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DOI: 10.2225/vol16-issue6-fulltext-1 *RESEARCH ARTICLE*

Guava *Psidium guajava* seed flour and dry *Aspergillus niger* mycelium as nitrogen sources for the production of biomass and antimicrobial compounds produced by *Weissella confusa*

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Abstract

Background: The fermentation substrate efficiency of glucose supplemented with guava seed flour (GGSF) or glucose supplemented with dry *Aspergillus niger* mycelium (GANM) was evaluated during the production of biomass and antimicrobial compounds by the lactic acid bacteria *Weissella confusa*.
Results: The fermentation substrate efficiency was measured by comparing the biomass formation, substrate consumption, substrate conversion, antimicrobial activity and product yield. The antimicrobial activity was measured against a commercial *Staphylococcus aureus* strain. The results were compared against fermentations performed in a commercial substrate (CS), the MRS (Man-Rogosa-Sharpe) substrate. The fermentations were performed discontinuously for 4 hrs at 100 rpm and 32ºC. The biomass production exhibited a statistically significant difference ($P \leq 0.05$) between treatments. The biomass production was 13.98% higher in the CS than in the GGSF and GANM substrates; however, there were no statistically significant differences for the specific growth rate.
Conclusions: The GGSF and GANM substrates favored an antimicrobial activity against *Staphylococcus aureus* during the second and third hours of fermentation (inhibition diameter was 6.11% and 4.72%, respectively). The GGSF, GANM and CS substrates did not present statistically significant differences for the production of antimicrobial substances against *Staphylococcus aureus*. Therefore, GGSF and GANM can be considered as viable and economical alternative nitrogen sources for the production of the antimicrobial compounds formed by *Weissella confusa* in submerged fermentations.

Keywords: agroindustrial residue; bacteriocin; biomass; inhibition.

INTRODUCTION

In the fermentation industry, the cost, availability and stability of fermentation substrates are topics of interest, as they can represent up to 68% of the production costs of fermentation products (Kwon et al. 2000; Serna-Cock and Rodríguez-De Stouvenel, 2007) and must have characteristics such as low contaminant levels, elevated fermentation rates, a high level of production and a low sub-product formation (Hurok et al. 2005; Serna-Cock and Rodríguez-De Stouvenel, 2007; Miller et al. 2011).

To achieve growth and the production of antimicrobial substances in a reasonable time, lactic acid bacteria (LAB) have stringent nutritional compound requirements such as carbohydrates, amino acids, peptides, nucleic acids and vitamins. These compounds have a high cost (Serna-Cock et al. 2010; Sica et al. 2010). Pure substrates that are utilized as carbon sources, such as lactose, glucose, starch and cellulose, require the addition of complex nitrogenized sources such as peptones and yeast extracts (Altaf et al. 2007; Serna-Cock and Rodríguez-De Stouvenel, 2007), which can be substituted for less

expensive renewable resources such as pigeon pea flour, red lentils, black chickpea, Bengal grain and soy (Hurok et al. 2005; Altaf et al. 2007; Rojan et al. 2009). However, Hofvendahl and Hahn-Hagerdal (2000) claim that the use of agroindustrial residues as the basis for fermentation substrates has the advantage of not contributing to the greenhouse effect. Agroindustrial residues that have been evaluated as fermentation substrates include juices extracted from the heart and leaves of sugar cane (*Saccharum officinarum* L.), red lentils, yeast cream and water from corn cooking (Ortíz et al. 2000; Altaf et al. 2007; Serna-Cock and Rodríguez-De Stouvenel, 2007). It is also possible to utilize low-cost renewable resources as alternative raw materials, such as starch and cellulose (Hofvendahl and Hahn-Hagerdal 2000).

Guava seed and the dry *Aspergillus niger* mycelium are agroindustrial residues that are potential alternative sources for the production of LAB, antimicrobial compounds, organic acids of interest, and other applications in the fermentation industry. Guava seed is a sub-product of the agroindustrialization of the guava *Psidium guajava* (Srivastava et al. 1997; Bernardino-Nicanoret al. 2006; Dhillon et al. 2011), and *Aspergillus niger* dry mycelium is a byproduct of the food and pharmaceutical industries (Cai et al. 2006). Guava agroindustrialization generates up to 120 kg of guava seeds per processed guava ton, and in the pharmaceutical industry, the final fermentation mass can generate up to 20 gL^{-1} of dry mycelium, which could constitute an alternative nitrogen source for other fermentations (Serna-Cock et al. 2012).

LAB, the extracts and metabolites produced by them, have been demonstrated to control diverse contaminating microorganisms and can thus extend food shelf life and provide security against bacteria that can affect the health of the consumer (Vásquez et al. 2009). The antimicrobial effects of these bacteria are mainly associated with a low pH, competition for nutrients and metabolite production (Sica et al. 2010). Najafian and Babji (2012) established the importance of discovering new antimicrobial substances that can be used as vaccines in the future, inactivate specific pathogens and be used as food preservatives.

Therefore, it is important to study agroindustrial residues as substitutes for some of the high-cost substrate components, such as peptones, yeast extract and vitamins.

The objective of this work was to evaluate the efficiency of the guava *Psidium guajava* seed and dry *Aspergillus niger* mycelium as alternative of nitrogen sources in submerged fermentations for the production of the LAB *Weissella confusa* and its compounds responsible for antimicrobial activity. The efficiencies were measured in terms of the biomass formation, substrate consumption, substrate conversion, antimicrobial activity and product yield (lactic acid). The antimicrobial activity was measured against a commercial *Staphylococcus aureus* strain.

MATERIALS AND METHODS

Agroindustrial residues

Guava seed flour and dry *Aspergillus niger* mycelium were selected as substrates because they provide a good source of nitrogen and vitamins, which can contribute to *Weissella confusa* growth. In addition, they could be used as low-cost alternative substrates for the production and commercialization of the microorganism (Serna-Cock et al. 2012).

Guava seeds *Psidium guajava,* which were provided by Dulces San Antonio La Tentación (Vélez-Santander County, Colombia), were used. The seeds were subjected to a hot air drying process (60ºC, 23 hrs) (Binder ED 115-UL, Germany). Next, a flour was obtained by grinding the seeds to a particle diameter of 0.5 mm (FRITSCH Pulverisette 14, Germany). The dry *Aspergillus niger* mycelium was provided by SUCROAL, Inc. (Valle del Cauca, Colombia). The agroindustrial residues were characterized by proximal analysis (moisture, nitrogen, protein, ash, ethereal content, fiber and reducing sugars) (AOAC, 1990) and iron, potassium, zinc, magnesium, calcium, phosphorus and vitamins A, C, B_2, B_3 and B_6 contents.

Fermentation

Microorganism. A *Weissella confusa* strain that was cryopreserved in glycerol (20% wv^{-1}, -20°C) was used. The strain was previously isolated in investigations performed by Serna-Cock et al. (2010). For these experiments, 10% (vv^{-1}) of the *Weissella confusa* culture was inoculated in 5 mL of MRS broth (De Mann-Rogosa-Sharpe, Scharlau Microbiology, Spain), supplemented with 40 gL^{-1} glucose (Merck, Germany) and incubated at 32ºC (INE400, Memmert, Germany) for 24 hrs.

Fermentation. Nine discontinuous fermentations were performed using the following substrates: glucose supplemented with guava seed flour (GGSF; 60 gL^{-1} of glucose and 222.77 gL^{-1} of guava seed flour), glucose supplemented with dry *Aspergillus niger* mycelium, (GANM; 60 gL^{-1} of glucose and 85.49 gL^{-1} of dry *Aspergillus* niger mycelium) and pure commercial substrate (CS; 60 gL^{-1} of MRS Broth and 20 gL^{-1} of glucose). As a CS, the MRS broth was used according to the methodology established by Serna-Cock et al. (2010). The GGSF and GANM substrates were prepared with distilled water. Glucose content of each of the substrates was adjusted to reach 60 gL^{-1}. The substrates were sterilized in an autoclave for 15 min at 121ºC. The fermentations were performed in 500-mL Erlenmeyer flasks, with a working volume of 250 ml. The speed was 100 rpm in an incubating orbital shaker (VWR -Incubating Orbital Shaker, EE.UU.), for 4 hrs at 32ºC. The strain was adapted to the fermentation conditions for 3 generations, and in each case, a 10% inoculation volume was used with respect to the substrate volume. Every half hour, the pH was adjusted to 6.0 (Mettler Toledo - SevenEasy, Switzerland) with 1 M NaOH (Mol Labs, Bogotá, Colombia).

Biomass formation and substrate consumption. To determine the substrate consumption and biomass formation kinetics for each substrate, 20 mL of substrate was aseptically taken at 0, 1, 2, 3 and 4 hrs of fermentation (time 0 represented the initial conditions for the LAB-inoculated substrate). The samples were centrifuged at 5000 rpm for 30 min at 4ºC (5840R Eppendorf Centrifuge, Germany) and subsequently passed through a 0.45-µm filter (Titan, USA).

Based in the kinetics was calculated to *Weissella confusa* the specific growth rate (µ). The substrate consumption (reducing sugars) was determined by spectrophotometry (Genesis 10UV, Thermo Scientific, USA) using the DNS method (3,5- dinitrosalicylic acid method) (Miller, 1959).

The biomass formation kinetics were determined by colony counting by utilizing depth seeding with 15 mL of Nutritive Agar (Scharlau 01-140, Spain), and the incubation was performed for 24 hrs at 32ºC (INE400, Memmert, Germany). Subsequently, the Colony Forming Units (CFU) were counted.

Substrate conversion and product yield. The substrate conversion (SC) expressed as a percentage (1) and product yield ($Y_{p/s}$) expresed in gg^{-1} (2) were calculated with the Equation 1 and Equation 2.

$$SC = \frac{(S_o - S) * 100}{S_o}$$

[Equation 1]

$$Y_{\frac{p}{s}} = \frac{P}{S_o - S}$$

[Equation 2]

Where S_0 is the initial reducing sugar concentration (gL^{-1}), S is the final reducing sugar concentration (gL^{-1}) up to the time in which P is the maximum, and P is the maximal lactic acid concentration (gL^{-1}).

Lactic acid concentration was performed in Reflectoquant (plus 10 RQflex Merck, Germany), using the lactic acid test (Method reflectometric with test strips from 3.0 to 60 mgL^{-1}).

***Weissella confusa* antimicrobial activity measurement.** *Staphylococcus aureus* (ATCC® 25923TM*) was used as an indicator strain of the antimicrobial activity. The previously described fermentation substrates were used. The antimicrobial activity tests were performed at each fermentation time and for each evaluated substrate using the method described by Serna-Cock et al. (2010). Plates with 5-mm-thick Baird-Parker Agar (Scharlau 01-030, Spain) were used. The plates contained specific nutrients for the growth of the pathogen *Staphylococcus aureus*. A central perforation was made in each plate with a sterile punch (1.5 cm in diameter). The plates were seeded with 1 x 10^6 CFU mL^{-1} of the pathogen, which was adapted to the growth conditions for 2 generations. In each case, a 10% inoculation volume with respect to the substrate volume was utilized. Similarly, for each fermentation time and for each substrate evaluated, sterile MRS agar circles (5 mm thick and 1.5 cm in diameter) were aseptically taken and inoculated with 0.1 mL of *Weissella confusa* (different concentrations depending on the fermentation time), which were obtained at each of the fermentation times and for each substrate evaluated. The inoculated MRS agar circles were introduced into the perforation of the Baird-Parker Agar plates (Scharlau 01-030, Spain). The plates were incubated at 32ºC for 2 hrs. After the incubation time, the pathogen growth inhibition halo was measured with a millimetric ruler. The inhibition tests for the pathogen were performed in triplicate for each of the times and for every fermentation substrate.

Experimental design and statistical analysis

A 3*5 factorial design was used, with two factors and three replicates. Thus, the substrate factor had three levels, as follows: glucose supplemented with guava seed flour (GGSF), glucose supplemented with *Aspergillus niger* dry mycelium (GANM) and the pure commercial substrate (CS) MRS broth. The fermentation time factor had five levels as follows: 0, 1, 2, 3 and 4 hrs of fermentation. The response variables were the following: biomass formation, substrate consumption, substrate conversion, antimicrobial activity (*Weissella confuse* towards *Staphylococcus aureus*) and product yield. The results were analyzed with SAS 9.2 statistical software for Windows (SAS Institute, Inc., Cary, NC, USA) (SAS, 1993). An average comparison was performed with the Duncan test with a 95% level of confidence ($P \leq 0.05$).

RESULTS AND DISCUSSION

Figure 1 presents the *Weissella confusa* biomass formation. Statistically significant differences were observed between the CS and GGSF treatments ($P \leq 0.05$). The maximal biomass production with CS was obtained at 4 hrs, with 8.94 Log_{10} CFU mL^{-1}, which was followed by GGSF and GANM, with values of 8.45 and 7.69 Log_{10} CFU mL^{-1}, respectively.

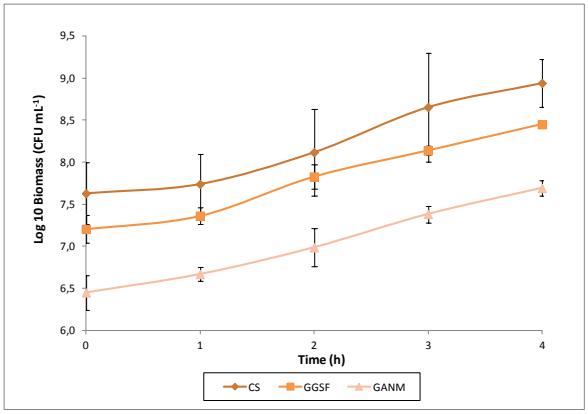

Fig. 1 The *Weissella confusa* biomass formation kinetics utilizing the commercial substrate (CS), glucose supplemented with guava seed flour (GGSF) or glucose supplemented with *Aspergillus niger* (GANM) dry mycelium.

The differences in biomass production observed in the agroindustrial residues evaluated (GGSF and GANM) are due to the presence of micronutrients in the guava seed flour such as phosphorous (0.16055 g), potassium (0.300 g), vitamins A (0.05013 g), C (0.0002 g), B_3 (0.00016 g) and B_6 (0.00042 g) (in 100 g of dry matter) that are essential for enzymatic reactions and the synthesis of cellular membranes and walls (Serna-Cock and Rodríguez-De Stouvenel, 2007; Lu et al. 2009; Serna-Cock et al. 2012); these elements are not found in the *Aspergillus niger* dry mycelium (GANM) (Serna-Cock et al. 2012), which is evidence of the complex nutritional requirements and the limited biosynthesis capacity of LAB. The specific growth rates (μ), did not present statistically significant differences among the substrates, with values of 0.35, 0.32 and 0.32 h^{-1} for CS, GGSF and GANM, respectively.

The results suggest that GGSF could become a low-cost alternative for *Weissella confusa* production, which is a microorganism with great potential for the control and treatment of bovine mastitis (Serna-Cock et al. 2010).

The substrate consumption rate (Figure 2) did not present statistically significant differences when comparing treatments ($P \geq 0.05$). The values for the residual substrate were 71.49%, 79.89% and 79.50% for the CS, GGSF and GANM substrates, respectively; these values close to those reported by Serna-Cock et al. (2010) for *Weissella confusa* production utilizing milk supplemented with yeast extract as a substrate.

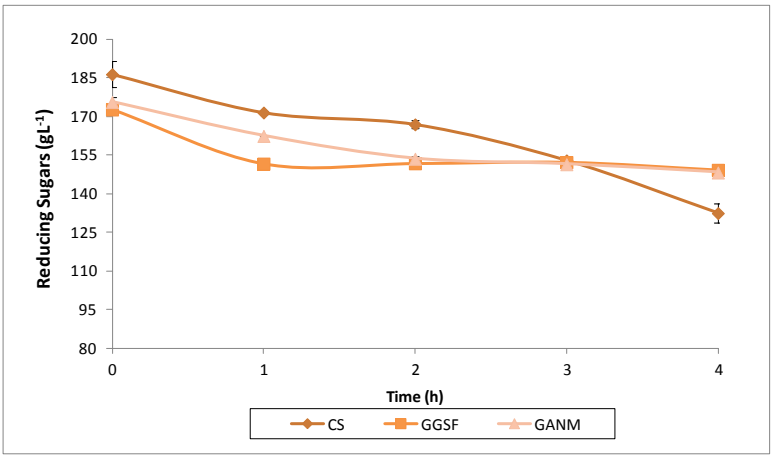

Fig. 2 The *Weissella confusa* substrate consumption kinetics utilizing the following: Commercial substrate (CS), glucose supplemented with guava seed flour (GGSF) and glucose supplemented with dry *Aspergillus niger* mycelium (GANM).

CS substrate containing yeast extract, peptone, meat extract, sorbitan monooleate, magnesium and manganese acetate; these compounds provide cofactors, which can inhibit the development of other microorganisms, and play an important role in the complete conversion of glucose, and lactic acid production (Hofvendahl and Hahn-Hagerdal, 2000). The statistics differences are directly related to the concentration of different nutrients provided by each substrate, reflecting the complex nutrient demand and the limited capacity of biosynthesis of lactic acid bacteria (Hofvendahl and Hahn-Hagerdal, 2000).

In the first two hours of fermentation, the GGSF and GANM substrates exhibited higher substrate conversion rates (Figure 3). The largest inhibition diameters against the pathogen relative to the CS substrate were also found during this time period (Figure 4). The substrate conversion during the first and second hours were 18.25% and 18.44%, respectively, for the GGSF substrate, 12.63% and 17.66%, respectively, for the GANM substrate and 8.01% and 10.25%, respectively, for the CS substrate. Figure 3 indicates that fermentation substrates formulated with agroindustrial residues result in an efficient use of the carbon source for up to the third fermentation hour by converting it into biomass.

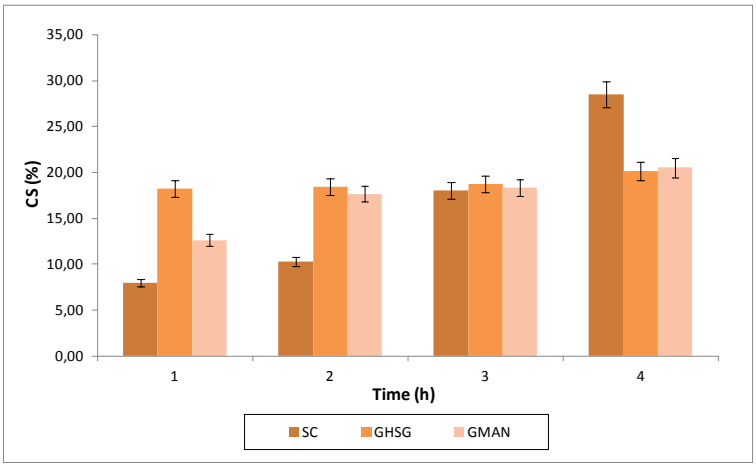

Fig. 3 The *Weissella confusa* substrate consumption kinetics utilizing the following: Commercial substrate (CS), glucose supplemented with guava seed flour (GGSF) or glucose supplemented with *Aspergillus niger* mycelium (GANM).

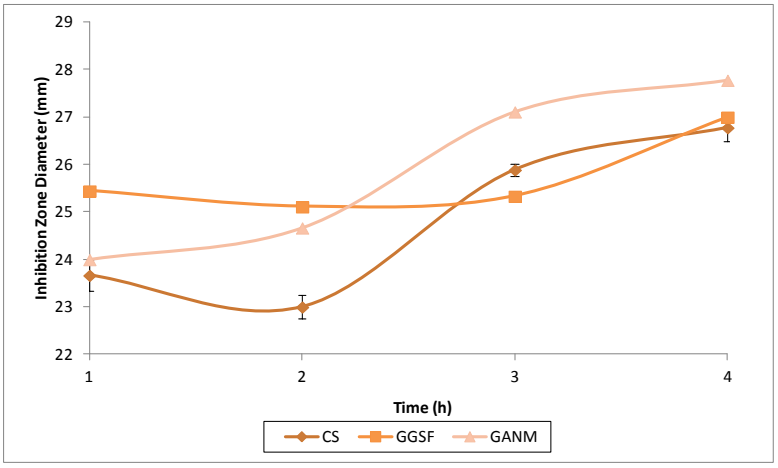

Fig. 4 The *Weissella confusa* antimicrobial kinetics against *Staphylococcus aureus* with the following substrates: Glucose supplemented with guava seed flour (GGSF), glucose supplemented with dry *Aspergillus niger* mycelium (GANM) and a pure commercial substrate (CS), MRS broth.

The antimicrobial activity did not present statistically significant differences for the three substrates during the 4 hrs of fermentation (Figure 4). *Weissella confusa* presented the largest antimicrobial activity against *Staphylococcus aureu*s at the fourth hour of fermentation in the GANM substrate, with an inhibition diameter of 28 mm, which was larger than the inhibition diameters reported by Serna-Cock et al. (2010) for the same fermentation time using a commercial substrate and milk supplemented with yeast extract. The effects of the *Weissella confusa* antimicrobial activity are due to the production of BLIS (bacteriocin-like inhibitory substance) protein compounds or the combined effect that the antimicrobial agents mediate (Cheikhyoussef et al. 2008; Serna-Cock et al. 2010).

Previous studies have reported *Weissella confusa* strains with antimicrobial and probiotic properties (Serna-Cock et al. 2010; Ayeni et al. 2011; Lee et al. 2012). These agroindustrial-based substrates could be used as a support matrix for LAB with probiotic potential, as the results of this investigation suggest that LAB can grow and develop its antimicrobial activity in this type of substrate. Studies conducted by Bernardino-Nicanoret al. (2006) indicate that because of their nutritional value (fiber and protein) and functional properties, guava seeds can be used for human and animal consumption (Bernardino-Nicanor et al. 2005).

The greatest *Weissella confusa* antimicrobial activity occurred during the exponential phase of microbial growth, indicating that the antimicrobial compound is associated with growth, which suggests that it could be synthesized as a primary metabolite (Serna-Cock et al. 2010). For medicinal applications, these antimicrobial metabolites are preferred over conventional bactericide antibiotics because they destroy bacteria faster and are not affected by antibiotic resistance mechanisms (Najafian and Babji, 2012).

Statistically significant differences were observed between the CS, GGSF and GANM substrates ($P \leq 0.05$). The highest yield of product (Figure 5) was obtained in CS substrate, followed by GGSF and GANM. The decrease in product yield in the first hours of fermentation, is caused by the accumulation of lactic acid because it generates inhibitory effect on cell growth, causing a decrease in conversion of substrate to product (Arias et al. 2009).

The lactic acid concentration (4 hrs of fermentation) was 4.6 gL^{-1} in CS, 0.8 gL^{-1} GGSF and 0.3 gL^{-1} GANM. These results were similar to those obtained with barley and wheat (0,8 gL^{-1}) as only source of nutrients (Oh et al. 2005). Altaf et al. (2005), obtained 0.88 gg^{-1} of lactic acid concentration from starch, and nitrogen sources of low cost, such as peptone and yeast extract.

As for the production costs, the utilization of guava seeds reduces the substrate cost by a factor of 8.5 compared to the CS cost.

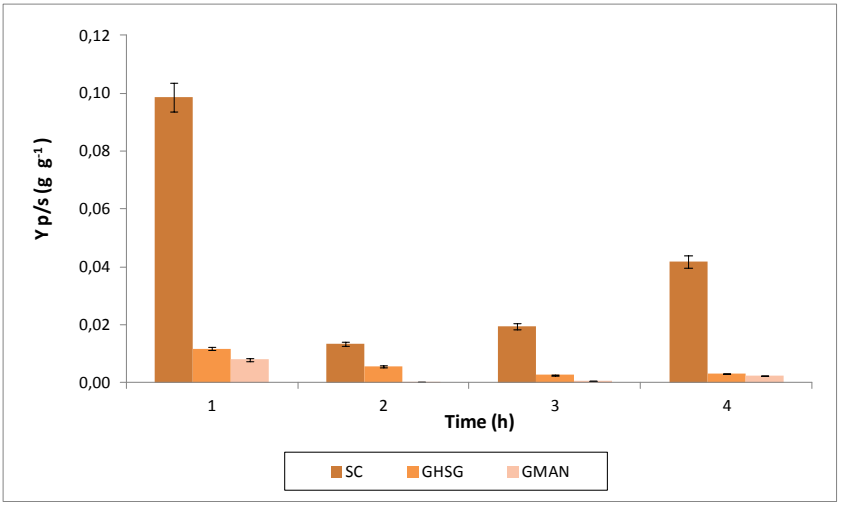

Fig. 5 Variation product yield utilizing commercial substrate (CS), glucose supplemented with guava seed flour (GGSF) or glucose supplemented with dry *Aspergillus niger* mycelium (GANM).

CONCLUDING REMARKS

The guava seed flour and dry *Aspergillus niger* mycelium have potential for use in the production of LAB for probiotic purposes and for the production of antimicrobial products to biopreserve food. When fermentation substrates are supplemented with guava seed flour and dry *Aspergillus niger* mycelium in submerged fermentations for the production of *Weissella confusa*, it is possible to obtain inhibition diameters against *Staphylococcus aureus* that are similar to those observed with commercial substrates. Therefore, these agroindustrial residues are a very interesting nitrogen source for the production antimicrobial compounds.

From an economic perspective, the use of a glucose substrate supplemented with guava seed flour for *Weissella confusa* biomass production is a viable alternative, as the seed is a product of the agroindustrialization of the guava and is available at a low cost (8.5 times lower than the CS cost); in addition, its contents include important growth factors.

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The commercial substrate contains as nitrogen source yeast extract, peptone and meat extract and elements such as sorbitan monooleate, magnesium, manganese and acetate, which provide cofactors that can inhibit the development of other microorganisms. These factors play an important role in the complete conversion of glucose and lactic acid production, which favors *Weissella confusa* development. The high cost of this substrate is a major obstacle for the industrial production of this bacterium.

Future research will be necessary to establish the behavior of substrates supplemented with guava seed flour and dry *Aspergillus niger* mycelium with other LAB that have a well-known commercial value.

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