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Antimicrobial Activity of Broth Fermented with Kombucha Colonies

Rodrigo José Santos Júnior[1], Rejane Andrade Batista[1], Sheyla Alves Rodrigues[2], Lauro Xavier Filho[1,2], Álvaro Silva Lima[1,2]*

[1]Universidade Tiradentes, Av. Murilo Dantas, 300, Prédio do ITP. CEP: 49032-490, Aracaju-Sergipe-Brazil
[2]Instituto de Tecnologia e Pesquisa, Av. Murilo Dantas, 300, Prédio do ITP. CEP: 49032-490, Aracaju-Sergipe-Brazil

Abstract

The kombucha is a consortium of yeast and bacterias originally from China, and able to produce a fermented broth, which presents antimicrobial activity against some pathogenic microorganisms. The goal of this work was to investigate the antimicrobial activity of fermented broth by kombucha colonies in the same condition used in a hospital in the Northeast of Brazil and to optimize the medium of kombucha growth. The fermented growth was efficient against *Microsporum canis* (LM-828), *Escherichia coli* (CCT-0355) and *Salmonella typhi* (CCT-1511). The best conditions of inhibition against *M. canis* (> 32mm) and *E. coli* (16 mm) was observed at pH 4.0, 55% of commercial sugar and 0.10 g/l of $MgSO_4$, and for *S. typhi* (32 mm) without $MgSO_4$. The conditions and time of fermentation used in the hospital are wrong.

Keywords: Kombucha; Antimicrobial; Fermentation; Microorganisms; Pathogens

Introduction

Kombucha is a symbiotic association of bacteria (*Acetobacter xylinum* and *Bacterium gluconicum*) and yeast strains (*Zygosaccharomyces kombuchensis*, *Pichia fluxum* and *Saccharomyces* sp.) (Kurtzman et al., 2001). The variation of its composition could be due to geographic, climatic and cultural conditions as well as diversity of local species of wild yeasts and bacteria (Teoh et al., 2004). These microorganisms are able to grow in culture medium formed of tea infusions (black, mate and green), supplemented by a carbon source. The broth fermented is called "tea fungus" and is originally from the northeast of China (Manchuria). The beverage was introduced in Russia by oriental merchants and then into Eastern Europe and Europe around the turn of this century. This refreshing beverage tasting like sparkling apple cider is often produced at home by fermentation using a tea fungus passed from house to house (Dufresne and Farnworth, 2000).

The fermentation and oxidation processes starts, when the tea fungus is placed in a freshly prepared infusion of tea and sugar. When grown in sucrose medium, colonies of yeast break the sucrose in glucose and fructose, than produce carbon dioxide and ethanol, which are oxidized to acetaldehyde by bacteria of the colonies. The tea fungus produces many other substances, like gluconic acid and vitamins, which with the supply of tea nutrients, give the drink its unusual flavor and healing properties. The glucose is polymerized and produces cellulose and hemicellulose (Greenwalt et al., 1998; Bauer-Petrovska and Petrushevska-Tozi, 2000). A wide range of flavor compounds, including alcohols, aldehydes, ketnes, esters and amino acids have been identified from fermented broth (Teoh et al., 2004).

The fermentation using kombucha colonies is composed of two portions: a floating cellulose pellicle layer, formed during the fermentation by *A. xylinum*, and the sour liquid broth (fermented broth) (Jayabalan et al., 2008). The fermentation using kombucha as a biological agent is conduced at ambient temperature for up to 7-10 days and produces a carbonated fermented broth, softly acid and with low concentration of ethanol. This broth presents beneficial effects, such as, antibiotic properties, regulation of gas-gastric, intestinal and glandular activities, relief of joint rheumatism, gout and hemorrhoids, positive influence on the cholesterol level, arteriosclerosis, toxin excretion and blood cleansing, diabetes, and aging problems, and it has been claimed to be a prophylactic and therapeutic beneficial agent to human health – from weight loss to curing cancer (Mayser et al., 1995; Cvetkovic et al., 2008). The beneficial effects of kombucha tea are attributed to the presence of tea polyphenols, gluconic acid, glucuronic acid, lactic acid, vitamins, aminoacids, antibiotics and a variety of micronutrients produced during the fermentation (Jayabalan et al., 2008). Many of these compounds are found in the tea's composition. Mate (*Ilex paraguariensis* st. Hill) is a plant originally from South America, which has 80% of its planted area in Brazil (Esmerilindro et al., 2002). The leaves are used in the production of tea, and presents in its composition: fiber (14.96-19.95%), fats (5.57-9.10%), protein (8.30-13.45%), glucose (1.30-6.14%), sucrose (3.60-6.90%), caffeine (0.97-1.79%) among other substances like tannin (Heinrichs and Malavolta, 2001).

Kombucha colonies grown in mate tea infusion with commercial sugar are used in *Nossa Senhora da Conceição* Hospital from Lagarto-SE, Brazil. After 7 days of fermentation, cellulose of fermented broth is separated, and then can be processed to obtain an artificial skin. Some authors use this skin to accelerate healing and as an antiseptic by adhering it to open injuries (Xavier Filho and Paulo, 1990), the so-called Bioskim (Vicente et al., 2001). On the other hand, some authors use the fermented broth

***Corresponding author:** Álvaro Silva Lima, Av. Murilo Dantas, 300, Farolândia, Aracaju-SE, Brazil. 49032-490, Tel/Fax: +557932182190; E-mail: aslima2001@yahoo.com.br

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as a prophylactic product or agent to decrease the gastrointestinal problems (Dufresne and Farnworth, 2000). Therefore, the goal of this work is to evaluate the antimicrobial activity of broth fermented with kombucha colonies, in the same conditions produced in the *Nossa Senhora da Conceição's* Hospital, against microorganisms that provide gastrointestinal and dermatomicoses disorders. For this matter, the culture medium composition (initial pH value, sugar concentration and magnesium sulphate concentration) was optimized to produce antimicrobial agents.

Material and Methods

Microorganism

The kombucha colonies used in this study were obtained from a local hospital (Nossa Senhora da Conceição) in Lagarto-Sergipe, Brazil. The colonies were maintained at the Laboratory of Bioprocess Engineering at The Institute of Science and Technology in Aracaju-Sergipe, Brazil. The kombucha was grown at room temperature in tea solution (0.5%, w/v) added with 35%

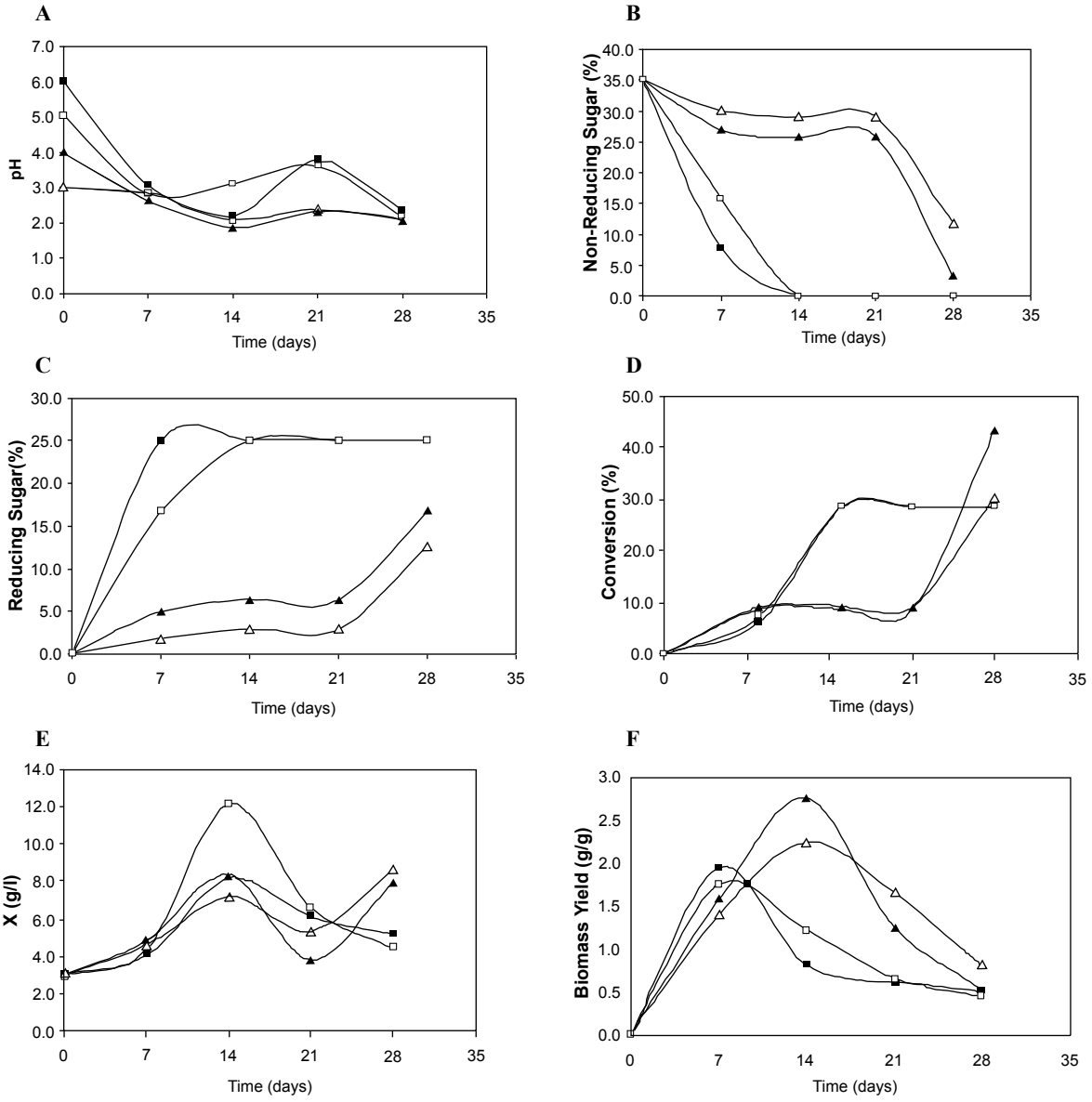

Figure 1: Influence of initial pH value in fermentation by kombucha at 25°C, 35% (w/v) of sugar concentration and without $MgSO_4$ (A- pH, B- non-reducing sugar, C- reducing sugar, D- conversion, E- biomass and F- biomass yield): (■) 3.0, (□) 4.0, (▲) 5.0, and (△) 6.0.

	Diameter of halo zone (mm)							
	Microsporum canis				*Salmonela typhi*			
Time (days)	3.0	4.0	5.0	6.0	3.0	4.0	5.0	6.0
0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
14	18	20	0	24	0	0	0	0
21	24	26	16	16	20	0	0	0
28	26	22	26	24	0	0	0	0

Table 1: Antimicrobial activity of fermented broth kombucha at different pH initial values, 35% (w/v) sugar concentration, without $MgSO_4$ and 25°C.

(w/v) of commercial sugar. The solution was sterilized at 121°C for 15min.

Fermentation process

First, 0.5% (w/v) mate tea was added to water and boiled for 15 min. The tea was then cooled to room temperature and filtered through a sieve. Subsequently, commercial sugar (25, 35, 45 and 55%, w/v) and $MgSO_4.7H_2O$ (0.10; 0.15; 0.20 and 0.25 g l^{-1}) were added into the tea, and the pH (3.0, 4.0, 5.0 and 6.0) was adjusted using acetic acid 4% (v/v) and NaOH 1% (w/v). The culture medium was sterilized at 121°C for 20 min. The kombucha colonies were cultivated in 500 ml Erlenmeyer flasks containing 200 ml of different culture medium. An initial amount of 6.0 (0.6 g in dry bases) of kombucha colonies were inoculated in each flask.

Assays

The pH value was determined using a digital pHmeter. Biomass was analyzed for its moisture content by drying the sample at 105°C until constant weight was obtained. Reducing and non-reducing sugar were determined by Lane-Eyon's methods. All determinations were performed in triplicate (the standard deviations were < 0.2) (AOAC, 1998). The biomass yield was assessed by mass measurement per gramme of commercial sugar used (g g^{-1}); of which the mass was formed from microorganisms and cellulose floating pellicle layer which were removed from the fermented liquid surface, rinsed with distilled water and dried with filter paper (Harta et al., 2004; Malbasa et al., 2008). The conversion of sucrose and other compounds were determined by the equation 1.

$$Conversion = \left[1 - \left(\frac{\text{Re}\,sidual \quad Sugar}{Initial \quad Sugar}\right)\right] \times 100 \qquad (1)$$

Antimicrobial activity

When the fermentation reached the desired endpoint, the broths were used to determine the antimicrobial activity. Ketoconazole (50 µg ml^{-1}) and Chloramphenicol (30 µg ml^{-1}) were used as antimicrobial compound for fungi and bacteria (control), respectively. They were chosen for inclusion in the antimicrobial assay based on their availability in the Centers of Basic Assistance in Health of the Brazilian Health Public System. The target microorganisms, *Microsporum canis* (LM828), *Escherichia coli* (CCT-0355), *Pseudomonas aeruginosa* (ATCC-27853), *Staphylococus aureus* (ATCC-6533), *Salmonela typhi* (CCT-1511), *Shigella sonnei* (CCT-1484), were grown in Bushell-Hass medium (Composition: g l^{-1}: $MgSO_4$ – 0.2; $CaCl_2$ – 0.02; KH_2PO_4 -1.0; $(NH_4)_2HPO_4$ – 1.0; KNO_3 – 1.0 and $FeCl_3$ – 0.05) to a similar optical density 0.5 (MacFallen scale). Suspensions (1 ml) of these target microorganisms were uniformly spread on the plates (Müller-Hinton medium and Sabouraud medium for bacteria and fungi, respectively) and wells of 6 mm of diameter were performed with a sterile glass tube by means of a vacuum pump. Samples of fermented broth (50 µl) were then transferred into the wells in the agar plates, previously inoculated with the target microorganisms (Silva et al., 2009). The plates were then incubated at 37°C. The diameter of inhibition halo was measured after 24-48 h (Seeramula et al., 2000). All determinations were performed in triplicate (the standard deviations were < 1.5).

Results

Influence of initial pH value

The results of fermentation process with different initial pH values are shown in the Figure 1. During the first time of fermentation the pH value decreased due to yeast and bacteria present in kombucha colonies, which metabolize sucrose into organic acids such as acetic acid and gluconic acid. At the end of fermentation (28 days) the pH values were approximately 2.17.

The non-reducing sugar (sucrose) decreased for all initial pH values, which was due to enzymatic hydrolysis of sucrose in glucose and fructose by action of yeast present in the colonies. The metabolism of kombucha colonies were most strongly evidenced for the lowest initial pH value. The concentration of reducing sugar increased continuously, and this was attributed to the balance between production of reducing sugar and consumption of non-reducing sugar in the biotransformation of ethanol in organic acid. For the lowest initial pH value, it was observed that the best conversion (28.57%) occurred on day 21 of fermentation, after this the best values of conversion were 30.36 – 40.33% at the highest initial pH value, as shown in Figure 1B and C.

The microorganism growth presents an exponential growth phase until 14 days of fermentation, when begins the stationary growth phase for all initial pH value. The best biomass concentration was 2.42 g/l (pH 4.0) at day 14 of fermentation. The highest biomass yield was verified for pH 3.0 and 4.0 (1.95 and 1.75 g/g, respectively) at day 7 and for pH 5.0 and 6.0 (2.77 and 2.24 g/g, respectively) at day 14, as shown in Figure 1E and F.

The fermented broth by kombucha colonies was active against *M. canis* (after 14 days) and *S. typhi* (at 21 days), as shown the Table 1, and it did not verify inhibition halo zone for the microorganisms target *E. coli, Ps. aeruginosa, Staph. aureus* and *Sh. sonnei.*. The inhibition halo zone for *M. canis* increased with the fermentation time and the acid pH value had less influence in the antimicrobial activity. At day 28 of fermentation, the final pH values for fermentations with different initial pH values were approximately 2.3 and the inhibition halo zone were 26, 22, 26 and 24mm for initial pH value 3.0; 4.0; 5.0 and 6.0, respectively. Ketoconazole inhibited *M. canis* with a 17 mm halo zone and Chloramphenicol presented an inhibition halo zone of 16 mm for *Sh. sonnei*.

The initial pH value chosen was 4.0 due to continues, fast and high antimicrobial activity to *M. canis*.

The influence of commercial sugar concentration

The influence of commercial sugar in fermentation using kombucha colonies are observed in the Figure 2. The pH values present the same trend of the previous experiments, which is to decrease with the course of fermentation.

During the fermentation, the conversion from non-reducing sugar to reducing sugar was highest with the increase of commercial sugar concentration; the best value (approximately 55%) was verified for 25% commercial sugar concentration. This observation is attributed to highest consumption of sucrose in the experiment with lower commercial sugar concentration, which is due to the enzymatic action produced by colony yeast, and also because of the high concentration of reducing sugar in the fermented broth.

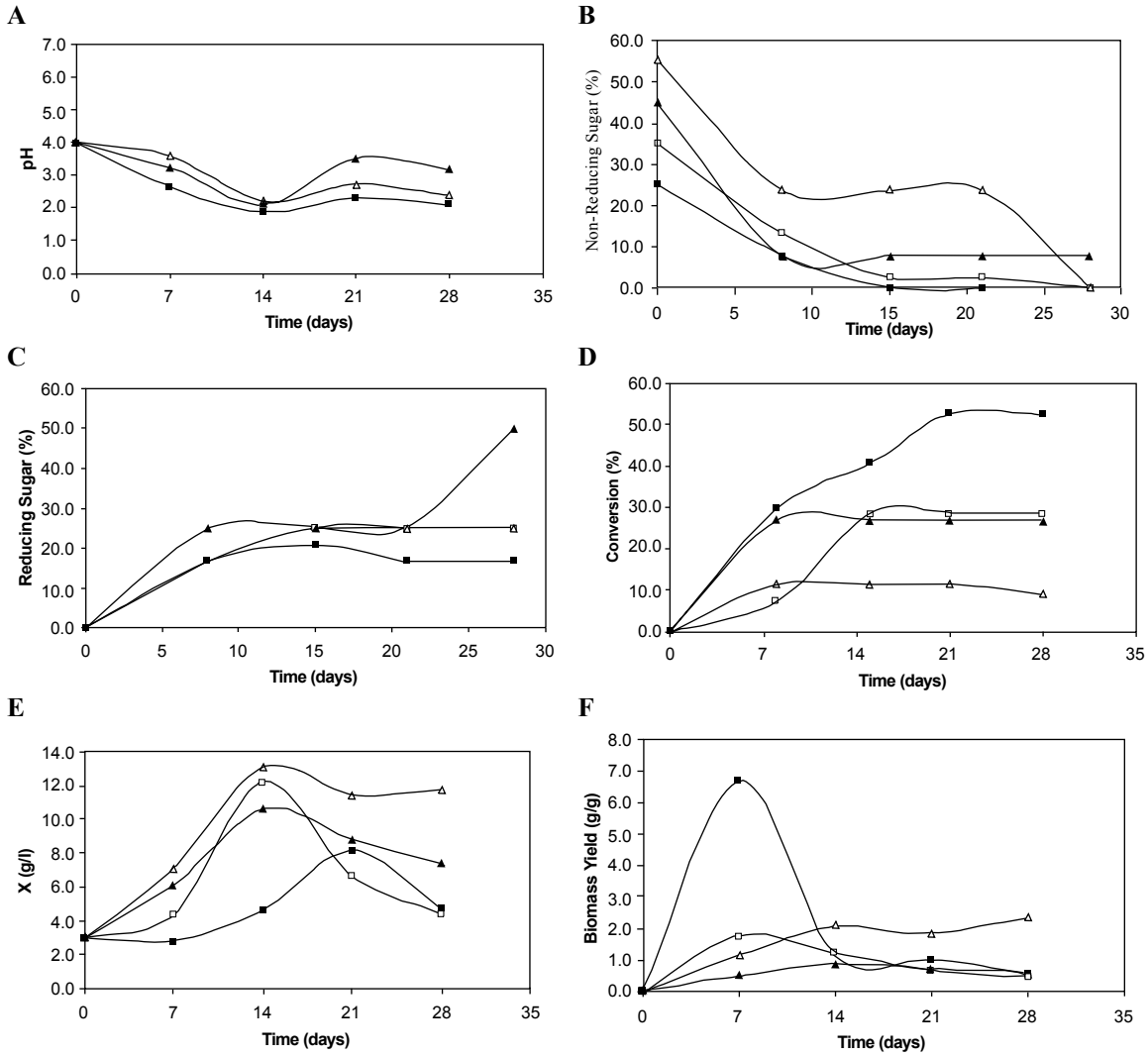

Figure 2: Influence of sugar concentration in fermentation by kombucha at pH 4.0, 25ºC and without $MgSO_4$ (A- pH, B- non-reducing sugar, C- reducing sugar, D- conversion, E- biomass and F- biomass yield): (■) 25%, (□) 35%, (▲) 45%, and (△) 55%.

	Diameter of halo zone (mm)											
	Microsporum canis				*Salmonela typhi*				*Escherichia coli*			
Time (days)	25	35	45	55	25	35	45	55	25	35	45	55
0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
14	20	14	16	22	0	0	0	0	12	0	0	16
21	26	28	24	24	0	0	30	30	0	0	0	0
28	22	20	18	24	0	0	0	32	0	0	0	0

Table 2: Antimicrobial activity of fermented broth of kombucha at different sugar concentration, without $MgSO_4$, pH 4.0 and 25ºC.

The growth curve for kombucha (Figure 2E) reached a peak of biomass between day 14 and 21 of fermentation with a range between 8 and 13 g/l. It was observed that the highest biomass concentration occurred for the highest commercial sugar concentration. However the highest biomass yield was verified for 25% commercial sugar concentration (6.68 g/g) due to high biomass concentration and less sugar consume at day 7, after this time the highest yield occurred for 55% commercial sugar concentration (2.35 g/g) at the end of fermentation.

Table 2 shows the action of fermentation broth on different pathogenic microorganisms, the observation of inhibition halo zone denote in general that the highest commercial sugar concentration the more activity is produced (except at day 21 for *M. canis*). The fermented broth presents antimicrobial activity only for *M. canis* (after 14 days of fermentation), *E. coli* (at day 14) and *Salm. typhi* (after 21 days. The highest inhibition halo zone occurred at the end of fermentation (28 mm for *M. canis* at 35% sugar, 32 mm for *Salm. typhi* at 55% sugar, for *E. coli* the highest inhibition was verified at day 14 at 55% sugar). The antimicrobial activities were better than those found with ketoconazole for fungal inhibition and chloramphenicol for bacterial inhibition.

To continue the experimental procedures it was used commercial sugar of 55% concentration due to the highest values of inhibition for the *Salm. typhi* and *E. coli*, as well as the high antimicrobial activity for *M. canis*.

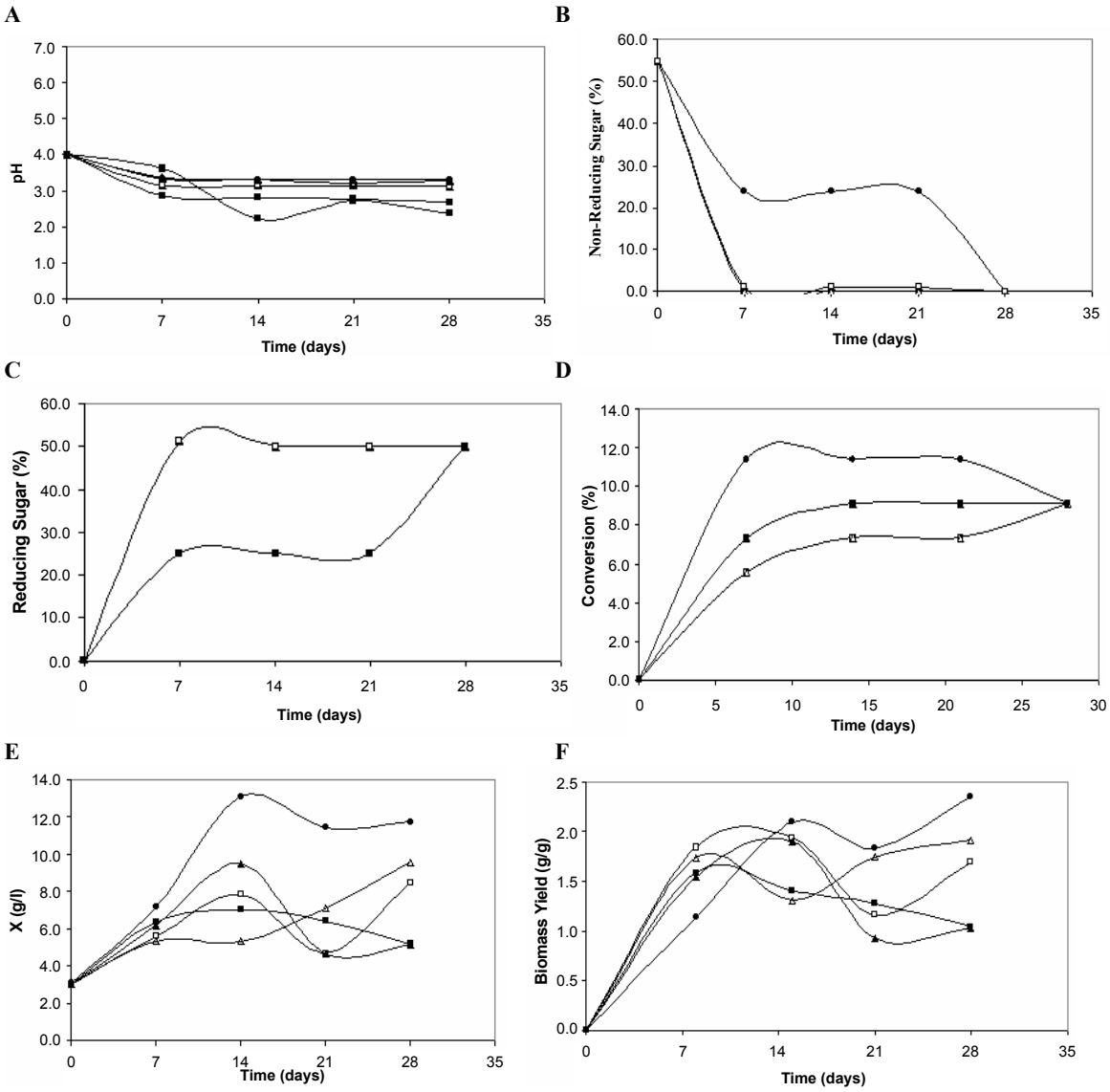

Figure 3: Influence of $MgSO_4$ concentration in fermentation by kombucha at pH 4.0, 25°C and 55% (w/v) of sugar concentration (A- pH, B- non-reducing sugar, C- reducing sugar, D- conversion, E- biomass and F- biomass yield): (●) 0.0 g l^{-1}, (■) 0.10 g l^{-1}, (□) 0.15 g l^{-1}, (▲) 0.20 g l^{-1}, and (△) 0.25 g l^{-1}.

	Diameter of halo zone (mm)														
	Microsporum canis					*Salmonela typhi*					*Escherichia coli*				
Time (dias)	0.0	0.10	0.15	0.20	0.25	0.0	0.10	0.15	0.20	0.25	0.0	0.10	0.15	0.20	0.25
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	>32	>32	>32	>32	0	14	0	0	0	0	16	0	0	0
14	22	>32	>32	>32	>32	0	16	0	0	0	16	16	0	0	0
21	22	22	30	30	20	30	16	0	0	0	0	16	0	0	0
28	24	16	20	20	20	32	12	0	0	0	0	0	0	10	0

Table 3: Antimicrobial activity of fermented broth kombucha at different $MgSO_4$ concentration, 55% sugar concentration, pH 4.0 and 25°C.

$MgSO_4$ concentration influence

In order to verify the effect of different $MgSO_4$ in the antimicrobial activity by kombucha colonies, it was used salts concentration between 0 and 0.25 g/l in the culture medium, the data of fermentation utilizing kombucha are show in the Figure 3. The pH values rapidly decay until day 7 of fermentation, after this time the decreases become slower.

As in previous experiments, the decrease of non-reducing sugar concentration was followed up by an increase of reducing sugars concentration, which was due to enzymatic action, but the reducing sugar was accumulated in the fermented broth, resulting in low conversions (7 and 11%). The microbial growth was inhibited by the addition of $MgSO_4$; this perception was evidenced by a high production of biomass (up to 13.07 g/l at 14 days) without addition of salt. On the other hand, the biomass concentration was small, when added MgSO4 into the medium. It can be showed that the experiments without salt were more effective for the biomass production, the maximum biomass concentrations were 7.03, 8.48, 9.47 and 9.57 g/l for 0.10, 0.15,

0.20 and 0.25 g/l of $MgSO_4$, respectively. Although the inhibitory action of $MgSO_4$ addition, the best results for biomass concentration in the experiments with salt were found at the highest value of salt concentration (9.57 g/l at 28 days). The biomass yield also followed the profile of the microbial growth.

We could verify that *Salm. typhi*, *M. canis* and *E. coli* were susceptible to antimicrobial agents present in the fermented broth by kombucha colonies in different $MgSO_4$ concentration (Table 3), because the enrichment of the culture medium which allowed the increase of inhibition halo zone, despite that the smallest addition was more efficient to inhibit the pathogenic microorganisms. The antimicrobial activities increased with the fermentation time, reaching a peak at day 14 of fermentation (> 32 mm, 16 mm and 16 mm for *M. canis*, *Salm. typhi* e *E. coli*, respectively).

Discussion

During the fermentation process, the conversion of sucrose in organic acids promotes the decrease of pH value (Seeramula et al., 2000; Loncar et al., 2006). Apparently, the fermentation broths presented a certain buffer capacity. Some authors attribute this phenomena to carbon dioxide dissociation and production of the amphiprotic hydrocarbonate anion (HCO_3^-), which easily reacts with hydrogen ions (H^+) from organic acids, preventing further changes in the H^+ concentration and contributing to a buffer typical of the system (Cvetkovic et al., 2008; Jayabalan et al., 2008).

Malbasa et al., (2008) verified low conversion (35 e 25%) of sucrose to glucose and fructose, then further ethanol and organic acid, thus the sucrose conversion may be used as a measure of the rate of fermentation. In medium containing molasses observed generally a lowest biomass yield for fermented broth. Silva et al., (2009) used kefir grains in culture medium containing molasses verified a biomass yield between 0.43 and 0.66 g/g.

The relation between pH value and antimicrobial activity, in fermented with kombucha, was observed by Greenwalt et al., (1998). At neutral pH value, Sreeramula et al., (2000) reported inhibition halo zone ranged between 20 and 25 mm to *E. coli* for fermented broth by kombucha colony with medium culture containing 10% (w/v) of sucrose and 2.5% of glucose (w/v). The fermented broth had a higher inhibition that ketoconazole, this feature is important because *M. canis* is a dermatophyte that frequently infects humans, with transmission of the infestation being observed in 30% of feline dermatophytosis cases (Cavalcanti et al., 2002). Comparatively to the extract of *Cladonia substellata* Vainio, the fermented broth is more efficient (38.46%) in inhabitation of *M. canis* (Ribeiro et al., 2000). On the other hand, a 21 mm of inhibition halo zone by *Staph. typhi* (20 mm) is interesting because this microorganism is responsible for human diseases that range from mild gastroenteritis to host-disseminated enteric fever (Orsi et al., 2006).

The inhibition to *E. coli* is important because this enteropathogenic microorganism is responsible for diarrheal disease, which is still the most prevalent and important public health problem in developing countries, causing mainly death in children with less than 5 years of age, despite advances in knowledge, understanding and management that have occurred over recent years (Fagundes Neto and Scaletsky, 2000). Silva et al., (2009) observed a halo zone between 8 and 12 mm for *Staph. aureus* (lower than in this work) by fermented broth with kefir grains. Greenwalt et al., (1998) found inhibition halo zone between 11 and 16 mm, when kombucha grew in black and green tea at day 9 and together with 10% (w/v) of sucrose for *E. coli* (values similar to this work). Generally, the increase of sugar concentration potentiate the organics acids productions and consequently a higher antimicrobial activity, however this observation has not been verified, thus the inhibition should not be associated with formation of organics acids.

Diniz et al., (2003) grew Tibetan mushroom (symbiotic culture of bacteria and fungi encapsulated into a polysaccharide matrix, like kombucha) and verified that the Tibetan mushroom present a exponential growth phase until day 10, in this work the exponential phase occurred until day 14, except for 0.25 g/l of $MgSO_4$.

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