leptospirosis in animals are not well characterized in Africa, although some common risk factors have been described. This includes exposure to rats, presence of other (reservoir) animals, pasture grazing and walking through rivers (Schoonman and Swai, 2010; Ngugi et al., 2019; Desa et al., 2021). These risk factors were also present in our study area. Studies concerning knowledge, attitudes and practices of people regarding leptospirosis have mainly been performed in South-America and Asia in areas where leptospirosis is known to be endemic and peoples' awareness might be higher than in Africa (De Araújo et al., 2013; Ricardo et al., 2018; Palma et al., 2022). Despite this, it is remarkable that majority of the respondents in our study area had not heard about the disease leptospirosis. However, people in a majority of the studied households were aware that contact with environmental water, rat's urine or cattle urine and excretions could transmit diseases. The majority of the households took protective measures, like using gloves, washing hands and asking for veterinary assistance. The presence of some knowledge regarding transmission of diseases and the presence of many risk factors reflects the gap between knowledge and daily practice concerning leptospirosis. This is consistent with previous reports from Ethiopia on leptospirosis and zoonotic diseases in general (Desa et al., 2021; Alemayehu et al., 2021). No significant relationship was found between the positive households and the investigated risk factors for zoonotic diseases and leptospirosis, although many of the commonly recognized risk factors were present in the households. The absence of statistical significance does not imply that non-significant potential factors pose no risk as the low prevalence observed in this study resulted in low statistical power for the logistic regression analysis.

Strengths and limitations of the study

This study is the first to investigate pathogenic *Leptospira* in peri-urban areas of the capital city of Ethiopia, where there is little knowledge about animal reservoirs of pathogenic *Leptospira* spp. The study responds to previous calls to investigate the presence of leptospirosis and pathogenic *Leptospira* in Ethiopia (Pieracci et al., 2016; Tulu, 2020). Even though the estimated prevalence was low (1.8%), this study has implications for public health given the zoonotic potential of pathogenic *Leptospira*. The detection of pathogenic *Leptospira* by a molecular assay is also the first of its kind in Ethiopia for detection of *Leptospira* in any species. In cattle, PCR on urine samples is more useful than serological tests, given that many shedders and carriers will not be detected through serology (Monti et al., 2023). This study confirms that a PCR assay with melting curve analysis – targeting the *lfb1* and *lipL32* gene and performed on the DNA extracted from the urine of cattle – can be used as a diagnostic method to detect pathogenic *Leptospira*. The DNA isolation method was tested by the investigators prior to this study with urine samples of known leptospirosis patients, which revealed positive PCR-results with both the lfb1 and lipL32 assay. Even at relatively low bacterial load levels (Ct values > 32), a distinct leptospiral specific PCR product with a melting peak of > 80°C was observed. Additionally, this study provided insights into the lack of awareness of leptospirosis in Ethiopian cattle-keeping households and presence of potential exposure pathways in peri-urban areas of Addis Ababa. These findings indicate that animal and human exposure to pathogenic *Leptospira* species is likely in peri-urban areas of Addis Ababa.

Nevertheless, there are several limitations which should be mentioned. The prevalence estimated in this study may underestimate the true prevalence given the low urine volume examined which may have affected the quantity of DNA and the fact that every animal was only sampled once, potentially missing intermittent shedding (Monti et al., 2023). Additionally, although the DNA isolation method was tested by the main investigators prior to the study on human urine samples, bovine urine samples were not available to test the effectiveness of our DNA extraction method. Furthermore, serological tests like microscopic agglutination test (MAT) would have provided additional information on *Leptospira* exposure of cattle in Addis Ababa and culture techniques would have been useful for providing further information on the leptospiral serovar. The use of more than one type of assay to detect *Leptospira* in urine from naturally infected cattle could have revealed a higher prevalence (Nally et al., 2020). Differentiation based on melting temperature (Tm) allows to differentiate between the most common pathogenic species, but is not able to unambiguously differentiate between *L. borgpetersenii* and other pathogenic species (Bourhy et al., 2011). Further proof of identification would require isolation of the bacteria and characterization by sequencing of the 16S ribosomal RNA gene.

Conclusion

Overall, this study confirmed the presence of pathogenic

Leptospira in cattle in peri-urban areas of Addis Ababa. Analysis of knowledge, attitude and practice among the households revealed that knowledge about leptospirosis is low and that exposure pathways for leptospirosis are widely present.

Further studies should investigate the presence of pathogenic *Leptospira* in cattle on a wider scale, as Ethiopia has the highest cattle population in Africa and cattle is the dominant species in most households. The presence of pathogenic *Leptospira* in cattle's urine indicates contamination of environment and potential exposure of humans. Further studies should therefore take other components of the "One Health" approach into consideration to understand the human and environmental burden in Ethiopia. This study highlights the need to educate cattle-keeping households and responsible veterinary and health professionals about the presence of pathogenic Leptospira.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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SUPPORTING INFORMATION
S1 Appendix. Questionnaire

QUESTIONNAIRE - Assessment of Knowledge, Attitudes and Practices

This questionnaire will take approximately 20 minutes to answer. Any information you provide will be anonymous and no personal information collected will appear in any documents or reports based on this survey.

Interview date:_____/____/_____(DD/MM/YYYY) Interviewer name: _____

_____

Sub-city: _____ Woreda:_____Household: _____

Respondent Status:

⎦ Female head of household ð Male head of household ð Other adult (>18)

Questions related to demographic characteristics of the household

⎦ Male ð Female

1. Sex:

2. Marital status:

⎦ Single ð Married ð Divorced ⎦ Widowed

3. Age: _____
4. What is the highest educational level you have attained?

⎦ No formal education ⎦ Read and write ⎦ Elementary
⎦ High school ⎦ College level ⎦ University level

5. How many people (including children) are in your household? _____

6. What is your occupation?

⎦ Employed: A) Government B) Private C) NGO ⎦ Builder/carpenter
⎦ Farmer ⎦ Unemployed/retired/housewife

7. Does your household have:

⎦ Electricity ⎦ Television / Radio
⎦ Refrigerator ⎦ Telephone/Mobile phone

8. What kind of toilet facility do members of your household usually use?

⎦ Flush, connected to latrines ⎦ Pit latrine with cement slab
⎦ Pit latrine, without cement slab ⎦ Canal or open defecation/bush/field
⎦ Other (please, specify)

9. What is the main water source of the household for the following activities?

	Piped water	Surface water	Ground/bore-hole	Rain water	River water	Bottled water
Drinking water for household						
Drinking water for animals						
Water for food preparation						
Water for cleaning house and utensils						
Water for hand washing and laundry						

10. Are there any times during the year when water is not readily available?

⌋ Yes: (please specify when) ⌋ No

11. Which of the following animal species do you have?

Animal species	Number of animals
Cattle	
Goat	
Sheep	
Pig	
Horse/Donkey/Mule	
Chicken	
Others (specify)	

Questions related to the knowledge, attitude and practices of households regarding leptospirosis, risk factors for leptospirosis and diseases in general

12. o you think that animals can be a source of human diseases?

Yes | No

If yes, how can humans get a disease from animals?

Direct contact with animals	Eating raw or undercooked meat/milk products
Touching urine of animals	Drinking raw or under boiled milk
Other (please, specify)	

13. Have you heard of diseases that you can get from contact with water?
⌋ Yes ð No

14. Have you heard of diseases transmitted by rat's urine to humans?
⌋ Yes ð No

15. Have you heard of diseases transmitted by urine of cattle to humans?
⌋ Yes ð No

16. Did you hear about a disease called leptospirosis?
⌋ Yes ð No
If yes, how did you hear about it? …….

17. If you suspect an animal having a disease, what do you do?
Seek veterinary assistance | Slaughter the animal
| Treat the animal self | Do nothing
| Sell the animal | Others (please, specify)

18. Do you take any specific action to protect yourself when dealing with a diseased animal?
Yes | No |
If yes, what kind of action (s) do you take?
Use gloves Wash hands
Others (please, specify)

19. Do you wash your hands with soap after contact with animals or their milk, manure or urine?
Yes No

20. The following symptoms can be found when someone has leptospirosis, which you can get from contact with water or urine of cattle or rats. Which of the following symptoms did you see in you or your family during the last month?

	Yes	No	I don't know
Fever			
Kidney diseases			
Jaundice			
Bleeding			

21. Indicate if you agree with the following statements:

	Yes	No	Sometimes
The area around my house is wet			
The area around my house is wet during the rainy season			
I walk without shoes or with open shoes through wet areas around the house			
I or my family walks through the water with the animals			
Rats come inside the house			
I use rat traps or rat poison around my house			

This is the end of the questionnaire. Thank you for agreeing to take part in this valuable study. Please feel free to mention any additional comments regarding the study or information you provided.

S2 Appendix. Real-time PCR using *Leptospira* specific lipL32 and lfb1 PCR detection assays

Real-time PCR using *Leptospira* specific lipL32 and lfb1 PCR detection assays

Developed by MRC-Holland and AMC, Department of Medical Microbiology and Infectio n Prevention, Expertise Centre for Reference and Research on Leptospirosis / OIE Reference Laboratory for Leptospirosis in the Netherlands.

1. Lfb1 PCR involving an Evagreen Real-Time PCR assay, in which a possibly correct lfb1 PCR product is revealed by a specific melting curve with a Tm of more than 80˚C, also allowing species identification.
2. LipL32 PCR detection involving a TaqMan probe hydrolysis assay that specificall y

detects the Real-Time formation of a lipL32 PCR product. No species identification possible.

PCR reactions

Per reaction:

1. 20 µl mix containing 0.3 µl Salsa polymerase and 19.7 µl of the mastermix

2. 5 µl of the extracted DNA sample is added to this 20 µl mix

Used primers, reverse primers and probes:

Lfb1

LFB1-F 5′-CATTCATGTTTCGAATCATTTCAAA-3′ LFB1-R 5′-GGCCCAAGTTCCTTCTAAAAG-3′
LipL32

LipL32-47Fd 5’-GCATTACMGCTTGTGGTG-3 LipL32-301Rd 5’-CCGATTTCGCCWGTTGG-3’

Controls:

A negative control with PCR-grade water was always used with the samples.
Purified leptospiral DNA control samples and patient urine, blood and serum samples were provided by the Expertise Centre for Reference and Research on Leptospirosis / OIE Reference Laboratory for Leptospirosis in the Netherlands.

	µl
10x SALSA PCR buffer	2.5
LFB1-F (100 µM)	0.1
LFB1-R (100 µM)	0.1
Evagreen	1
dNTPs (4mM)	1.2
DNA	5
SALSA TAQ polymerase	0.3
H_2O	14.8000
Total volume	25.0000

LipL32 PCR Reaction with TAQ-Man probe and degenerate primers

	µl
10x biolabs buffer	2.5
LipL32-47Fd (100 µM)	0.175
LipL32-301Rd (100 µM)	0.175
LipL32 Probe (50 µM)	0.075
dNTPs (4mM)	1.2
DNA	5
SALSA TAQ polymerase	0.3
H_2O	15.5750
Total volume	25.0000

CFX96 real-time PCR detection system (BIO-RAD) with BIO-RAD software

Biorad PCR detection system settings: Lid 105 °C. 4/10°C per cycle.
Step 1 95˚C for 1 min

Step 2 95°C for 0:10 min

Step 3 58°C for 0:30 min

Step 4 72°C for 0:30 min

Step 5 45 times

Step 6 40°C for 3:00 min

Step 7 40°C for 0:05 min

Step 8 95°C = END

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Effect of sanitizers combined with ultrasound on adhesion of microorganisms isolated from fish from the Amazon region

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Attachment of undesirable microorganisms to surfaces that contact food is a source of concern, since it can result in product contamination leading to serious economic and health problems. Bacteria aggregated to form biofilms are more resistant to environmental stress than planktonic cells. The objective of this paper was to evaluate the bactericidal effect of sodium hypochlorite and peracetic acid associated with ultrasound (40 Hz) to control the adhesion of *Staphylococcus aureus*, *Staphylococcus hominis,* and *Pseudomonas aeruginosa* isolated from two fish species from the Amazon region: butterfly peacock (*Cichla ocellaris*) and piramutaba (*Brachyplatystoma vailantii*). After incubation at 30°C for 24 h, stainless steel coupons were treated for 10 min by different concentrations of sodium hypochlorite (50, 100 and 150 mg/L) and peracetic acid (40, 60 and 80 mg/L) at 25°C. The sodium hypochlorite (150 mg/L) and peracetic acid (80 mg/L) treatments were also combined with ultrasound (40 Hz) for 10 min at 25°C. The results showed that the recommended treatment based on this study was the use of peracetic acid combined with ultrasound.

Key words: Sanitizer, adhesion, *Staphylococcus aureus*, *Staphylococcus hominis, Pseudomonas aeruginosa.*

INTRODUCTION

The surfaces that come into contact with foods are important sources of microbial contamination in food-processing plants, which may be associated with food quality and safety (Vogel-Fonnesbech et al., 2001). This happens because some pathogenic bacteria are able to adhere to food-contact surfaces and remain viable even after cleaning and disinfection (Ammor et al., 2004). One of the most common ways for bacteria to live is by adhering to surfaces and forming biofilms in which they are embedded in an organic extracellular polymeric matrix (Chae and Schraft, 2000).

The surface characteristics of the microorganisms themselves and the various environmental conditions encountered in agri-food industries (organic materials, pH, temperature, water activity, etc.) influence microbial attachment to inert surfaces (Giovannacci et al., 2000; Gross et al., 2001). Adhesion of undesirable microorganisms to these surfaces is a source of concern, since it can result in product contamination leading to serious economic and health problems.

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According to Costerton et al. (1999), biofilms are cell aggregates embedded in an organic extracellular polymeric matrix that confers resistance to the microorganisms involved. Bacteria aggregated to form biofilms are more resistant to environmental stress than their planktonic counterparts, including sensitivity to sanitizers (Fux et al., 2004; Spoering and Lewis, (2001).

To remove biofilm organisms, the sanitizing solution must penetrate the exopolymer matrix and gain access to the microbial cells, which causes biofilm inactivation and removal. Chlorinated products such as hypochlorite salts (Meyer, 2003; Srey et al., 2012) constitute the most widely used group of sanitizing compounds. However, there has been some concern regarding the use of hypochlorite and other chlorine salts considered precursors in the formation of organic chloramines, which are harmful to health due to their high carcinogenic potential (Andrade, 2008).

In order to reduce the incidence of microorganisms in foods, the industry has used several sanitizers such as chlorates, peracetic acid (PAA), and quaternary ammonium, among others.

The most widely used chlorate compounds are: sodium hypochlorite (NaClO), lithium hypochlorite, calcium hypochlorite, and chlorine dioxide (inorganic) and chloramine-T, dichloramine-T, dichloroisocyanuric acid, and dichloro dimethyl hydantoin (organic) (Srebernich, 2007).

NaClO in aqueous medium dissociates into hypochlorous acid and hypochlorite. The bactericidal power of the chlorate compounds is usually based on the release of hypochlorous acid in its non-dissociated form when in aqueous solution, except for chlorine dioxide, which does not hydrolyze in aqueous solution and the whole molecule is considered the active agent (Andrade, 2008).

The use of hypochlorite and of the other chlorine salts considered precursors in the formation of organic chloramines has raised a lot of concern since they are harmful to health due to their high carcinogenic potential (Andrade, 2008). Thus, several sanitizing agents have been proposed to replace NaClO.

The use of PAA has many advantages when compared with NaClO (Kunigk and Almeida, 2001). One important advantage is that it does not produce toxic residues when decomposed and therefore does not affect the final product or the waste treatment process. PAA can be used over a wide temperature spectrum (0 to 40°C) in clean-in-place (CIP) processes (Leaper, 1984). PAA can also be used with hard water and protein residues do not affect its efficiency. Up until now, no microbial resistance to PAA has been reported and it is efficient over a wide pH range (3.0 to 7.5) (Block, 1991; Lenahan, 1992).

Ultrasound (US) was adopted by the electronic industry to decontaminate surfaces and its use has recently been recommended as an alternative sanitization step in the food industry (Nascimento et al., 2008; Adekunte et al., 2010; Cao et al., 2010; Sagong et al., 2011). When applied to liquids, ultrasonic waves promote cavitation, which consists on the formation, growth, and collapse of air bubbles. These bubbles generate localized mechanical and chemical energies that are capable of inactivating microorganisms such bacteria as virus (Valero et al., 2007; Gogate and Kabadi, 2009; Adekunte et al., 2010). Bubble collapse causes pressure changes, which is considered the main cause of microbial cell disruption (Patil et al., 2009). US has been frequently studied in research aimed at interrupting the biofilm or even at inactivating microorganisms.

The objective of this paper was to evaluate the bactericidal effect of NaClO (50, 100, and 150 mg/L) and PAA (40, 60, and 80 mg/L) associated with US (40 Hz) to control the surface adhesion of *Staphylococcus aureus*, *Staphylococcus hominis*, and *Pseudomonas aeruginosa* isolated from fish species from the Amazon region.

MATERIALS AND METHODS

Bacterial strains

The pure cultures were isolated from fish species butterfly peacock [*Cichla ocellaris*] and piramutaba [*Brachyplatystoma vailantii*] from the Amazon region. The bacteria were isolated through seeding in Agar surface using violet red bile glucose (VRBG) (Kasvi, Spain) for *P. aeruginosa* strains and Baird-Parker with egg-yolk tellurite (Kasvi, Spain) for *S. aureus* and *S. hominis*, both with incubation at 36°C/48 h. Next, colonies were selected to be striated in VRBG or Baird-Parker agar plates to obtain pure cultures. After another incubation at 36°C/48 h, these colonies were transferred to BHI (brain-heart infusion) with 10% glycerol (Kasvi, Spain) and stored in a freezer to be used in further tests.

The bacteria isolated were previously identified with Gram stain tests. Next, *P. aeruginosa* strains were identified using the API 20E kit (Enterobacteria) while *S. aureus* and *S. hominis* strains, with the API Staph kit (Staphylococci). This procedure was in accordance with the manufacturer's recommendations (Biomérieux, France) (Harrigan, 1998).

Surface

Stainless steel coupons (6 cm^2) were used as test surfaces. The coupons were individually cleaned and sterilized according to Marques et al. (2007).

Adhesion to surfaces, quantification of adhered cells and sanitizers application

Strains were reactivated in nutrient broth for 72 h at 36°C and replicated to another nutrient broth (Himedia, India) for 24 h at 36°C. Then, 2 mL of activated contents were transferred to 300 ml of a new nutrient broth, in which the stainless-steel coupons were immersed and incubated at 30°C/24 h. After this last incubation period, the population density (planktonic cells) in the bacterial suspension was estimated using nutrient agar (Himedia, India). Next, all coupons were aseptically removed, rinsed three times with sterile distilled water to remove unattached cells, and dried in a laminar flow cabinet (DELEQ MA1500/90) for 30 min.

Afterwards, the coupons were immersed in sterile distilled water at 25°C (control group) for 10 min. Finally, microorganisms were

Table 1. Population density of planktonic and sessile cells of the tested organisms in nutrient broth with stainless steel coupons.

Bacteria	Planktonic cells (log CFU/mL)	Sessile cells (log CFU/cm^2)
S. aureus	7.09±0.05^a	4.11±0.09^a
S. hominis	6.82±0.78^a	4.95±0.85^b
P. aeruginosa	6.99±0.17^a	4.14±0.17^a

Means followed by the same letter in the column did not differ by Tukey's test at 5% probability. Values represent the mean of three repetitions.

quantified using a swab rubbed 20 times onto two coupons and then immersed in 0.1% peptone water for subsequent plating.

The remaining coupons were immersed for 10 min in sanitizer solutions at 25°C at three different concentrations: commercial NaClO (50, 100, and 150 mg/L) and PAA (40, 60, and 80 mg/L). Two coupons were used for each sanitizer concentration. The sanitizing effect was neutralized with the aid of a 0.1% sodium thiosulfate solution. The microorganisms were quantified using the swab technique.

All coupons were plated in duplicate on nutrient agar and incubated at 37°C for 48 h. Next, the NaClO and PAA concentrations that obtained the greatest decimal reduction in the microorganism population were associated with the US treatment in an ultrasound bath (QSONICA Q700) for 10 min following the same procedures mentioned earlier.

The effectiveness of the disinfectant agent expressed as germicidal effect or decimal reduction (DR), was determined by the equation:

$$DR = \log N_i - \log N_f \quad (1)$$

where N_i is the cell count in the control group (no sanitizer treatment) (CFU/cm^2) in nutrient agar and N_f is the count after exposure to sanitizer.

Reproducibility and statistical analysis

All analyses were carried out in duplicate with three repetitions on separate occasions, and the results are expressed as the average of the assays. Counts were converted into decimal logarithmic values (log CFU/cm^2). The test results before and after sanitizer application were compared using Tukey's test. Data were analyzed using the software Statistica 7.0. A probability value $p<0.05$ was accepted as indicating significant differences.

RESULTS

Bacterial adherence to surfaces

The population density of the bacterial suspensions in nutrient broth is not significantly different ($p>0.05$) after 24 h/30°C. However, the number of *S. hominis* cells adhering to stainless steel coupons was higher ($p<0.05$) compared to the species *S. aureus* and *P. aeruginosa*, which shows their greater adhesion capacity in the test conditions (Table 1).

Effect of sanitizers

Counts of *S. aureus, S. hominis*, and *P. aeruginosa* cells adhered to stainless steel surfaces after application of PAA (40, 60, and 80 mg/L) and NaClO (50, 100, and 150 mg/L) are as shown in Figures 1 to 3. These results are significantly different ($p<0.05$) when submitted to analysis of variance (ANOVA) (Table 2).

The different NaClO concentrations (50 to 150 mg/L) yielded a significant difference ($p<0.05$) when applied to adhered cells of *S. aureus* and *S. hominis*, reaching reductions between 1.57-2.20 and 1.52-2.35 log cycles, respectively. The application of PAA (40-80 mg/L) yielded reduction values between 2.09-2.64 log cycles for *S. aureus* and 3.22-4.34 for *S. hominis*. These values differed among themselves at a 95% significance level.

DISCUSSION

All species evaluated were able to adhere to stainless steel surfaces, reaching values between 4.11 and 4.95 log CFU/cm^2 (Table 1). Parizzi et al. (2014) found results of approximately 5.0 log CFU/cm^2 for *S. aureus* on stainless steel after 12 h of contact at 30°C. Another study showed that the adhesion of *S. aureus* reached 6.10 log CFU/cm^2 (Silva et al., 2009). Also, Jeromino et al. (2012) showed adhesion of 6.9 log CFU/cm^2 for *S. aureus* on stainless steel after 24 h at 28°C. Krolasik et al. (2010) observed the adhesion of *S. hominis* in stainless steel after incubation for 4 h at 20°C, while Vanhaecke et al. (1990), Cloete and Jacobs (2001), Figueiredo et al. (2009), and Caixeta et al. (2012) also observed the adhesion capacity of *P. aeruginosa* to stainless steel.

The different NaClO concentrations (50 to 150 mg/L) reached reductions between 1.57-2.20 and 1.52-2.35 log cycles (*S. aureus* and *S. hominis*). The application of PAA (40-80 mg/L) yielded reduction values between 2.09-2.64 log cycles for *S. aureus* and 3.22-4.34 for *S. hominis*.

The United States Environmental Protection Agency's (EPA) Scientific Advisory panel has stated that any treatment which can reduce microbial contamination by 2 log cycles is significant (Michaels et al., 2003). That shows that PAA was more efficient than NaClO in reducing *S. aureus* and *S. hominis* populations since lower PAA concentrations (40 mg/L) yielded population reductions equivalent to those observed for the highest

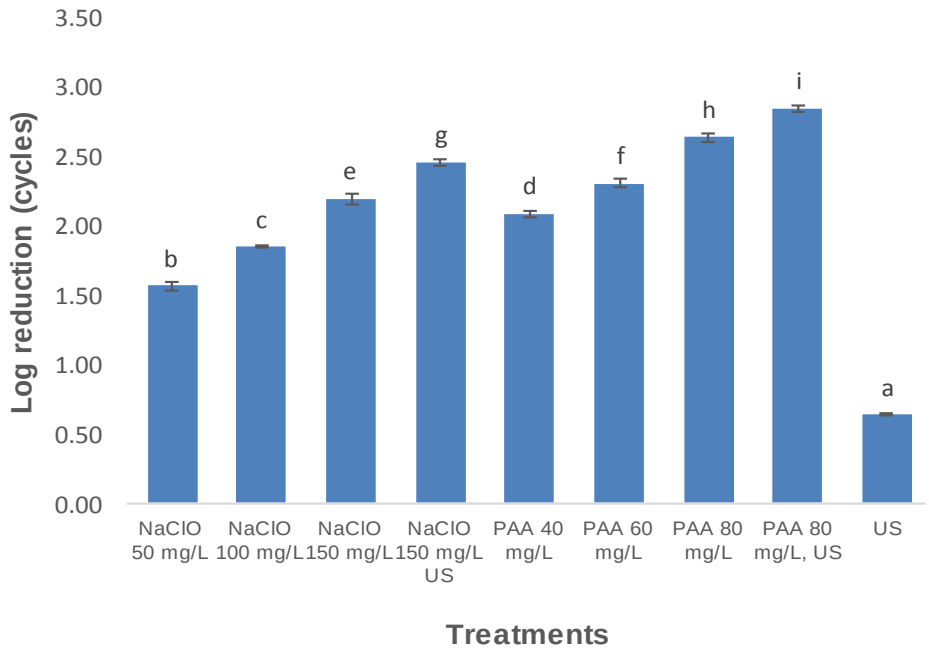

Figure 1. Effect of applying peracetic acid (PAA) and sodium hypochlorite (NaClO) combined with ultrasound (US) to control *S. aureus* adhesion. Treatments indicated with the same letter did not differ (p>0.05) among themselves.

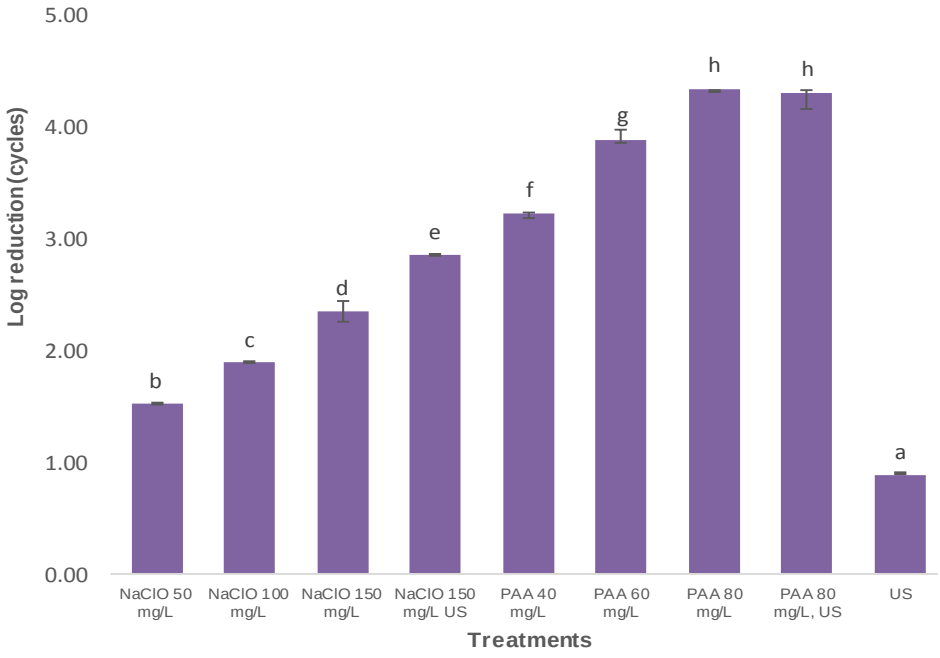

Figure 2. Effect of applying peracetic acid (PAA) and sodium hypochlorite (NaClO) combined with ultrasound (US) to control *S. hominis* adhesion. Treatments indicated with the same letter did not differ (p>0.05) among themselves.

NaClO concentrations (150 mg/L).

When associated with US (40 Hz, 10 min, NaClO (150 mg/L) and PAA (80 mg/L)) allowed the reduction of 2.46 and 2.85 cycles, respectively, of adhered *S. aureus* cells. For *S. hominis*, this association yielded reductions of 2.85 and 4.30 cycles, respectively. In other words, PAA at 80

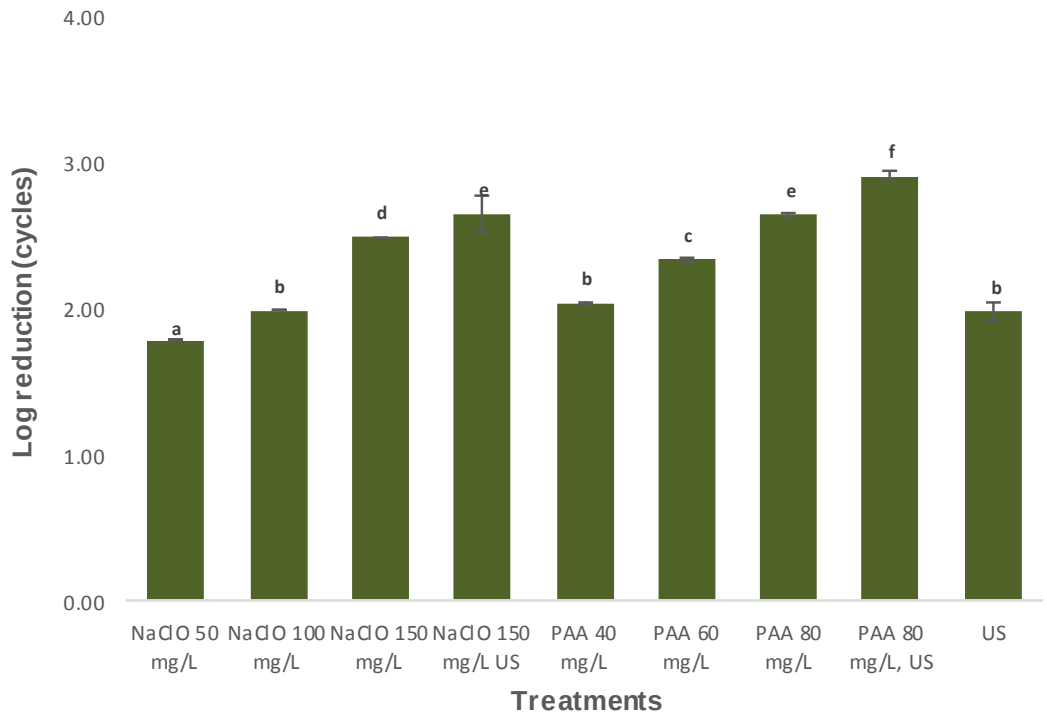

Figure 3. Effect of applying peracetic acid (PAA) and sodium hypochlorite (NaClO) combined with ultrasound (US) to control *P. aeruginosa* adhesion. Treatments indicated with the same letter did not differ (p>0.05) among themselves.

Table 2. ANOVA for sanitizer treatments applied to *S. aureus*, *S. hominis*, and *P. aeruginosa*.

Bacteria/Treatment	SS	S	F	p
S. aureus	10.4888	1,311	1,913.50	0.00
S. hominis	36.6267	4,578	1,334.94	0.00
P. aeruginosa	3.5450	0,433	168.51	0.00

mg/L and NaClO at 150 mg/L associated with US yield significant values (at a 95% level) in the adhesion of *S. aureus* and *S. hominis* compared to the application of these sanitizers alone.

However, US (40 Hz, 10 min) applied alone yielded a reduction of only 0.64 and 0.89 cycles for *S. aureus* and *S. hominis*, respectively. These results suggest that no synergistic or additive effect occurred between the sanitizers (NaClO and PAA) and US in the conditions studied.

Therefore, US might help aqueous sanitizers penetrate inaccessible sites (hydrophobic pockets and folds in leaf surfaces on fruits and vegetables), which makes such sanitizers more effective (Seymour et al., 2002; Gogate and Kabadi, 2009; Sagong et al., 2011).

Studies have combined US with other sanitizers such as organic acids (Sagong et al., 2011), hydrogen peroxide (São José and Vanetti, 2012), and chlorine dioxide (Huang et al., 2006) and have found an additive or even synergistic bactericidal effect compared to the individual treatments (Ding et al., 2015). São José and Vanetti (2015) observed no synergistic effect of applying US with sodium dichlorocyanurate (50 and 200 mg/L) and PAA (40 mg/L) to remove *Salmonella* from cherry tomato surfaces.

In fact, US is a clean technology (Rahman et al., 2010) with potential to be used in bacteria inactivation. However, it is not very effective alone in killing microorganisms in food at ambient or sub-lethal temperatures (Sengül et al., 2011). Microorganism reduction by US is mainly due to the physical phenomenon called cavitation (Alegria et al., 2009; Piyasena et al., 2003; Seymour et al., 2002).

Lee et al. (2014) suggested that the treatment with US alone may not be effective for application in the food industry. Piaysena et al. (2003) reported that bactericidal effects on food treated with US alone is localized and does not affect a large area.

Others studies have examined the inactivation of

pathogenic bacteria by chemical disinfection treatments such as NaClO *in vitro*. Ha and Ha (2012) reported strong resistance of *S. aureus* against NaClO. NaClO has also been reported to be a potential antimicrobial agent against *S. aureus* in biofilm (Toté et al., 2010). Bodur and Cagri-Mehmetoglu (2012) noted that NaClO (250 mg/L) was not efficient in completely removing *S. aureus* cells adhered to stainless steel surfaces. Meira et al. (2012) found similar results when studying *S. aureus* biofilm formation on stainless steel surfaces.

Rossini and Gaylarde (2000) stated PAA has an important advantage because this compound does not pose an environmental risk and does not produce toxic compounds after reaction with organic materials. Marques et al. (2007) confirmed that PAA was the most effective in removing adhered *S. aureus* cells. Meira et al. (2012) reported that PAA (30 mg/L) was more effective than NaClO (250 mg/L) in reducing the viable cell count of *S. aureus* in the biofilm matrix. Vázquez-Sánchez et al. (2014) noted PAA (100-750 mg/L) was more effective against *S. aureus* biofilms and planktonic cells when compared with NaClO (500-1,000 mg/L) treatment.

Nonetheless, more studies on the inactivation of *S. hominis* by the application sanitizers are required given the scarce literature data on the subject.

The different NaClO (50 to 150 mg/L) and PAA (40-80 mg/L) concentrations yielded a significant difference ($p<0.05$) when applied to adhered *P. aeruginosa* cells, reaching reductions between 1.78-2.49 and 2.04-2.64 log cycles, respectively. When associated with US, only PAA yielded reductions (2.91 log cycles) that are significantly different at a 95% level when compared with the treatment with PA alone.

The individual application of US yielded a reduction of 1.98 cycles and can be compared to the efficiency of NaClO (100 mg/L). Moreover, it can be considered an efficient treatment to control the adhesion of this microorganism according to the EPA since it alone yielded a reduction of approximately 2 log cycles.

A synergistic effect ($p<0.05$) was also observed between the treatments with NaClO (150 mg/L) and PAA (80 mg/L) when combined with US.

Herceg et al. (2012) noted that Gram-negative bacteria are more susceptible to the US treatment than Gram-positive ones. Gram-positive bacteria, especially *S. aureus*, usually have a thicker and more tightly adherent layer of peptidoglycan than Gram-negative bacteria, and this morphological feature did seem to be a differentiating factor in ranking the microorganisms according to the percentage of bacteria killed by US treatment.

Conclusion

The results in the present study showed that *S. hominis* is quite sensitive to the treatment with PAA and may reach reductions of up to 4 log cycles. Furthermore, the results showed that the best treatment combination both for *S. aureus* and *S. hominis* and *P. aeruginosa* was PAA at 80 mg/L associated with US. The use of US at 40 Hz to remove adherent *P. aeruginosa* can be considered efficient and has an effect comparable to that of NaClO (100 mg/L).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Growth, root colonization and yield attribute responses of five groundnut (*Arachis hypogaea* L.) varieties toward arbuscular mycorrhizal fungal inoculation in a Senegalese agricultural soil

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The aim of this study was to isolate the most specific and effective arbuscular mycorrhizal fungi (AMF) for groundnut and to determine the degree of variability in the response of groundnut varieties to inoculation. The seeds of five varieties: 55-437, Fleur 11, Sunu Gaal, Amoul Morom, and Essamaay were inoculated individually with five AMF (*Funneliformis mosseae*, *Rhizophagus aggregatus* and *Rhizophagus fasciculatus*, the indigenous isolates, and *Rhizophagus irregularis* and *Gigaspora rosea*, isolated from Canada). Plants were grown under greenhouse conditions in a mixture of non-sterile sandy soil and sterilized soil at 120°C for 20 min (1:1, v/v). The results obtained in terms of root AMF colonization and nodule formation showed a positive effect of AMF inoculation in all varieties. Furthermore, we showed that inoculation efficacy did not depend on the origin of the inoculated AMF and no clear relationship was found between the fact that the varieties used were traditional or modern. However, our data indicated that Amoul Morom, Essamay, and 55-437 were more responsive to AMF inoculation, showing the greatest increase in plant growth, leaf chlorophyll content, and yield parameters. The results therefore confirm the functional variation among the inoculated AMF, which is crucial for establishing potential formulations of AMF inoculants to improve groundnut productivity. According to this study, further selection of compatible AMF partners would be useful to improve inoculation success with Fleur 11 and Sunu Gaal.

Key words: Peanut (*Arachis hypogaea*), bioferlilizers, arbuscular mycorrhizal fungi (AMF), symbiotic performance, plant growth, yield parameters.

INTRODUCTION

Soil microbial communities are involved in several functions in agroecosystems, such as nutrient availability, pathogen control, and resilience to abiotic stresses (Aguégué et al., 2023; Lu et al., 2023; Sharma et al., 2023). Arbuscular mycorrhizal fungi (AMF) are among these important soil-dwelling microorganisms and can

have a strong influence on plant growth and productivity. They form mutualistic associations with over 80% of all vascular plants, affecting plant fitness and competitive interactions (Johnson et al., 1997; Aguégué et al., 2023). They are well known for assisting host plants with phosphorus uptake (Smith and Read, 2008; Lu et al., 2023), but can also provide other benefits such as protection from pathogens (Cardoso and Kuyper, 2006; Sharma et al., 2023), assistance with the uptake of other nutrients such as nitrogen and copper, and improved water relations (Smith and Read, 2008; Sene et al., 2010; Lu et al., 2023; Sharma et al., 2023). AMF hyphae also play a role in the formation and structural stability of soil aggregates (Miller and Jastrow, 2000; Zhang et al., 2023) and contribute to the composition of plant community structures (van der Heijden et al., 2015; Chen et al., 2023). In return, AMF receive photosynthetic products from the host plant (Smith and Read, 2008; Lu et al., 2023).

Soil microorganisms are now being promoted as smart fertilizers for a new green revolution in the 21st century (Sene et al., 2010; Fortin et al., 2015; Lesueur et al., 2016; Mohanty and Swain, 2018; Rocha et al., 2019; Sene et al., 2021, 2023). Microbial inoculants offer low-cost alternatives to expensive mineral fertilizers and provide a means to maintain or improve soil fertility (Hart et al., 2015; Itelima et al., 2018; Begum et al., 2019). A large body of scientific evidence demonstrates not only improved crop yield and resistance to environmental stress in AMF crops, but also improvements in many food quality attributes, such as increased levels of desirable antioxidants, vitamins and minerals (Sene et al., 2010; Calvo et al., 2014; Fortin et al., 2015; Hart et al., 2015; Rocha et al., 2019).

Groundnut, also known as peanut (*Arachis hypogaea* L.), is an important grain legume grown in the tropics and subtropics, including sub-Saharan Africa. It is an important source of oil and protein and also contains vitamin B as well. Groundnut is consumed worldwide for human and animal feeding (Noba et al., 2014). In Senegal, groundnut has been a cash crop for more than a century, contributing to 60% of the country's agricultural gross domestic product (GDP) and about 80% of its export earnings (Sene et al., 2010; Noba et al., 2014). It is the most important oil-producing crop, and the four oil factories established in the country formed the backbone of the national industrial fabric. After a long period of decline, groundnut yields have increased in the last five years. However, the factors that determine these increases, that is, soil fertility, have steadily deteriorated, with a reduction in fallow land and low levels of fertilizer use (Sene et al., 2010). Various agricultural practices, including the use of chemical fertilizers, have been adopted to increase yields and alleviate food shortages. However, the high cost of chemical fertilizer and the need for sustainable alternative sources have increased the strategic importance of microbial inoculation. The study was undertaken to isolate the most specific and effective AMF inoculants for five modern and traditional Senegalese groundnut varieties and to use elite strains as inoculants. Our hypothesis was that the response of groundnut to AMF inoculation would vary between varieties and that this variability would differ between modern and traditional varieties.

MATERIALS AND METHODS

Plant

Five local groundnut (*A. hypogaea* L.) varieties obtained from the Centre National de Recherche Agronomique (CNRA) in Bambey, Senegal, were used in this experiment. These varieties were selected on the basis of the taste desired by the local population and their characteristics are shown in Table 1.

Arbuscular mycorrhizal fungal materials

The arbuscular mycorrhizal fungal (AMF) inoculants used in this study are from the collection of the Laboratoire Commun de Microbiologie (LCM) IRD/ISRA/UCAD, Dakar, Senegal. Three of them (*Funneliformis mosseae*, *Rhizophagus aggregatus* and *Rhizophagus fasciculatus*) are indigenous and isolated from Senegalese soils (Table 2). In this experiment, they were tested against two exotic AMF inoculants (*Rhizophagus irregularis* and *Gigaspora rosea*).

Greenhouse experimental design

The experiment was set up in the greenhouse (Bel Air experimental station, 14°44'N, 17°30'W in Dakar) using a non-sterile soil from Sangalkam, 30 km east of Dakar, mixed with sterilized soil at 120°C for 20 min (1:1, v/v). This soil has a pH of 6.5 with 58.15, 32.8 and 3.6% of sand, loam and clay, respectively and contains 0.06% total N, 0.54% total C, 39 mg P kg^{-1} total P, 4.8 mg P kg^{-1} available P. It was sieved (< 1 mm), homogenized and used to fill up the pots. Seeds of selected groundnut varieties (listed in Table 1) were first surface sterilized (to avoid seed-borne diseases) with 5% sodium hypochlorite (NaOCl) for 5 min, 70% ethanol for 3 min and thoroughly rinsed with sterile distilled water. The seeds were then placed on Petri dishes containing moist filter paper for germination under sterile conditions and kept in the dark at 25°C. The germinated seeds were manually transplanted to a depth of 2-3 cm into 1.5 L plastic pots disinfected with a solution containing 1.81% of calcium hypochlorite and filled with the soil substrate. Two germinated seeds were planted in each pot. The plants were dismantled on the 3rd day after planting and before inoculation to one plant per pot. The pots were arranged in randomized blocks, with a single inoculation and five replications. The pots were placed at 10 and 40 cm within and between the rows for the varieties Fleur 11, 55-437 and Sunu Gaal. The distance between the pots was 10

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Table 1. Characteristics of the groundnut varieties used in the study.

Variety	Type	Growth habit	Growth cycle (days)	Registration in Senegal
Fleur 11	Spanish	Erect	90	Traditional, since 1955
55-437	Spanish	Erect	90	Traditional, since 1993
Sunu Gaal	Spanish	Erect	95	New, since 2017
Essamay	Virginia	Semi-erect	105	New, since 2017
Amoul Morom	Virginia	Semi-erect	120	New, since 2017

Table 2. Characteristics of the arbuscular mycorrhizal fungal (AMF) strains used in the study.

AMF strains	Origin of AMF strain isolation
Funneliformis mosseae (formerly *Glomus mosseae*)	Diokoul -Senegal
Rhizophagus aggregatus (formerly *Glomus aggregatum*) R13	Djignaki - Senegal
Rhizophagus fasciculatus (formly *Glomus fasciculatum*) R10	Kabrousse - Senegal
Rhizophagus irregularis (formerly *Glomus intraradices*)	Exotic, Canada
Gigaspora rosea	Exotic, Canada

and 60 cm for the varieties Amoul Morom and Essamay. The plants were grown for 65 days under greenhouse conditions (temperature of 27-35°C, relative humidity of 70-80% and 12 h of light) and were watered every two days with tap water without added nutrients.

Inoculant preparation and inoculation

The greenhouse experiment was composed of six treatments: three with application of indigenous AMF inoculants compared with two exotic AMF, and a negative control without inoculation for each variety. The AMF inoculants were propagated on *Zea mays* L for 12 weeks under greenhouse conditions on sterilized substrate (soil and sandy 1:1 v/v). For AMF inoculation, 10 g of the substrate containing an average of 40 spores g^{-1} soil and root fragments with 85% of colonized roots length, were placed adjacent to roots of seedlings. Treatments without AMF inoculants received 10 g of autoclaved inoculants in order to avoid differences in soil nutrient content linked to the addition of AMF inoculants.

Collection of growth and yield variables

Data on growth variables (plant height and number of branches) and leaf chlorophyll content for each variety were collected at flowering [30 days after planting (DAP)], pod filling (45 DAP) and pod maturity periods (60 DAP). Plant height (cm) was measured with a ruler from the base of the stem to the apex, while the number of branches was counted manually. Leaf chlorophyll content was quantified at 30, 45 and 65 DAP using a SPAD-502Plus chlorophyll meter (Konica-Minolta). At harvest, whole groundnut plants were uprooted. The soil adhering to the roots was removed under running tap water and nodules were picked and counted. The pods were manually stripped from the plants to record the yield components. For each variety, above-ground and root biomass, root colonization (intensity and frequency of AMF in the roots) and the yield attributes (number of pods per plant, pod weight) were determined. Above-ground and root biomass, nodule weight and the yield attributes were determined by weighing sample parts after over-drying to constant weight at 65°C.

Root arbuscular mycorrhizal fungal colonization

The roots obtained at 65 DAP were washed properly and used to examine the level of AMF colonization. Randomly selected lateral roots, which are more likely to form mycorrhizae, were collected, cleared in KOH [10% (w/v)] at 80°C for 30 min and stained with trypan blue (0.05% (w/v) in 0.8% acetic acid solution) at 80°C for 35 min (adapted from Phillips and Hayman, 1970). Roots were cut into 1-2 cm pieces and placed on slides for microscopic observation (x250). A total of 100 root pieces were taken randomly from each sample. AMF colonization was quantified according to the method of Mcgonigle et al. (1990).

Data analyses

All data were tested for normality and homogeneity using the Shapiro-Wilk and Levene tests, respectively. Data for plant growth and yield parameters were statistically analyzed using univariate analysis with one-way analysis of variance (ANOVA) using the R software v3.4.4 (R Core Team, 2020). AMF colonization data for each treatment and plot were square-root transformed and subjected to a two-way ANOVA followed by Tukey's multiple means tests to analyze how the response variable varied between treatments. Significantly different means were separated using the Tukey (HSD) test at the 5% probability threshold. Means and standard errors are presented throughout and $P < 0.05$ is considered significant.

RESULTS

Mycorrhizal root colonization and root nodulation

Groundnut plants of all varieties were naturally colonized by autochthonous AMF as shown for the control root plants. However, root AMF colonization levels at 65 DAP were generally low in the uninoculated plants, ranging from 9.96 ± 1.84 to 13.06 ± 2.3%. Furthermore, the

Table 3. Root arbuscular mycorrhizal fungal (AMF) colonization and nodulation (number of nodules) of five groundnut varieties (*A. hypogaea*) under single inoculation with AMF 65 days after planting.

Treatments	Groundnut varieties									
	Amoul Morom		Essamaye		55-437		Fleur11		Sunu Gaal	
	IRC (%)	NNum	IRC (%)	NNum	IRC (%)	NNum	IRC (%)	NNum	IRC (%)	NNum
F. mosseae	33.1 ± 2.52^{a}	59.3 ± 4.72^{a}	38.4 ± 4.03^{b}	44.5 ± 4.12^{b}	24.55 ± 3.05^{b}	33.0 ± 4.94^{bc}	18.73 ± 1.16^{b}	68.0 ± 10.7^{ab}	23.46 ± 2.28^{a}	43.8 ± 11.4^{b}
R. aggregatus	27.01 ± 2.47^{b}	34.0 ± 4.82^{bc}	24.6 ± 2.55^{c}	72.8 ± 6.02^{a}	42.73 ± 3.07^{a}	27.8 ± 5.56^{c}	28.6 ± 1.86^{a}	80.3 ± 16.29^{a}	26.95 ± 2.40^{a}	57.8 ± 11.7^{ab}
R. fasciculatus	18.06 ± 2.63^{c}	41.3 ± 4.11^{b}	27.9 ± 2.84^{c}	34.3 ± 6.40^{bc}	21.0 ± 2.66^{b}	37.7 ± 4.62^{bc}	26.31 ± 2.98^{a}	60.8 ± 11.3^{abc}	27.39 ± 2.11^{a}	41.8 ± 9.54^{b}
G. rosea	21.14 ± 1.98^{c}	64.3 ± 11.0^{a}	23.8 ± 2.36^{c}	66.7 ± 5.49^{a}	24.3 ± 1.05^{b}	42.3 ± 5.71^{b}	19.43 ± 1.15^{b}	72.0 ± 12.2^{ab}	24.09 ± 2.34^{a}	74.0 ± 7.55^{a}
R. irregularis	38.4 ± 3.78^{a}	60.0 ± 11.3^{a}	62.1 ± 5.61^{a}	48.0 ± 10.8^{b}	44.6 ± 4.65^{a}	61.8 ± 10.5^{a}	29.59 ± 2.35^{a}	64.8 ± 8.85^{ab}	27.73 ± 2.60^{a}	43.3 ± 8.3^{b}
Control	9.96 ± 1.84^{d}	26.7 ± 3.06^{c}	13.06 ± 2.3^{d}	29.3 ± 2.22^{c}	12.13 ± 1.25^{c}	14.0 ± 3.73^{d}	11.74 ± 1.96^{c}	43.5 ± 7.23^{c}	13.05 ± 2.17^{b}	14.3 ± 3.8^{c}

NNum: Nodule number; IRC: intensity of root AMF colonization; Values (mean ± standard error) are an average of five replications; means ± standard error within the same column followed by the same superscript letters are not statistically different at the 5% probability according to Tukey test.

results showed that there was a significant difference ($p < 0.05$) in AMF colonization (ranging from 18.06 ± 2.63% to 62.1 ± 5.61%) between the inoculated and non-inoculated plants (Table 3), irrespective of the groundnut variety. Interestingly, the highest root AMF colonization occurred with the exotic strain *R. irregularis*, especially in the modern groundnut varieties. In addition, the indigenous AMF *R. aggregatus* also showed high root AMF colonization (42.73 ± 3.07%) in the traditional variety 55-437, whereas the root colonization was still low in the modern varieties Amoul Morom, Essamay and Sunu Gaal. Furthermore, the variety Essamay showed an overall higher AMF root colonization rate than the other varieties, irrespective of the AMF inoculated. In contrast, *R. fasciculatus* and *G. rosea* showed relatively low root AMF colonization compared to the other AMF inoculants (Table 3).

The results showed that there was a significant difference ($p < 0.05$) in the number of nodules between the inoculated and uninoculated treatments, except for *R. fasciculatus* when inoculated on Essamay and Fleur 11 and for *R. aggregatus* when inoculated on Amoul Morom (Table 3). In this case, the inoculated plants showed a higher number of nodules, including those inoculated with *G. rosea*, although such an increase was not clear for root AMF colonization. Irrespective of the groundnut variety, plants in the *F. mosseae*, *G. rosea* and *R. irregularis* treatments were more nodulated than the uninoculated plants (Table 3), indicating that these AMF inoculants had a high capacity to increase root nodule occupancy. The native AMF *R. aggregatus* also showed high nodulation on plants of the varieties Essamay and Fleur 11.

Leaf chlorophyll content

For the six varieties, leaf chlorophyll content at 30, 45 and 65 DAP after inoculation with AMF ranged from 29.8 ± 6.84 to 40.5 ± 2.72 (Table S1), 31.9 ± 3.23 to 44.0 ± 2.40 (Table S2), and 33.9 ± 2.86 to 46.7 ± 2.07 (Table 4), respectively, and was higher for groundnut variety Amoul Morom followed by the variety Sunu Gaal. At 30 and 45 DAP, the data showed no significant difference between the inoculated and non-inoculated plants, irrespective of the variety (Tables S1 and S2). However, leaf chlorophyll content increased significantly at 65 DAP for both Amoul Morom and Sunu Gaal when plants were inoculated with all native AMF and *G. rosea* for the former and only native AMF for the latter (Table 4).

Growth response of groundnut varieties to AMF inoculation

Plant growth parameters affected by AMF inoculation with the indigenous and exotic inoculants are shown in Table 5. Overall, the results showed that Amoul Morom, 55-437 and Essamay were more responsive to AMF inoculation in terms of plant height and collar diameter. Inoculation with 80 and 50% of our AMF collection showed the ability to improve plant height and collar diameter in Amoul Morom and 55-437, respectively. Interestingly, the plant height at 65 DAP showed a significant difference ($p < 0.05$) with the highest height and collar diameter observed on the *R. aggregatus* and *G. rosea* inoculation treatments. Only *R. irregularis* showed

Table 4. Leaf chlorophyll content at 65 days after planting in response to groundnut varieties (*A. hypogaea*) single inoculation with AMF strains.

Treatments	Groundnut varieties				
	Amoul Morom	Essamaye	55-437	Fleur11	Sunu Gaal
F. mosseae	45.3 ± 2.54^{a}	39.6 ± 1.17^{a}	36.0 ± 2.70^{a}	38.0 ± 1.52^{b}	40.2 ± 3.32^{a}
R. aggregatus	44.8 ± 2.23^{a}	36.6 ± 4.86^{ab}	38.1 ± 1.29^{a}	38.1 ± 1.82^{b}	40.6 ± 2.10^{a}
R. fasciculatus	45.5 ± 2.73^{a}	39.2 ± 1.67^{a}	37.6 ± 0.95^{a}	43.0 ± 1.71^{a}	42.3 ± 2.05^{a}
G. rosea	46.7 ± 2.07^{a}	35.4 ± 3.08^{ab}	36.0 ± 1.94^{a}	38.0 ± 1.59^{b}	39.4 ± 3.71^{ab}
R. irregularis	42.4 ± 3.24^{ab}	35.3 ± 1.69^{b}	39.2 ± 1.45^{a}	40.2 ± 2.67^{ab}	39.4 ± 3.47^{ab}
Control	41.3 ± 1.01^{b}	34.3 ± 1.80^{b}	36.0 ± 2.55^{a}	37.7 ± 1.30^{b}	33.9 ± 2.86^{b}

Values (mean ± standard error) are an average of five replications; means ± standard error within the same column followed by the same superscript letters are not statistically different at the 5% probability according to Tukey test.

Table 5. Growth (plant height and collar diameter) response of groundnut varieties (*A. hypogaea*) to single inoculation with AMF 65 days after planting.

Treatments	Groundnut varieties									
	Amoul Morom		Essamaye		55-437		Fleur11		Sunu Gaal	
	Height (cm)	CD (mm)	Height (cm)	CD (mm)	Height (cm)	CD (mm)	Height (cm)	CD (mm)	Height (cm)	CD (mm)
F. mosseae	14.3 ± 1.04^{ab}	5.45 ± 0.63^{a}	16.8 ± 1.19^{ab}	5.24 ± 1.03^{a}	21.4 ± 2.50^{ab}	4.32 ± 0.32^{ab}	18.5 ± 2.67^{a}	5.03 ± 0.79^{a}	23.0 ± 2.16^{a}	5.28 ± 0.59^{a}
R. aggregatus	15.7 ± 1.15^{a}	6.68 ± 0.32^{a}	18.8 ± 1.55^{ab}	5.12 ± 0.26^{a}	22.1 ± 1.89^{a}	4.76 ± 0.37^{a}	21.6 ± 2.50^{a}	4.52 ± 0.77^{a}	23.3 ± 1.71^{a}	4.33 ± 1.00^{a}
R. fasciculatus	16.0 ± 1.41^{a}	5.65 ± 0.95^{a}	16.6 ± 1.11^{ab}	5.24 ± 1.03^{a}	17.8 ± 1.26^{b}	4.73 ± 0.58^{ab}	19.4 ± 1.60^{a}	4.69 ± 0.31^{a}	24.6 ± 1.49^{a}	4.71 ± 0.39^{a}
G. rosea	15.9 ± 1.03^{a}	6.01 ± 0.28^{a}	18.4 ± 2.25^{ab}	5.08 ± 0.50^{a}	24.4 ± 2.45^{a}	4.85 ± 0.78^{a}	21.8 ± 2.22^{a}	4.56 ± 0.45^{a}	23.2 ± 1.58^{a}	4.57 ± 0.29^{a}
R. irregularis	16.0 ± 1.50^{a}	5.32 ± 0.18^{a}	19.5 ± 1.87^{a}	5.15 ± 0.26^{a}	21.0 ± 2.24^{ab}	4.65 ± 0.04^{ab}	19.4 ± 1.18^{a}	4.65 ± 0.61^{a}	23.1 ± 1.65^{a}	4.75 ± 0.42^{a}
Control	13.5 ± 0.71^{b}	5.18 ± 0.85^{a}	15.6 ± 1.38^{b}	5.04 ± 0.32^{a}	16.7 ± 1.76^{b}	3.41 ± 0.68^{b}	19.6 ± 2.14^{a}	4.41 ± 0.45^{a}	22.7 ± 2.08^{a}	4.26 ± 0.47^{a}

CD: Collar diameter; Values (mean ± standard error) are an average of five replications; means ± standard error within the same column followed by the same superscript letters are not statistically different at the 5% probability according to Tukey test.

a significant increase in plant height with Essamay (19.5 ± 1.87 cm plant^{-1}) compared to the uninoculated treatment (15.6 ± 1.38 cm plant^{-1}). However, no significant difference was found in the varieties Fleur 11 and Sunu Gaal (two genetically close varieties, Faye I. personal communication) when comparing the growth parameters of inoculated and uninoculated plants, but inoculated plants performed better than uninoculated plants for all groundnut varieties (Table 5).

Groundnut dry matter and yield parameters

The varieties Amoul Morom and Essamay responded better in terms of biomass production. For these varieties, all inoculated AMFs increased both shoot dry weight (SDW) and root dry weight (RDW). However, the differences observed were only significant for SDW. Furthermore, we found no significant difference in biomass production between plants inoculated with *R. irregularis* and the uninoculated plants, regardless of the variety used. The RDW was increased in three AMF treatments (*R. aggregatus*, *R. fasciculatus* and *G. rosea*), but the SDW was increased only when *G. rosea* was inoculated. Only the SDW was increased in the *R. fasciculatus* and *F. mosseae* treatments for the varieties Fleur 11 and Sunu Gaal, respectively. Both varieties were less responsive to AMF inoculation (Table 6).

Yield characteristics were improved in 40% of the treatments for each of the varieties Amoul Morom and 55-437. However, an improvement in

Table 6. Biomass production (above-ground and root biomass) of groundnut varieties at harvest.

Treatments	Groundnut varieties									
	Amoul Morom		Essamaye		55-437		Fleur11		Sunu Gaal	
	SDW (g)	RDW (g)	SDW (g)	RDW (g)	SDW (g)	RDW (g)	SDW (g)	RDW (g)	SDW (g)	RDW (g)
F. mosseae	3.27 ± 0.53^{a}	1.31 ± 0.24^{a}	2.82 ± 0.75^{a}	1.13 ± 0.12^{ab}	1.52 ± 0.40^{ab}	0.51 ± 0.07^{ab}	1.72 ± 0.18^{b}	0.73 ± 0.16^{ab}	3.38 ± 0.45^{a}	0.88 ± 0.17^{a}
R. aggregatus	2.72 ± 0.30^{ab}	1.05 ± 0.15^{ab}	2.83 ± 0.31^{a}	1.33 ± 0.24^{a}	1.58 ± 0.48^{ab}	0.67 ± 0.07^{a}	1.78 ± 0.17^{b}	0.77 ± 0.23^{ab}	2.54 ± 0.21^{ab}	0.81 ± 0.14^{ab}
R. fasciculatus	2.37 ± 0.31^{b}	1.11 ± 0.15^{ab}	2.76 ± 0.49^{a}	1.32 ± 0.14^{a}	1.53 ± 0.22^{ab}	0.75 ± 0.36^{a}	2.54 ± 0.14^{a}	0.87 ± 0.11^{a}	2.62 ± 0.65^{ab}	0.75 ± 0.11^{ab}
G. rosea	2.73 ± 0.52^{ab}	1.27 ± 0.22^{a}	2.88 ± 0.54^{a}	1.06 ± 0.16^{ab}	1.95 ± 0.73^{a}	0.68 ± 0.21^{a}	2.17 ± 0.62^{ab}	0.86 ± 0.16^{ab}	2.28 ± 0.34^{ab}	0.68 ± 0.11^{ab}
R. irregularis	2.22 ± 0.12^{bc}	0.92 ± 0.11^{ab}	2.58 ± 0.39^{ab}	1.07 ± 0.18^{ab}	1.42 ± 0.28^{ab}	0.48 ± 0.11^{ab}	1.66 ± 0.22^{b}	0.71 ± 0.10^{ab}	2.51 ± 0.82^{ab}	0.62 ± 0.01^{ab}
Control	1.52 ± 0.27^{c}	0.68 ± 0.32^{b}	1.56 ± 0.29^{b}	0.82 ± 0.11^{b}	0.60 ± 0.11^{b}	0.16 ± 0.09^{b}	1.65 ± 0.21^{b}	0.52 ± 0.12^{b}	1.96 ± 0.46^{b}	0.59 ± 0.04^{b}

SDW: Shoot dry weight; RDW: root dry weight; Values (mean ± standard error) are an average of five replications; means ± standard error within the same column followed by the same superscript letters are not statistically different at the 5% probability according to Tukey test.

yield attributes was observed in 20% of the treatments for the varieties Sunu Gaal and Essamay, whereas none of the inoculated AMFs showed a significant improvement in yield parameters for the variety Fleur 11. This suggests that the response of groundnut inoculation in terms of yield attributes is variety dependent, but not related to the fact that the varieties are traditional or modern. Among theinoculated AMF strains, only *G. rosea* showed a significantly better agronomic performance for variety Sunu Gaal and no significant difference was observed for variety Fleur 11. However, Amoul Morom showed a significant yield improvement when plants were inoculated with *F. mosseae* or *R. aggregatus*. Improved pod number and weight were also observed in variety 55-437 inoculated with *R. aggregatus*, while *R. irregularis* improved pod number. Only inoculation with *G. rosea* showed an improvement in pod number for varieties Essamaye and Sunu Gaal, but no significant difference was found for variety Fleur 11. Essamaye had a higher yield than the other four varieties, with a maximum of 1.38 ± 0.22 g plant^{-1} in the *G. rosea* treatment (Table 7).

DISCUSSION

Legume crops are closely associated with symbiotic microbial communities that influence plant traits related to plant growth and yield (Cardoso and Kuyper, 2006; Calvo et al., 2014; Lesueur et al., 2016; Begum et al., 2019; Xiang et al., 2022; Aguégué et al., 2023). In the present study, five traditional and modern groundnut varieties were tested for requirements with or without indigenous and exotic arbuscular mycorrhizal fungi (AMF) inoculants. The efficacy of the AMF inoculants was assessed in terms of their ability to increase root AMF colonization, plant growth, leaf chlorophyll content and yield parameters. The results confirm the functional variation among the inoculated AMF, which is crucial in establishing potential formulations of AMF inoculants to enhance groundnut productivity. The efficacy of inoculated AMF was specifically dependent on the groundnut genotype used, with the varieties Amoul Morom, 55-437 and Essamay being more responsive to AMF inoculation than the varieties Fleur 11 and Sunu Gaal.

High root colonization ability is an important requirement for the selection of AMF inoculants in crop production (Calvo et al., 2014; Hart et al., 2015; Aguégué et al., 2023). The fact that groundnut is a root-hairless crop (Nambiar et al., 1983; Wissuwa and Ae, 2001) suggests that its dependence on AMF for nutrient uptake would be high, highlighting the importance of AMF fertilizers in groundnut. In this study, as predicted, the AMF inoculants tested appeared to be infective even in the presence of native AMF. In the inoculated treatments, there was a significant increase in the rate of root AMF colonization of all groundnut varieties compared to the control plants. Evidence of increased root AMF colonization by mycorrhizal inoculation has been reported previously (Cely et al., 2016; Thioub et al., 2019; Adeyemi et al., 2021; Sene et al., 2021, 2023) and our results are consistent with such previous findings. The inoculated strains may compete with indigenous AMF for colonization sites and spread rapidly within the host roots.

Furthermore, the results showed that root AMF colonization varied greatly depending on the groundnut variety used. Specifically, inoculation

Table 7. Yield attributes (number of pods per plant, pod weight) of groundnut varieties at harvest.

Treatments	Groundnut varieties									
	Amoul Morom		Essamaye		55-437		Fleur11		Sunu Gaal	
	No. of pods	Wt. of Pods (g)	No. of pods	Wt. of Pods (g)	No. of pods	Wt. of Pods (g)	No. of pods	Wt. of Pods (g)	No. of pods	Wt. of Pods (g)
F. mosseae	5.00 ± 1.63^{a}	0.27 ± 0.03^{a}	6.00 ± 3.83^{ab}	0.56 ± 0.12^{b}	4.25 ± 1.50^{ab}	0.44 ± 0.15^{ab}	4.75 ± 0.96^{a}	0.59 ± 0.04^{a}	4.00 ± 1.41^{b}	0.49 ± 0.14^{a}
R. aggregatus	3.25 ± 1.50^{ab}	0.15 ± 0.02^{b}	5.75 ± 2.87^{ab}	0.52 ± 0.14^{b}	7.25 ± 1.71^{a}	0.69 ± 0.07^{a}	4.75 ± 1.26^{a}	0.58 ± 0.11^{a}	5.75 ± 0.96^{ab}	0.46 ± 0.07^{a}
R. fasciculatus	3.00 ± 0.00^{ab}	0.10 ± 0.01^{bc}	6.75 ± 1.50^{ab}	1.11 ± 0.20^{a}	4.33 ± 2.31^{ab}	0.55 ± 0.24^{ab}	5.25 ± 2.22^{a}	0.53 ± 0.15^{a}	4.25 ± 1.26^{b}	0.50 ± 0.15^{a}
G. rosea	3.00 ± 1.00^{ab}	0.10 ± 0.01^{bc}	10.0 ± 1.41^{a}	1.38 ± 0.22^{a}	4.00 ± 2.00^{ab}	0.37 ± 0.11^{ab}	6.50 ± 2.38^{a}	0.75 ± 0.25^{a}	7.50 ± 1.73^{a}	0.65 ± 0.09^{a}
R. irregularis	2.50 ± 0.58^{b}	0.10 ± 0.02^{bc}	4.25 ± 2.06^{b}	0.51 ± 0.04^{b}	7.25 ± 1.71^{a}	0.56 ± 0.20^{ab}	5.00 ± 1.15^{a}	0.56 ± 0.02^{a}	3.33 ± 1.15^{b}	0.50 ± 0.09^{a}
Control	2.50 ± 0.58^{b}	0.08 ± 0.01^{c}	3.75 ± 0.50^{b}	0.44 ± 0.13^{b}	3.00 ± 0.00^{b}	0.35 ± 0.03^{b}	4.75 ± 0.96^{a}	0.55 ± 0.10^{a}	4.00 ± 0.00^{b}	0.50 ± 0.21^{a}

No. of pods (number of pods per plant); Wt. of Pods (weight of pods per plant); Values (mean ± standard error) are an average of five replications; means ± standard error within the same column followed by the same superscript letters are not statistically different at the 5% probability according to Tukey test.

with *R. irregularis* resulted in the highest root AMF colonization in the varieties Amoul Morom, Essamay and 55-437. The increased root AMF colonization with *R. irregularis* is consistent with the report of Köhl et al. (2016). These authors reported that the *R. irregularis* has a broad niche with the ability to successfully compete with native AMF, and thus can successfully colonize root plants in a wide range of soils. In the case study of the present study, such high root AMF colonization was not observed in the varieties Fleur 11 and Sunu Gaal. In addition, *R. aggregatus* also showed a higher colonization of variety 55-437 than the other varieties. This indicates a discrepancy in the ability of AMF inoculants to compete and colonize the groundnut varieties, and supports a report by Jie et al. (2013) on soybean (*Glycine max* L.). To date, there is no convincing evidence of AMF host specificity, but host preference and selectivity have been widely reported (Torrecillas et al., 2012; Bender et al., 2016; Köhl et al., 2016), and variability amongst different AMF species in root AMF colonization has been investigated in several studies (Wagg et al., 2015; Berruti et al., 2017; Thioub et al., 2019).

It has been previously reported that inoculation causes a change in the root system morphology in groundnut through lateral root development (Yano et al., 1996; Gutjahr and Paszkowski, 2013). Such changes in the root system are generally considered to have a large uptake capacity (Smith and Read, 2008; Fortin et al., 2015; Aguégué et al., 2023). Although root length was not assessed in the present study, there is sufficient evidence that AMF inoculation had a positive effect on this parameter, as root dry weight (RDW) increased significantly in almost all inoculated treatments. This could lead to an increase in the volume of root tissue that can be colonized by AMF or rhizobia. Therefore, a very clear difference in nodule formation was observed between the inoculated and uninoculated plants. In this case, the inoculated plants showed a significantly higher number of nodules, regardless of the groundnut variety. In contrast, no such increase in root AMF colonization was observed in any of the varieties.

Leaf chlorophyll content was generally higher in inoculated than in uninoculated plants, irrespective of the variety used. The AMF association has been reported to affect the host plants in terms of stomatal movement and leaf photosynthesis. This has been shown to increase chlorophyll content and the rate of transpiration and photosynthesis (Sheng et al., 2008; Ye et al., 2022). On the other hand, the highest leaf chlorophyll content could be due to the highest nodule formation in the inoculated plants, suggesting a potential synergistic effect between inoculated AMF and indigenous rhizobia and thus the basic function of rhizobia in N_2 fixation. The efficiency of N_2 fixation in groundnut may result in the accumulation of nitrogen in plant tissues, which in turn reflects the synthesis of chlorophyll.

The results of this study also showed that different varieties responded differently to the AMF applied in terms of plant growth and yield parameters. Significantly higher plant growth and yield parameters for Amoul Morom, Essamay and 55-437 varieties were reported with AMF inoculation. This could be attributed to a higher responsiveness of these groundnut varieties to the inoculated AMF strains. The inoculation of efficient and compatible AMF may help to establish symbioses earlier than the indigenous AMF populations, resulting in increased plant growth benefits. Indeed, studies using P^{32}-labelled phosphate have clearly shown that P is translocated from the soil to the root by the AMF

mycelium (Qin et al., 2022), and perhaps the efficiency differs between plant genotypes. In contrast, the genetically closely related varieties Fleur 11 and Sunu Gaal were less responsive to the AMF inoculation in the case study of this present study. This suggests functional differences between AMF inoculants and is consistent with a number of recent studies reporting differences in host genotypes in response to AMF inoculation (Calvo-Polanco et al., 2016; Duc et al., 2017; Bazghaleh et al., 2018; Frew, 2020). Furthermore, the increased nodule numbers in the *R. irregularis* and *G. rosea* treatments did not translate into plant growth or yield parameters in the Fleur 11 and Sunu Gaal varieties. This was not expected and indicates the need for further selection of highly efficient and appropriate AMF inoculants for successful inoculation of Fleur 11 and Sunu Gaal. Similar negative or neutral effects after AMF inoculation were observed by Chotangui et al. (2022) on two groundnut varieties in the Western Highlands of Cameroon. However, the potential of inoculated AMF in our case study may be underestimated as the confined space of the pots does not allow for maximum root development.

Conclusion

The demand for microbial inoculants is increasing, driven by the need for sustainable and environmentally friendly agricultural practices and safer and healthier food. To select the best arbuscular mycorrhizal fungi (AMF) inoculants for five traditional and modern Senegalese groundnut varieties, we hypothesized that the response of groundnut to indigenous and exotic AMF inoculation is cultivar dependent and that there is a different degree of variability between traditional and modern cultivars. The results of this study showed that the AMF inoculants tested promoted increases in various parameters analyzed. In particular, inoculation efficacy did not depend on the origin (exotic or indigenous) of the inoculated AMF and no clear relationship was found between the fact that the varieties used were traditional or modern. However, the response to AMF inoculation differed between varieties, demonstrating the differential feedback between groundnut genotypes and AMF partners. Groundnut varieties such as Essamay, Amoul Morom and 55-437 responded better than the closely related genotypes Fleur 11 and Sunu Gaal. These results confirm the functional variation among inoculated AMF, which is crucial for establishing potential formulations of AMF inoculants to improve groundnut productivity. According to this study, further selection of compatible AMF partners would be useful to improve inoculation success with these latter varieties.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table S1. Leaf chlorophyll content at 30 days after planting in response to groundnut varieties inoculation with arbuscular mycorrhizal fungi

	Groundnut varieties				
Treatments	**Amoul morom**	**Essamaay**	**55-437**	**Fleur 11**	**Sunu Gaal**
F. mosseae	41.1 ± 3.79[a]	35.8 ± 4.37[a]	34.2 ± 4.92[a]	34.8 ± 1.31[a]	37.9 ± 3.34[a]
R. aggregatus	41.5 ± 2.72[a]	31.6 ± 4.70[a]	31.3 ± 4.63[a]	37.0 ± 2.61[a]	39.0 ± 2.76[a]
R. fasciculatus	38.5 ± 2.55[a]	32.3 ± 3.34[a]	33.8 ± 4.10[a]	39.5 ± 5.97[a]	36.0 ± 5.81[a]
G. rosea	38.9 ± 1.40[a]	33.5 ± 6.06[a]	35.0 ± 3.29[a]	35.3 ± 1.10[a]	35.8 ± 6.59[a]
R. irregularis	38.9 ± 3.28[a]	34.5 ± 1.66[a]	31.6 ± 1.82[a]	37.3 ± 3.46[a]	40.0 ± 1.86[a]
Control	37.8 ± 1.68[a]	29.8 ± 6.84[a]	30.5 ± 3.33[a]	34.7 ± 2.00[a]	33.2 ± 5.45[a]

In columns, means with identical superscript letters are statistically equivalent at the 5% probability level.

Table S2. Leaf chlorophyll content at 45 days after planting in response to groundnut varieties inoculation with arbuscular mycorrhizal fungi

	Groundnut varieties				
Treatments	**Amoul morom**	**Essamaay**	**55-437**	**Fleur 11**	**Sunu Gaal**
F. mosseae	43.2 ± 2.40[a]	32.5 ± 3.64[a]	35.2 ± 1.73[a]	35.0 ± 1.73[a]	36.2 ± 5.19[a]
R. aggregatus	44.0 ± 2.40[a]	37.5 ± 3.24[a]	35.7 ± 1.90[a]	35.7 ± 1.58[a]	36.8 ± 2.17[a]
R. fasciculatus	43.2 ± 1.11[a]	32.7 ± 5.12[a]	35.9 ± 2.30[a]	36.3 ± 0.67[a]	37.8 ± 2.43[a]
G. rosea	43.1 ± 3.12[a]	36.0 ± 3.40[a]	34.4 ± 3.16[a]	36.4 ± 1.54[a]	37.0 ± 1.68[a]
R. irregularis	41.8 ± 2.06[a]	32.1 ± 1.44[a]	36.4 ± 2.44[a]	34.4 ± 2.28[a]	37.8 ± 3.73[a]
Control	40.8 ± 2.33[a]	31.9 ± 3.23[a]	33.4 ± 3.40[a]	33.0 ± 1.16[a]	33.0 ± 1.95[a]

In columns, means with identical superscript letters are statistically equivalent at the 5% probability level.

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