Microbiological quality analysis of inoculants based on *Bradyrhizobium* spp. and *Azospirillum brasilense* produced “on farm” reveals high contamination with non-target microorganisms

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Abstract

The use of inoculants carrying diazotrophic and other plant growth–promoting bacteria plays an essential role in the Brazilian agriculture, with a growing use of microorganism-based bioproducts. However, in the last few years, some farmers have multiplied microorganisms in the farm, known as “on farm” production, including inoculants of *Bradyrhizobium* spp. for soybean (*Glycine max* L. Merrill.) and *Azospirillum brasilense* for corn (*Zea mays* L.) or co-inoculation in soybean. The objective was to assess the microbiological quality of such inoculants concerning the target microorganisms and contaminants. In the laboratory, 18 samples taken in five states were serial diluted and spread on culture media for obtaining pure and morphologically distinct colonies of bacteria, totaling 85 isolates. Molecular analysis based on partial sequencing of the 16S rRNA gene revealed 25 genera of which 44% harbor species potentially pathogenic to humans; only one of the isolates was identified as *Azospirillum brasilense*, whereas no isolate was identified as *Bradyrhizobium*. Among 34 isolates belonging to genera harboring species potentially pathogenic to humans, 12 had no resistance to antibiotics, six presented intrinsic resistance, and 18 presented non-intrinsic resistance to at least one antibiotic. One of the samples analyzed with a shotgun-based metagenomics approach to check for the microbial diversity showed several genera of microorganisms, mainly *Acetobacter* (~32% of sequences) but not the target microorganism. The samples of inoculants produced on farm were highly contaminated with non-target microorganisms, some of them carrying multiple resistances to antibiotics.

Keywords Inoculation · Biological nitrogen fixation · Plant growth–promoting bacteria · Pathogenic microorganisms · On farm fermentation

Introduction

Soybean (*Glycine max* L. Merr.) and corn (*Zea mays* L.) are the main Brazilian grain crops [1], with a production ~125 million tons in ~37 million hectares of soybean, ~102.5 million tons in ~18.5 million hectares of corn [2]. The symbiosis between soybean and elite *Bradyrhizobium* strains can supply the most part of the required N via biological nitrogen fixation (BNF) [3] and grain yield increases by 8% due to inoculation [4]. In corn, yield increase due to inoculation with *Azospirillum brasilense* has been attributed to bacterial phytohormones [5, 6]. Co-inoculation of soybean with *Bradyrhizobium* spp. and *A. brasilense* has doubled the benefits compared with single inoculation [7, 8].

Brazil has a long tradition in research with inoculants containing rhizobia and *Azospirillum*, and legislation for quality control of inoculants. According to the standards established by the Ministry of Agriculture, Livestock and Food Supply (MAPA), commercial inoculants must have the minimal concentration of $10^9$ viable cells of
Bradyrhizobium and 10^8 cells of Azospirillum per gram or milliliter of inoculant, no contaminants at the 10^-5 dilution, and must carry only elite strains with recognized agronomic efficiency [9, 10].

The industrial production of inoculants is a complex process, but improvements in the last two decades have resulted in high-quality products in terms of cell concentrations, no contaminants, and very low cost, probably the cheapest inoculant in the world [11]. However, in the last five years, some farmers have tried to produce their own bioproducts, including inoculants in the farm, using simplified biofactories, known as “on farm” production. In most cases, the production system is rudimentary and varies in terms of installations, equipment, microbiological standards, and technical capacity. Very often the bioproducts are produced in fermenters, open tanks, or even water tanks, without appropriate control of contaminations, which may result in highly contaminated, non-effective products [12, 13].

The objective of this study was to assess the microbiological quality of inoculants based on Bradyrhizobium spp. and A. brasilense produced on farm in Brazil, concerning the intended microorganisms, presence, and characterization of probable contaminants.

### Materials and methods

#### Sampling

Sampling and transportation kits containing Styrofoam box, sterile 50-mL Falcon-type conical tubes, sterile 30-mL disposable syringes, disposable gloves, Parafilm M® for sealing the tubes, and cooling packs were sent to farmers interested to know the microbiological quality of their inoculants produced on farm. The kit included a protocol for sampling, emphasizing aseptic procedures and an identification form. Immediately after sampling, two aliquots per tank or fermenter were packed with cooling packs in the Styrofoam box and sent back by express postal service or personally delivered in the Laboratory for Soil Biotechnology at Embrapa Soja. A total of 18 samples were obtained during 2019/20 cropping season, six aiming Bradyrhizobium and 12 aiming Azospirillum as target microorganisms (Table 1). These samples were obtained from five states: São Paulo (six), Bahia (two), Paraná (five), Rio Grande do Sul (three), and Mato Grosso (two). For comparative purposes, commercial inoculants containing A. brasilense strains Ab-V5 and Ab-V6 (C1, lot 1,108,718), B. diazoefficiens strain SEMIA 5080 and B. japonicum strain SEMIA 5079 (C2, lot 0,135,218),

| Sample | Municipality-State | Target microorganism | pH | Electrical Conductivity (μS/cm) | Odor | Type of multiplication | Growth time |
|--------|--------------------|----------------------|----|--------------------------------|------|------------------------|------------|
| 1      | Presidente Bernardes-SP | Bradyrhizobium | 5.7 | 4000 | Putrid | Open tanks | 2 days |
| 2      | Presidente Bernardes-SP | Azospirillum | 4.0 | 2900 | Sour | Open tanks | 1 day |
| 3      | Barreiras-BA | Bradyrhizobium | 4.9 | 2100 | Sour | Open tanks | 10 days |
| 4      | Marilândia do Sul-PR | Azospirillum | 4.4 | 890 | Sour | Fermenter | 2 days |
| 5      | Mauá da Serra-PR | Azospirillum | 5.9 | 800 | Sour | Open tanks | 4 h |
| 6      | Mauá da Serra-PR | Azospirillum | 3.6 | 1030 | Sour | Open tanks | 7 days |
| 7      | Luís Eduardo Magalhães-BA | Azospirillum | 7.2 | 2060 | Fecal | Open tanks | 5 days |
| 8      | Panambi-RS | Azospirillum | 3.9 | 1620 | Urine | Open tanks | 1 day |
| 9      | Palotina-PR | Bradyrhizobium | 5.3 | 6890 | Sour | Open tanks | 2 days |
| 10     | Palotina-PR | Azospirillum | 5.0 | 8390 | Garbage | Open tanks | 2 days |
| 11     | Sorriso-MT | Azospirillum | 3.9 | 5930 | Sour | Open tanks | 3 days |
| 12     | Sorriso-MT | Azospirillum | 4.4 | 4640 | Fecal | Open tanks | 3 days |
| 13     | Panambi-RS | Bradyrhizobium | 4.7 | 1870 | Yeast | Fermenter | 2 days |
| 14     | Panambi-RS | Azospirillum | 4.8 | 2200 | Yeast | Fermenter | 1 day |
| 15     | Salto Grande-SP | Bradyrhizobium | 4.0 | 3830 | Sour | Open tanks | 3 days |
| 16     | Salto Grande-SP | Azospirillum | 5.5 | 7020 | Fecal | Open tanks | 1 day |
| 17     | Lutécia-SP | Bradyrhizobium | 5.5 | 2760 | Fecal | Not informed | Not informed |
| 18     | Lutécia-SP | Azospirillum | 5.1 | 2910 | Sour | Not informed | Not informed |
| C1     | – | Azospirillum | 7.1 | 9810 | Vinegar | Industrial fermenter | – |
| C2     | – | Bradyrhizobium spp. | 7.1 | 1960 | Yeast | Industrial fermenter | – |
| C3     | – | B. elkanii | 7.2 | 2200 | Yeast | Industrial fermenter | – |
and *Bradyrhizobium elkanii* strains SEMIA 587 and SEMIA 5019 (C3, lot 19,014,223) were included. It is worth mentioning that, although not mandatory, commercial inoculants in Brazil usually contain two bacterial strains.

**Physical–chemical and organoleptic properties**

The samples and the commercial inoculants were evaluated for pH using a pH-meter model FiveEasy Plus pH-meter FP20 (METTLER TOLEDO, Ohio, USA) and electrical conductivity in a digital conductivity-meter Tec-4MP (TECNAL, Piracicaba, Brazil). A sensorial analysis was based on the “odor wheel” described by McGinley and McGinley [14], which highlights eight categories of odors.

**Isolation of morphotypes**

Under aseptic conditions, serial dilutions were made in sterile 0.85% NaCl saline and 100-μL aliquots of the 10⁻⁵, 10⁻⁶, and 10⁻⁷ dilutions were spread on five different culture media: modified YMA (Yeast Mannitol Agar) for *Bradyrhizobium* [15]; RC (Rojo Congo) [16] for *Azospirillum*; LB (Luria Bertani) [17]; NA (Nutrient Agar) [18]; and Sabouraud [19]. The different culture media aimed to check the morphology of colonies. Finally, morphologically distinct isolates in each culture medium were streaked again to standardize the isolation in NA medium were cryopreserved in NA broth with 30% glycerol at −80°C in the inverted position in a growth room and were daily observed for 7 days. The morphologically distinct colonies in each culture medium were streaked again on the same culture medium to select single colonies. To avoid morphologically distinct isolates due to the growth medium, all isolates were streaked on NA to standardize the morphology of colonies. Finally, morphologically distinct isolates in NA medium were cryopreserved in NA broth with 30% glycerol at −80°C for further analysis.

Prior to cryopreservation, all isolates were observed at 400× magnification under an optical microscope (AxioLab A1, Zeiss) coupled to an AxioCam ERC 5 s digital video camera system (Zeiss) for recognition of typical yeast traits such as nucleus, vacuole, and cell dimensions. Isolates identified as yeasts were not submitted to further analysis.

**Molecular identification of isolates**

Total DNA of morphologically distinct isolates was extracted with the DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer’s instructions. After extraction, the integrity of DNA was verified by electrophoresis in 1% agarose gel. The 16S rRNA gene was amplified as described [20] with universal primers fD1 (5′-AGAGTT TGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATC CAGCC-3′) for phylogenetic studies of bacteria, flanking nearly the entire region of the 16S rRNA gene (~ 1,500 bp) [21]. The PCR products were purified with the PureLink™ Quick PCR Purification Kit (Invitrogen), according to the manufacturer’s instructions. Sequencing was performed in an ABI3500XL analyser (Applied Biosystems) as described [22]. Fragment sequences ranging from 484 to 1139 bp were analyzed using the software BioNumerics version 7.6 and identification was based on comparison with the NCBI GenBank database using the BLAST tool for nucleotides (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Metagenome analysis**

To have a broader view of the diversity of microorganisms that might not have grown on the culture media, or occurring at low concentrations in the sample, metagenomic analysis was performed in sample 10, from Palotina, PR. We used the shotgun approach, sequencing all DNA fragments extracted from the sample, without previous amplification of any specific region, as described before [23]. The shotgun approach detects higher diversity in a sample as well microorganisms in all domains of life and, if required, can also be used for functional analysis. For the metagenomics analysis, total DNA was extracted with the DNeasy blood and tissue kit (Qiagen) and used to build the library with the Nextera XT kit, according to the manufacturer’s procedure. The library was processed on the MiSeq platform (Illumina) at Embrapa Soja, and the sequences were assembled with the A5-miseq pipeline (de novo) version 20,140,604. The sequenced fragments were uploaded to the MG-RAST v.4.0.4 (RAST—http://metagenomics.anl.gov) and submitted to automatic annotation in the server based on the NCBI BLAST and SEED databases [24].

**Susceptibility to antimicrobials**

After molecular identification, isolates belonging to potentially pathogenic genera were subjected to evaluation of susceptibility to antimicrobials by the Disk-Diffusion Test [25]. Cells grown for 24–48 h on NA medium were suspended in sterile saline (0.85% NaCl) until a turbidity compatible with the McFarland scale 0.5 (~ 1.5×10⁸ CFU mL⁻¹). The suspension was then inoculated on the Müller-Hinton [26] agar plate using a sterile swab. Then, paper disks impregnated with antimicrobials were added, as indicated in the annual updates of the Clinical and Laboratory Standards Institute (CLSI) [27].

The antimicrobials and their concentrations per disk were as follows: amikacin 30 μg, amoxicillin + clavulanate 20/10 μg, ampicillin 10 μg, ampicillin + sulfactam 10/10 μg, aztreonam 30 μg, cefazolin 30 μg, cefepime 30 μg,
The cell concentration in the commercial inoculant C1 (A. brasilense) presented the highest electrical conductivity. In the inoculants, pH was slightly alkaline and the one containing Bradyrhizobium spp. SEMIA 5079 and SEMIA 5080) was 6.30. The physical–chemical and organoleptic properties, type of equipment used for multiplication (open tanks or fermenters), and growth time (from inoculation up to sampling) of 18 samples are shown in Table 1. The pH ranged from 3.6 (sample 6) to 7.2 (sample 7), the latter was the only one with slightly alkaline pH, whereas the others were acidic, below pH 6.0. The electrical conductivity ranged from 800 (sample 5) to 8390 μS cm−1 (sample 10). Among the commercial inoculants, pH was slightly alkaline and the one containing A. brasilense presented the highest electrical conductivity. The cell concentration in the commercial inoculant C1 (A. brasilense Ab-V5 and Ab-V6) was 1.01×10⁹ CFU mL⁻¹; in C2 (Bradyrhizobium spp. SEMIA 5079 and SEMIA 5080) was 6.30×10⁴ CFU mL⁻¹; and in C3 (B. elkanii SEMIA 587 and SEMIA 5019) was 8.47×10⁹ CFU mL⁻¹. No contaminants were found in the commercial inoculants.

In the sensorial analysis [14], only two samples were classified as “yeast” (samples 13 and 14), whereas the others presented odors classified as “offensive,” which might be attributed to putrefaction processes. The commercial inoculants, however, presented odors classified as “vinegar” and “yeast” for Azospirillum and Bradyrhizobium, respectively (Table 1). Among 18 samples, three were declared as multiresistant to antibiotics, e.g., isolates 1.5 and 2.4, which showed high similarity with deposited sequences in the GenBank showed 44 isolates with similarity ≥99% and 28 between 99 and 97.2% with deposited sequences, and coverage between 95 and 100%. Finally, 12 isolates were identified as yeasts based on the cell morphology (size, presence of nucleus, and budding) and were not sequenced.

Among the 84 bacterial isolates, 41 had similarity with species or genera containing at least one species reported as potentially pathogenic to humans (49%): Enterococcus (10), Acinetobacter (seven), Citrobacter (six), Klebsiella (three), Stenotrophomonas (three), Enterobacter (three), Burkholderia (two), Atlantibacter (one), Bacillus (one), Escherichia (one), Kocuria (one), Paenibacillus (one), Pseudomonas (one), and Staphylococcus (one) (Table 2).

The shotgun approach of the sample no. 10 revealed a total of 2,467,209 sequences. After removal of the low-quality sequences and artificial duplicate reads, a total of 679,917,634 bp with average length of 276 bp was obtained. The rarefaction curve indicated that the number of sequences submitted was capable of detecting the existing diversity in the sample (not shown). Among the good-quality sequences, 1% contained ribosomal RNA genes, 90.68% encoded for proteins with known functions, and 8.14% proteins with unknown functions. Considering the automatic annotation in the MG-RAST v.4.0.4 server, the taxonomic classification of all shotgun sequences indicated that 99.23% belonged to the domain Bacteria, 0.2% to Eukaryota, 0.01% to Archaea, and 0.56% to Viruses (not shown). Among the 14 dominating genera identified in the sample, Acetobacter and Leuconostoc represented more than 50% of the sequences in the microbiome, whereas Azospirillum, the target microorganism in that sample, was not found (Fig. 2).

The test of susceptibility to antimicrobials was carried out according to [73–75] only with 36 isolates considered of clinical relevance. Considering the CLSI protocol, 12 isolates presented no resistance to at least one antibiotic; six presented intrinsic resistance to at least one antibiotic; and 18 isolates presented single or multiple resistance (Table 3). Noteworthy, some isolates showed multiresistance to antibiotics, e.g., isolates 1.5 and 2.4, which showed high 16S rRNA gene homology with Enterococcus faecalis, and showed resistance to all and to five tested antibiotics, respectively.
Discussion

Among 84 isolates, 25 genera were identified, 44% of which are known to harbor potential human pathogens, whereas only one isolate (5.2) showed 16S rRNA gene homology with the target microorganism *A. brasilense*. That was a case in which the sample was taken only 4 h after the tank had been inoculated with a commercial inoculant. Thus, the isolate probably originated from the commercial inoculant used as inoculum, not from the multiplication, since the short time between the addition of inoculum and the sampling may still have allowed the microorganism to survive. No other sample provided colonies identified as *Azospirillum*, showing that the target microorganism is eliminated or suppressed as the growth media become dominated by contaminating microorganisms. In addition, among the six samples aiming to multiply *Bradyrhizobium*, no isolate corresponded to the target bacteria.

Multiplication of microorganisms must assure several minimal microbiological procedures to guarantee that the target microorganism prevails in the culture medium. In the case of *Azospirillum* and mainly *Bradyrhizobium*, a slow-growing bacterium [15], several other microbial contaminants dominate the culture medium as they have shorter
Table 2  Similarity based on partial sequencing of the 16S rRNA gene of bacterial isolates obtained from samples of inoculants produced on farm, and commercial inoculants, in the 2019/20 growth season aiming the multiplication of *Bradyrhizobium* spp. and *Azospirillum brasilense*, and their potential as human pathogens

| Sample | DNA fragment (bp)* | Likely species/genus | Cover, %* | Identity, %* | GenBank access number | Potentially human pathogen | Reference |
|--------|-------------------|----------------------|-----------|--------------|-----------------------|---------------------------|-----------|
| 1.1 *** 1139 | *Citrobacter braakii* | 99 | 99.56 | LR134214.1 | Yes | Hirai et al. [28] |
| 1.2 1139 | *Enterobacter bugandensis* | 99 | 99.68 | CP039453.1 | Yes | Pati et al. [29] |
| 1.3 1134 | *Acinetobacter baumanii* | 100 | 100 | CP044356.1 | Yes | McConnell et al. [30] |
| 1.4 1134 | *Rummeliibacillus pyccus* | 100 | 100 | JF833091.2 | No | Her; Kim [31] |
| 1.5 1058 | *Enterococcus faecalis* | 98 | 100 | CP041738.1 | Yes | Poulsen et al. [32] |
| 2.1 1134 | *Bacillus megaterium* | 99 | 98.82 | CP032527.2 | No | Faccin et al. [33] |
| 2.2 1139 | *Citrobacter sp.* | 99 | 98.91 | MN521452.1 | Depends on the species | Brenner et al. [34]; Hasan; Sultana; Hossain [35] |
| 2.3 1127 | *Escherichia coli* | 99 | 99.82 | CP044314.1 | Yes | Forson et al. [36] |
| 2.4 1009 | *Enterococcus faecalis* | 100 | 100 | MN420846.1 | Yes | Poulsen et al. [32] |
| 2.5 1110 | *Lactococcus lactis* | 99 | 99.73 | AJ132470.1 | Depends on the species | Camargo et al. [42] |
| 2.6 1134 | *Kurthia gibsonii* | 95 | 100 | KJ872770.1 | No | Dworkin et al. [38] |
| 3.1 1097 | *Acetobacter syzygii* | 99 | 99.72 | NR_113850.1 | No | Aghazadeh; Pouralibaba; Yari Khosroushahi [39] |
| 3.2 1127 | *Lactobacillus farraginis* | 98 | 100 | NR_041467.1 | No | Endo; Okada [40] |
| 3.3 1134 | *Lactobacillus lactis* | 100 | 99.86 | AM944595.1 | No | Guerra [37] |
| 3.4 1132 | *Acinetobacter nosocomialis* | 99 | 100 | CP042994.1 | Yes | Knight et al. [43] |
| 3.5 1058 | *Raoultella sp.* | 99 | 100 | MK999972.1 | Yes | Kus; Burrows [46] |
| 3.6 1110 | *Stenotrophomonas maltophilia* | 100 | 97.02 | L C438378.1 | No | Delgado et al. [56] |
| 3.7 1107 | *Enterococcus sp.* | 100 | 97.02 | CP045918.1 | Yes | Poulsen et al. [32] |
| 3.8 1083 | *Burkholderia contaminans* | 99 | 99.25 | MW195002.1 | Yes | Power et al. [57] |
| 3.9 1104 | *Stenotrophomonas maltophilia* | 99 | 97.02 | LC438378.1 | No | Delgado et al. [56] |
| 4.1 1134 | *Lactobacillus sp.* | 100 | 99.37 | CP028899.1 | Yes | Kasper et al. [48] |
| 4.2 1132 | *Lactococcus lactis* | 100 | 99.86 | AM944595.1 | No | Guerra [37] |
| 4.3 – Yeast** | – | – | – | | | Depends on the species | Moyad [44]; Hafed et al. [45] |
| 4.4 812 | *Enterobacter sp.* | 100 | 99.88 | MK999972.1 | Yes | Kus; Burrows [46] |
| 4.5 – Yeast** | – | – | – | | | Depends on the species | Moyad [44]; Hafed et al. [45] |
| 4.6 1132 | *Klebsiella pneumoniae* | 99 | 99.91 | CP030931.1 | No | Selvakumar et al. [51] |
| 5.1 1130 | *Streptococcus agalactiae* | 99 | 99.91 | LR134214.1 | Yes | Hirai et al. [28] |
| 5.2 1134 | *Acinetobacter baumanii* | 100 | 100 | CP044356.1 | Yes | McConnell et al. [30] |
| 5.3 1134 | *Rummeliibacillus pyccus* | 100 | 100 | JF833091.2 | No | Her; Kim [31] |
| 5.4 1058 | *Enterococcus faecalis* | 98 | 100 | CP041738.1 | Yes | Poulsen et al. [32] |
| 6.1 1139 | *Citrobacter sp.* | 99 | 98.91 | MN521452.1 | Depends on the species | Brenner et al. [34]; Hasan; Sultana; Hossain [35] |
| 6.2 1132 | *Lactococcus lactis* | 100 | 99.37 | AM944595.1 | No | Guerra [37] |
| 6.3 1107 | *Enterococcus sp.* | 100 | 97.02 | LC438378.1 | No | Delgado et al. [56] |
| 7.1 1134 | *Streptococcus agalactiae* | 99 | 99.91 | CP030931.1 | No | Guerra [37] |
| 7.2 1089 | *Staphylococcus aureus* | 99 | 99.37 | AM944595.1 | No | Guerra [37] |
| 7.3 1083 | *Burkholderia contaminans* | 99 | 99.25 | MW195002.1 | Yes | Power et al. [57] |
| 7.4 1084 | *Streptococcus pyogenes* | 99 | 99.91 | CP042941.1 | Yes | Ioannou [55] |
| 7.5 1104 | *Streptococcus agalactiae* | 99 | 99.37 | AM944595.1 | No | Guerra [37] |
| 8.1 1104 | *Enterococcus sp.* | 100 | 97.02 | LC438378.1 | No | Delgado et al. [56] |
| 8.2 – Yeast** | – | – | – | | | Depends on the species | Moyad [44]; Hafed et al. [45] |
| 8.3 1039 | *Lactobacillus johnsonii* | 100 | 97.02 | CP016603.1 | No | Dai et al. [53] |
| 8.4 1083 | *Burkholderia contaminans* | 99 | 99.25 | MW195002.1 | Yes | Power et al. [57] |
| 8.5 1104 | *Enterococcus faecalis* | 100 | 99.08 | CP045918.1 | Yes | Poulsen et al. [32] |
| 9.1 – Yeast** | – | – | – | | | Depends on the species | Moyad [44]; Hafed et al. [45] |
| 9.2 1058 | *Acinetobacter sp.* | 99 | 99.34 | LN609302.1 | No | Kommanee et al. [58] |
| 9.3 1107 | *Enterococcus sp.* | 100 | 98.65 | AJ626904.1 | Depends on the species | Camargo et al. [42] |
| Sample | DNA fragment (bp)* | Likely species/genus | Cover, %* | Identity, %* | GenBank access number | Potentially human pathogen | Reference |
|--------|------------------|----------------------|-----------|--------------|----------------------|---------------------------|-----------|
| 9.4    | 1129             | Lactococcus lactis   | 100       | 99.67        | AM944595.1           | No                        | Guerra [37] |
| 9.5    | 1138             | Bacillus subtilis     | 99        | 99.59        | MN415973.1           | No                        | Van Dijl; Hecker [52] |
| 9.6    | 1074             | Kocuria sp.          | 99        | 98.32        | AM179882.1           | Depends on the species    | Kandi et al. [59] |
| 9.7    | 1062             | Terrabacillus goriensis | 99      | 99.10        | DQ519571.1           | No                        | Krishnamurthi; Chakrabarti [60] |
| 9.8    | –                | Yeast **             | –         | –            | –                    | Depends on the species    | Moyad [44] Hafed et al. [45] |
| 9.9    | 1119             | Lactobacillus sp.    | 100       | 98.21        | NR_028658.1          | No                        | Delgado et al. [56] |
| 10.1   | 1133             | Enterococcus faecalis | 99       | 99.76        | CP045918.1           | Yes                       | Poulsen et al. [32] |
| 10.2   | 1045             | Acetobacter sp.      | 98        | 99.33        | LN609302.1           | No                        | Bommareddy et al. [58] |
| 10.3   | 1076             | Lactobacillus sp.    | 99        | 99.81        | NR_028658.1          | No                        | Delgado et al. [56] |
| 10.4   | –                | Yeast **             | –         | –            | –                    | Depends on the species    | Moyad [44] Hafed et al. [45] |
| 11.1   | 706              | Bacillus sp.         | 100       | 98.45        | GQ181150.1           | Depends on the species    | Tuazon et al. [61] Amin; Amin; Ahmady [62] |
| 11.2   | 637              | Paenibacillus sp.    | 100       | 98.90        | MW555628.1           | Depends on the species    | Sáez-Nieto et al. [63] |
| 11.3   | 919              | Enterococcus hirae   | 100       | 99.59        | MN420858.1           | Rarely                    | Bourafa et al. [64] |
| 11.4   | –                | Yeast **             | –         | –            | –                    | Depends on the species    | Moyad [44] Hafed et al. [45] |
| 11.5   | 1064             | Rummeliibacillus sp. | 99        | 98.85        | MT512031.1           | No                        | Her; Kim [31] |
| 12.1   | 582              | Acinetobacter sp.    | 100       | 98.31        | MK210236.1           | Depends on the species    | Chagas [49] |
| 12.2   | –                | Yeast **             | –         | –            | –                    | Depends on the species    | Moyad [44] Hafed et al. [45] |
| 12.3   | 1018             | Burkholderia vietnamiensis | 100   | 99.21        | MH547402.1           | Yes                       | Ieranò et al. [65] |
| 13.1   | 1081             | Lactococcus lactis   | 100       | 97.72        | AM944595.1           | No                        | Guerra [37] |
| 13.2   | –                | Yeast **             | –         | –            | –                    | Depends on the species    | Moyad [44] Hafed et al. [45] |
| 13.3   | 1072             | Gluconobacter japonicus | 100    | 99.12        | AB253433.1           | No                        | Callete-Rodríguez et al. [66] |
| 13.4   | 975              | Acetobacter sp.      | 100       | 98.87        | MW261886.1           | No                        | Kandi et al. [59] |
| 14.1   | 1094             | Enterococcus faecalis | 99       | 99.45        | CP041738.1           | No                        | Poulsen et al. [31] |
| 14.2   | –                | Yeast **             | –         | –            | –                    | Depends on the species    | Moyad [44] Hafed et al. [45] |
| 14.3   | 1050             | Weiella paramesenteroides | 100  | 99.60        | AY342326.1           | No                        | Libonatti et al. [67] |
| 15.1   | 1021             | Lactobacillus rhamnosus | 100     | 98.53        | CP044228.1           | No                        | Jung et al. [41] |
| 15.2   | 1046             | Staphylococcus epidermidis | 100 | 99.18        | EF522128.1           | Yes                       | Nguyen; Park; Otto [68] |
| 16.1   | 1097             | Citrobacter sp.      | 99        | 98.63        | KY630556.1           | Depends on the species    | Brenner et al. [34] Hasan; Sultana; Hossain [35] |
| 16.2   | 1062             | Klebsiella pneumoniae | 98        | 99.18        | AB614122.1           | Yes                       | Bosszczewski et al. [54] |
| 16.3   | 1052             | Enterobacter sp.     | 100       | 98.86        | MW412560.1           | Yes                       | Kus; Burrows [46] |
| 16.4   | 1015             | Pseudomonas aeruginosa | 100     | 99.81        | LR509473.1           | Yes                       | Morello et al. [69] |
| 16.5   | 1073             | Acinetobacter baumannii | 100 | 98.21        | CP044356.1           | Yes                       | McConnell et al. [30] |
| 17.1   | 1033             | Citrobacter sp.      | 99        | 99.13        | MT229332.1           | Depends on the species    | Brenner et al. [34] Hasan; Sultana; Hossain [35] |
| 17.2   | 1128             | Enterococcus sp.     | 100       | 97.87        | MZ229962.1           | Depends on the species    | Camargo et al. [42] |
| 17.3   | 1047             | Acinetobacter baumannii | 100 | 99.24        | CP042931.1           | Yes                       | McConnell et al. [30] |
| 17.4   | 1082             | Klebsiella pneumoniae | 100       | 99.08        | CP034420.1           | Yes                       | Bosszczewski et al. [54] |
| 17.5   | –                | Yeast **             | –         | –            | –                    | Depends on the species    | Moyad [44] Hafed et al. [45] |
| 18.1   | 1119             | Acinetobacter baumannii | 100 | 98.75        | CP045541.1           | Yes                       | McConnell et al. [30] |
| 18.2   | 1081             | Enterococcus faecalis | 100       | 99.35        | CP045918.1           | Yes                       | Poulsen et al. [32] |
| 18.3   | 484              | Sienotrophomonas maltophilia | 100 | 99.17        | CP040440.1           | Yes                       | Almeida et al. [70] |

Table 2 (continued)
generation times, i.e., higher growth rates than the target bacteria. In many cases, the carbon source in the culture medium used for on-farm production is not appropriate. For example, the use of sucrose provided as molasses for growth of *Bradyrhizobium* is not appropriate, as the preferred carbon sources are glycerol or mannitol [15]. Besides competition with contaminating microorganisms, the physical–chemical characteristics in the culture medium are also inappropriate for growth of the target microorganisms. For example, the adequate range of pH for *Bradyrhizobium* and *Azospirillum* is between 6.8 and 7.0 [15, 16, 76]; however, 94.4% of the samples had pH ranging from 3.6 to 5.9. The low pH can also favor the growth of contaminating microorganisms adapted to low pH and thus contributing to suppress the target microorganisms.

The lack of standardization in the incubation time is another problem in the samples taken from on-farm production in this study. The average growth time of the recommended *Bradyrhizobium* strains to reach the ideal concentration (at least 1 × 10^9 cells mL\(^{-1}\)) in the inoculant is approximately 7 days [76–79]. In contrast, many contaminants have much shorter generation times, and dominate the culture medium.

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### Table 2 (continued)

| Sample | DNA fragment (bp)* | Likely species/genus | Cover, %* | Identity, %* | GenBank access number | Potentially human pathogen | Reference |
|--------|-------------------|----------------------|-----------|--------------|-----------------------|---------------------------|-----------|
| 18.4   | 995               | *Citrobacter* sp.    | 100       | 99.90        | MT229332.1            | Depends on the species    | Brenner et al. [34] Hasan; Sultana; Hossain [35] |
| 18.5   | –                 | Yeast **             | –         | –            | –                     | Depends on the species    | Moyad [44] Hafed et al. [45] |
| 18.6   | 1033              | *Comamonas* sp.      | 100       | 99.52        | MT765012.1            | No                        | Ghanbarinia; Kheirbadi; Mollania [72] |
| C1     |                   | *Azospirillum brasilense* | 100       | 100          | SAMN08346097         | No                        | Hungria et al. [71] |
| C1     |                   | *A. brasilense*      | 100       | 100          | SAMN08354664         | No                        | Hungria et al. [71] |
| C2     |                   | *Bradyrhizobium japonicum* | 100       | 100          | AF234888             | No                        | Menna et al. [20] |
| C2     |                   | *B. diazoefficiens*  | 100       | 100          | AF234889             | No                        | Menna et al. [20] |
| C3     |                   | *B. elkanii*         | 100       | 100          | AF234890             | No                        | Menna et al. [20] |
| C3     |                   | *B. elkanii*         | 100       | 100          | AF237422             | No                        | Menna et al. [20] |

*DNA fragment (bp) sequenced; Coverage: percentage of the sequence of interest aligned with a sequence deposited at GenBank; identity: maximum identity obtained with the highest alignment scores

**The isolates identified as “yeast” under microscope observation were not subjected to molecular identification

***The isolates were numbered using the sample numbering as received in the laboratory followed by the number of the isolated colony. For example, isolate 1.5 is the 5th isolate of the sample 1

Commercial inoculants: C1, *Azospirillum brasilense* (strains Ab-V5 and Ab-V6); C2, *Bradyrhizobium japonicum* (SEMIA5079) and *B. diazoefficiens* (SEMIA5080); C3, *B. elkanii* (SEMIA587 and SEMIA5019), respectively

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**Fig. 2** Occurrence of prevailing genera based on metagenome analysis performed with the sample no. 10 produced on farm in Palotina, Paraná, Brazil. *Azospirillum*, the target microorganism, was not detected in the sample.
Table 3  Antimicrobial resistance test applied to isolates with pathogenic potential to humans obtained from samples of inoculants produced on farm aiming the multiplication of *Bradyrhizobium* spp. and *Azospirillum brasilense* in the 2019/20 growth season

| Sample Likely species/genus | Resistant to | Susceptible to |
|-----------------------------|--------------|----------------|
| 1.1 *Citrobacter* sp.       | FOX, AMC, AMP* | GEN, AMI, CPM, CFZ, CTR, CIP, SXT, IMI, AZT, CAZ, CHL, TET |
| 1.2 *Enterobacter* sp.      | AMP, FOX, AMC | CFZ, GEN, AMI, CPM, CFZ, CTR, CIP, SXT, IMI, AZT, CAZ, CHL, TET |
| 1.3 *Acinetobacter baumannii* | CTX, SXT | PTZ, CIP, CAZ, IMI, LEV, CPM, GEN, SAM, MRP, AMI |
| 1.5 *Enterococcus faecalis* | GEN, LNZ, AMP, STR, VAN, PEN | |
| 2.2 *Citrobacter* sp.       | IMI, AZT, AMP*, CFZ*, AMC** | GEN, AMI, CPM, FOX, CTR, CIP, SXT, CAZ, CHL, TET |
| 2.3 *Escherichia coli*      | – | ERT, MRP, CIP, FOX, IMI, SXT, CTX, AMP, GEN, TET, AMC |
| 2.4 *Enterococcus faecalis* | LNZ, AMP, STR, VAN, PEN | GEN |
| 3.4 *Enterococcus* sp.      | LNZ, PEN | AMP, GEN, STR, VAN |
| 4.2 *Acinetobacter nosocomialis* | CTR** | SAM, CAZ, CIP, LEV, IMI, MRP, GEN, AMI, PTZ, CPM, SXT |
| 4.4 *Enterobacter* sp.      | AMI, AMC, CHL, SXT, AMP*, CFZ* FOX*, GEN**, CPM** | AZT, CAZ, CIP, IMI, TET |
| 4.7 *Stenotrophomonas maltophilia* | – | LEV, SXT |
| 7.1 *Citrobacter* sp.       | AMI, AZT, GEN, IMI, AMP*, CFZ*, CTR** | AMC, CPM, FOX, CIP, SXT, CAZ, CHL, TET |
| 7.3 *Klebsiella pneumoniae* | AMP* | CFZ, GEN, AMI, AMC, CPM, FOX, CTR, CIP, SXT, IMI, AZT, CAZ, CHL, TET |
| 7.4 *Stenotrophomonas* sp.  | – | LEV, SXT |
| 7.5 *Atlantibacter hermannii* | AMP | CFZ, GEN, AMI, AMC, CPM, FOX, CTR, CIP, SXT, IMI, AZT, CAZ, CHL, TET |
| 8.4 *Enterococcus faecalis* | – | AMP, LNZ, PEN, STR, VAN |
| 10.1 *Enterococcus faecalis* | STR | AMP, LNZ, PEN, VAN |
| 11.3 *Enterococcus hirae*   | – | AMP, LNZ, PEN, STR, VAN |
| 12.1 *Acinetobacter* sp.    | – | CAZ, SXT, CIP, IMI, LEV, MR, PTZ, CTR, AMI, SUL, CPM, GEN |
| 14.1 *Enterococcus faecalis* | – | AMP, LNZ, PEN, STR, VAN |
| 15.2 *Staphylococcus epidermidis* | ERY | GEN, CLI, CIP, OXA, TET, CHL, LNZ, PEN |
| 16.1 *Citrobacter* sp.      | CFZ*, AMC* | SXT, AMI, CHL, TET, IMI, AMP, CIP, CAZ, CFZ, GEN, CTR, AZT, CPM, AMC |
| 16.2 *Klebsiella pneumoniae* | AMP* | AMC, SXT, AMI, CHL, TET, IMI, CFZ, CIP, CAZ, CFZ, GEN, CTR, AZT, CPM, AMC |
| 16.3 *Enterobacter* sp.     | AMC*, AMP*, CFZ* | SXT, CHL, TET, IMI, CFZ, CIP, CAZ, GEN, CTR, AZT, CPM |
| 16.4 *Pseudomonas aeruginosa* | – | GEN, CFZ, LEV, MRP, IMI, CPM, CIP, AZT, PTZ |
| 16.5 *Acinetobacter baumannii* | CTR | CAZ, SXT, CIP, IMI, LEV, MRP, PTZ, AMI, SUL, CPM, GEN |
| 17.1 *Citrobacter* sp.      | AMP*, CFZ*, AMC* | AME, SXT, AMI, CHL, TET, IMI, CIP, CAZ, CFZ, GEN, CTR, AZT, CPM |
| 17.2 *Enterococcus* sp.     | – | GEN, LNZ, AMP, STR, VAN, PEN |
in less than 24 h. Contaminating microorganisms compete for resources in the growth medium that becomes nutritionally poor and can also release inhibiting byproducts [81]. Therefore, it is reasonable to conclude that the high multiplication rates of the contaminating microorganisms, in addition to the low growth rates of the target microorganisms, result in the rapid depletion of the culture medium and enrichment with metabolites that inhibit the development of slow-growing microorganisms, like Bradyrhizobium and Azospirillum.

Multiplication of microorganisms without strict quality control can be risky to humans, animals, crops, and environment. Many contaminants are potentially pathogenic to humans and may cause various diseases, posing risks to the health of individuals who handle these products, or even final consumers if applied to products consumed in natura. Although potentially pathogenic microorganisms are found in the environment, they usually do not cause risk due to the low potential of inoculum in the environment. However, the multiplication of this microbial population in contaminated culture media could also magnify risks of infections or contaminations. For example, microorganisms from genera like Enterococcus, for which similar sequences were found in 61.1% of the samples, are frequently related to bacteremia, septicemia, urinary tract infections, abscesses, meningitis, and endocarditis [32, 82–84]. Some isolates also presented high genetic similarity with Citrobacter freundii [85], Enterobacter cloacae [86, 87], and Paenibacillus polymyxa [88], which are also potentially pathogenic to plants [86–88].

The possibility to carry genes of resistance to antimicrobials is a further concern in magnifying the population of potentially pathogenic contaminants in the on farm production. The spread of such genes in the environment may restrict the resources to fight infections. Some opportunist pathogens like Stenotrophomonas maltophilia are intrinsically resistant to several antimicrobials and collaborate to spread genes of resistance in the environment [70]. In this study, 12 isolates presented non-intrinsic resistance to antimicrobials, and 10 isolates presented resistance to two or more antimicrobials (1.1, 1.2, 1.3, 1.5, 2.2, 2.4, 3.4, 4.4, 7.1, and 18.4), what is an additional concerning issue.

Isolates identified microscopically as yeasts were not sequenced for genetic comparisons with sequences deposited in ribosomal databanks. However, some genera of yeasts can also cause injuries to humans and animals. Although yeasts are used in the manufacture of breads and beer, without any risk to humans and animals, like Saccharomyces cerevisiae, the genus Candida is the main pathogenic yeast and comprises approximately 200 species [89].

The approach based on metagenome for sample no. 10 showed that only contaminating microorganisms prevailed in the on farm sample. Although four morphologically distinct

| Sample | Likely species/genus | Resistant to | Susceptible to |
|--------|----------------------|-------------|----------------|
| 17.3   | Acinetobacter baumannii | CAZ, SXT    | AMC, SXT, AMI, CIP, CTR, CPM, GEN |
| 17.4   | Acinetobacter baumannii | AMP        | AMC, CTR, AZT, AMI, CTR, CPM, GEN |
| 18.1   | Enterococcus faecalis  | AMI*        | CTR, AMP, AMC, CIP, CTZ, AZT, AMI |
| 18.2   | Enterococcus faecalis  | AMI, CTR    | AMC, CTR, CPM, GEN, IMI |
| 18.3   | Staphylococcus aureus   | SXT         | AMC, SXT, CIP, CTR, CPM, GEN |
| 18.4   | Gram-positive sp.      | SXT         | AMC, SXT, CIP, CTR, CPM, GEN |

* Intrinsic resistance (natural of the microorganism)

| Intermediate resistance (natural of the microorganism) |
|-------------------------------------------------------|
| AMI, CTR, AMP, AMC, CTZ, AZT, AMI, GEN, IMI |

* Intrinsically resistant (natural of the microorganism)
colonies were isolated from that sample based on the culture medium approach, the metagenome approach revealed more than 10 genera, including the ones isolated based on the cultivation method. This indicates that the amount of contaminating microorganisms in the on-farm multiplications can be far higher than revealed by the culture-based method. In addition, even using a more sensitive method, the target microorganism was not found in that sample.

Studies on inoculants produced on farm and their impacts on production systems and potential risks to public health are scarce. However, our findings corroborate previous studies on bioinsecticides produced on farm, which revealed low concentration or absence of the target microorganisms Bacillus thuringiensis [12], and absence of Chromobacterium subsutgae and Saccharopolyspora spinosa [13]. However, there was high prevalence of contaminants in the samples, some of them potentially pathogenic to humans [12, 13].

The negative effect of low-quality bioproducts produced on farm goes beyond the risk to Brazilian quality of agricultural products, crops, and environment, because the benefits to the crops cannot be reached with its use. The lack of effect for not containing the target microorganism might put in doubt consolidated technologies that are important to the sustainability of cropping systems like the BNF in soybean by inoculation with Bradyrhizobium [3, 4], and more recently inoculation of grasses and co-inoculation of soybean with Azospirillum [7, 8, 11].

In conclusion, the samples of inoculants produced on farm assessed in this study were highly contaminated with several non-target microorganisms, whereas the target microorganisms Azospirillum and Bradyrhizobium were not detected in the great majority of the samples. In addition, the occurrence of contaminants presenting high genetic similarity with potentially pathogenic microorganisms, some of them carrying non-intrinsic resistance or multi-resistance to antimicrobials, may indicate risk to human health.

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Declarations

Conflict of interest The authors declare no competing interests.

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