GALACTOMYCES GEOTRICHUM ISOLATED FROM WATER KEFIR: INTERACTION WITH *LACTOBACILLUS KEFIR*

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ABSTRACT

In this study, a yeast-like fungus strain wk1 isolated from water kefir grains was identified as *Galactomyces geotrichum*, which had great adhesion ability with *Lactobacillus kefir*. Exposure to lithium chloride aroused the marked loss of attached bacteria, while acetic acid treatment led to a thicker adhesion. Heat and sodium hydroxide treatment had non-significant effect on the interaction. Furthermore, *G. geotrichum* wk1 showed its hydrophobic and basic phenotype with strong electron donor property, contributing to the extremely strong auto-aggregation ability. These properties were greatly weakened after all pretreatments, except LiCl treatment, coincided with the low auto/co-aggregation ability. The results in this study may be helpful to understand the interactions of participating bacteria and fungi in the kefir grains and its role for the grain formation.

Keywords: aggregation, interaction, *Galactomyces geotrichum*, hydrophobicity, kefir, *Lactobacillus kefir*

1. INTRODUCTION

Water kefir, or sugary kefir, is a fizzy and cloudy lacto-fermented homemade beverage, low acid, somewhat sweet and slightly alcoholic, which is prepared by inoculation of sucrose solution or fruit juice with kefir grains containing different species of health-promoting bacteria and fungi (GULITZ *et al*., 2011; ALTAY *et al*., 2013). Nowadays, water kefir with great probiotic characteristics has been taken great attention from scientists and consumers due to the trends towards natural, nutritional, health-promoting and flavor rich beverages, and also the advantages of sucrose breakdown compared to common soft drinks, making it a perfect alternative for those who wish to avoid dairy or soft drinks, or just want another tasty addition to their probiotic menu (MOREIRA *et al*., 2008; SILVA *et al*., 2009).

However, unlike milk kefir, there are only few data available about the analysis of the water kefir consortia and the interactions of participating microbes, especially fungi. Several reports have explored the microbes present in water kefir. GULITZ *et al*. (2011) obtained a collection of 453 bacterial isolates from water kefir with the viable cell numbers (cells/g) ranging from 1.2×10^{6} to 5.6×10^{8}, and the viable yeast cells from 5.8×10^{6} to 2.7×10^{7}. Among yeasts, *Saccharomyces cerevisiae*, *Lachancea fermentati*, *Hanseniaospora valbyensis* and *Zygotorulaspora florentina* were identified, and *Z. florentina* is the yeast species which is the predominant yeast species. Other researchers (WITTHUHN *et al*., 2005; MAGALHAES *et al*., 2011) also reported similar results. Lactic acid bacteria and yeasts were considered to be important for microbiota and grain formation.

In an unpublished previous study, we collected water kefirs of different long term traditional household preparations. Microorganisms in water kefir grains were isolated using standard microbiological media, and one strain isolate of *Galactomyces geotrichum* was obtained. To the best of our knowledge, this is the first time that *Galactomyces geotrichum* was isolated from water kefir. In addition, interestingly, during the isolation, we found that the strain always appeared with the adhesion of lactic acid bacteria around its surface, which indicated it may play an important role to the formation of stable microbiota in kefir grains due to the interaction with lactic acid bacteria.

In general, the current studies on the microbial population and symbiotic interactions are carried out and laid special emphasis on the role of lactic acid bacteria on the basis of the biofilm production (WANG *et al*., 2012), lectin-like adhesion (GOLOWCZYC *et al*., 2009) and surface layer collagen binding protein (YADAV *et al*., 2013), and so on. Up to date, there's no detailed report on the interaction between *G. geotrichum* and lactic acid bacteria. On the other hand, previous researches on the adhesion or biosorption ability of fungi were mainly focused on the removal of dye, pesticide, heavy metals, and so on (BHATTI and AMIN, 2013; ALMEIDA and CORSO, 2014; HAROUNE *et al*., 2014; TANG *et al*., 2014). Few studies were carried out to evaluate the interaction occurred between fungi and bacteria. Herein, the objective of the present study was to describe preliminary investigation on the interaction of the isolate *G. geotrichum* and *Lactobacillus kefir* 12-2, which was also isolated from water kefir, to evaluate its possible contribution to the formation of kefir grains.

2. MATERIALS AND METHODS

2.1. Strains, media and cultivation

Water kefir grains were collected from ordinary families in Wuhan, Hubei province, China. Water kefir was prepared in sucrose solution (100 g/L), and the fermentation

carried out at 25°C for three days. The supernatant was discarded and the grains were used for further study. 10 g water kefir grains were diluted with 90 mL 0.1% (w/v) sterile peptone water, and then mechanically homogenized by a stomacher for 60 s. Serial dilutions were prepared by mixing 1 mL grain suspension with 9 mL of peptone water. The different serial dilutions were plated on MRS (Difco, USA) and YPD agar plates to isolate lactic acid bacteria and fungi in grains respectively. *Lactobacillus kefir* 12-2 (identification data not shown) was obtained and cultured in MRS broth at 37°C without shaking. The fungus (strain number wk1) was cultured in YPD broth at 28°C with shaking (150 rpm).

2.2. DNA extraction and sequence analysis of PCR amplicon

For the DNA extraction from overnight cultures of single colony, the Yeast DNAiso Kit (Takara Bio, China) was used according to the instruction. The DNA was amplified with primers NS1 (GTA GTC ATA TGC TTG TCT C) and NS8 (TCC GCA GGT TCA CCT ACG GA) spanning 18S rDNA gene (White *et al*., 1990). A total of 50 μL PCR reaction system contained 2 μL of the extracted DNA solution, 5 μL of 10×PCR buffer (50 mM KCL, 15 mM $MgCL_2$, 100 mM Tris-HCL, pH9.0), 2.5 U Taq polymerase, 0.2 mM each deoxyribonucleoside triphosphate, 25 pmol of each primer.
The amplification program was carried out under following conditions: 94 °C for 5 min for initial denaturation, then 34 cycles of 40 s at 94°C, 40 s at 50°C, and 40 s at 72°C, followed by a final extension step for 5 min at 72°C. The PCR products were electrophoretically analyzed by 1% (w/v) agarose gel. Direct sequencing of the PCR product was performed on a DNA sequencer. Sequence was submitted and deposited in GenBank database, and was compared as a query sequence with those deposited in GenBank database by means of blast algorithm.

2.3. Adhesive interaction assay

The adhesion ability of the fungus was evaluated using *Lactobacillus kefir* 12-2 as a model microorganism. The microorganism cultures were centrifuged at 3000×*g* for 5 min to remove the supernatant. The cells were washed twice and resuspended in sterile saline solution (0.75% w/v NaCl). The OD_{600} was adjusted to 0.8-1.0. Equal volumes of the fungus and *L. kefir* 12-2 cell suspension were mixed and incubated for 3 h at 30°C. After being spread on slide glass and stained with 1% crystal violet solution for 30s, the cell mixture was examined under a light microscope (Nikon Eclipse E100) with 10× ocular lens and 100× oil immersion lens.
In order to evaluate the adhesion activity of the nonviable or partially damaged cells, different pretreatments were used. Heat treatment at 121°C for 20 min was used to inactivate the fungus cells. Nonviable cells were then tested according to the method as above. For the assay to obtain partially damaged cells through the extraction of cell wall polysaccharide like glucans with hot-water (CORRADI DA SILVA *et al*., 2008), 1 M sodium hydroxide (NaOH) or 0.5 M acetic acid (AA), instead of saline solution, were used to resuspend the fungus cells, followed by incubation at 90°C for 3h. Besides, 5 M lithium chloride (LiCl) was also used but incubated at 37°C for 1 h. After pretreated in the three solutions, the fungus cells were centrifuged and re-suspended twice in 0.75% (w/v) NaCl solution, and then exposed to bacteria to evaluate the effect of cell damage on the adhesion ability of the fungus. Native cells without any treatment were defined as viable control.

2.4. Auto-aggregation and co-aggregation assay

Auto-aggregation and co-aggregation abilities were studied spectrophotometrically according to the method described before (COLLADO *et al*., 2008) with some modifications.
Microorganism suspensions were prepared as described before. The cell suspension was incubated at 30°C for 5 h. The supernatant was separated and the absorbance at 600 nm was measured using a spectrophotometer (Mapada UV-1800, Shanghai, China). Auto-aggregation (%) was expressed as $[1 - (A_T / A_0) \times 100]$, where A_T represents the absorbance of the supernatant at different times and A_0 is the initial absorbance at time T=0.
For the co-aggregation assay, 2 ml of cells of the fungus and *L. kefir* 12-2 were mixed and incubated at 30°C for 5 h. Absorbance values at 600 nm were measured at different times. Co-aggregation (%) was calculated as $[1 - A_{mix} /(A_1 + A_2)/2] \times 100$, where A_1 and A_2 represent the absorbance in control tubes containing only wk1 or the lactobacilli strain respectively, and A_{mix} represents the absorbance of the mix suspension at certain monitored times.

2.5. Cell surface hydrophobicity

Cell suspensions were prepared as described above. 3 mL of cell suspensions were mixed by vortexing with 1 mL of xylene, chloroform and ethyl acetate, respectively, and then incubated for 30 min at room temperature. The absorbance of the aqueous phase was measured at 600 nm. The hydrophobicity (%) was calculated as $(1\text{-}A_T/A_0) \times 100$, where A_T and A_0 represent the absorbance at time T = 30 min and 0, respectively.

2.6. Statistical analysis

All the experiments were carried out three times independently. The data are presented as means ± standard deviation. Analysis of variance (ANOVA) was carried out with SPSS Statistics software v20.

3. RESULTS AND DISCUSSION

3.1. Morphological and molecular characteristics of the yeast

The strain wk1 was cultured in YPD agar at 28°C for 5 days and the morphological characteristics of the fresh microculture were observed under microscopic observation. The strain presented a whitish and powdery circular colony without pigments (Fig. 1A). Mycelia are hyaline, septate, branched, 4.5-15 μm in width. Conidia, single or in chain, are long cylindrical and tip dulled, with the size of 4.7-10 μm × 8.1-22 μm (Fig. 1B).
Wk1 was further identified by amplication and sequencing of 18S rDNA with primers NS1 and NS8. An amplicon of 1694 bp was obtained and submitted to NCBI GenBank database. The sequence was deposited with the accession number KM357929. Sequence comparison showed the strain sharing 99% similarity with *Galactomyces geotrichum*. Molecular identification evidenced the microscopic characteristics very well. *G. geotrichum* is commonly the dominant species in milk products like yoghurt, cheese and so on (CHAVES-LÓPEZ *et al*., 2012; AKABANDA *et al*., 2013). DE HOOG and SMITH (2004) considered that *G. geotrichum* is a closely related, but a different teleomorph of the well-known yeast-like fungus, *Geotrichum candidum*. Since 2004, a recent taxonomic revision concluded that the old *Galactomyces geotrichum* complex contained four separate species

(POTTIER *et al*., 2008). *Galactomyces geotrichum* is wildly considered as teleomorph of *Galactomyces candidum*. ALPER *et al*. (2011) obtained 18 *G. candidum* strains from various environmental niches. The phylogenetic lineage analysis presented that all these 18 strains and *Galactomyces geotrichum* were located within a well-supported clade with 99% bootstrap value. The microbiota in milk/water kefir grains was related to its origin, to a great extent. The local endemic environment is very important to the biodiversity of the kefir grains. To the best of our knowledge, this is the first time to report that *G. geotrichum* was found in water kefir.

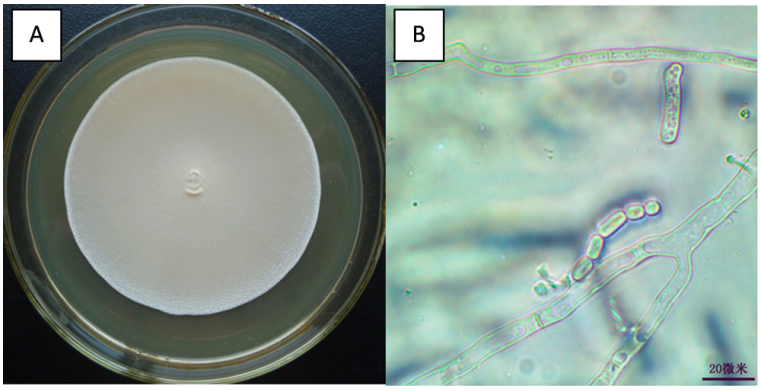

Figure 1: Colony on YPD agar plate (A) and micrograph (B) of *G. geotrichum* wk1. Scale bar 20 μm.

3.2. Adhesive interaction with lactic acid bacteria

During the isolation of microbes from kefir grains, the binding between *G. geotrichum* wk1 and lactic acid bacteria was observed. To investigate the attachment of LABs on the surface of *G. geotrichum* wk1, the two kinds of microbes isolated from water kefir grains were mixed and co-incubated. *G. geotrichum* wk1 exhibited the surprising interaction ability to all the tested LABs (data not shown). The adhesion with *L. kefir* 12-2 in this study was shown in Fig 2. *L. kefir* 12-2 arranged in lines and layers around the surface of *G. geotrichum* wk1, seems like forming a bacterial film outside.

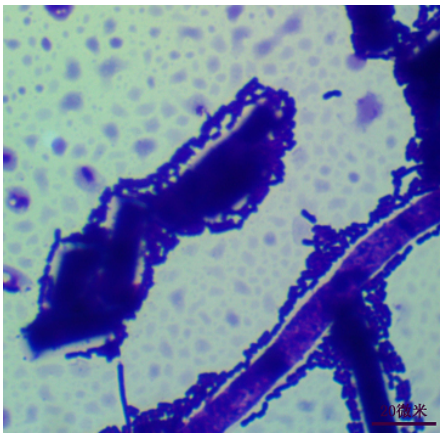

Figure 2: Interaction between *G. geotrichum* wk1 and *L. kefir* 12-2. Scale bar 20 μm.

3.3. Effect of pretreatment on the adhesion capacity

In order to investigate the interaction between inactivated or partially damaged *G. geotrichum* wk1 and *L. kefir*, different pretreatments including heating and treated with NaOH, AA and LiCl were performed. Results showed that adhesive patterns were significantly different between native and damaged fungal cells.

Pretreatment of heating at 121°C for 20 min inactivated the cells, and changed the cell morphology mildly, causing the blunting of the ends of conidia and the decrease of the binded bacteria, to some extent (Fig. 3A). Treatment with NaOH and AA, offering basic and acid conditions to *G. geotrichum* respectively, led to totally different effects on the interaction of *G. geotrichum* and *L. kefir*.

Based on the fact that fungal cell walls are mainly composed of polysaccharides such as α- and β-glucans, mannans and chitin (AHRAZEM *et al*., 2002), and alkali or acid were often used to extract the polysaccharides from the fungal cell wall at high temperature (CORRADI DA SILVA *et al*., 2013; CHEN *et al*., 2013), we deduced that exposure of *G. geotrichum* to NaOH led to the degradation of the components in cell wall, mostly alkali-soluble polysaccharides. CORRADI DA SILVA *et al*. (2008) obtained three D-glucans from the mycelium of the fungus *Botryosphaeria rhodina* MAMB-05 by sequential extraction with hot-water and hot aqueous KOH (2% w/v). In our experiment, we set the high temperature condition at 90°C. After exposure to NaOH at 90°C, *G. geotrichum* was observed to have a weaker binding with crystal violet, which was reflected by the obviously weak appearance of the cells in the microscopic figure. But the damage of cell in NaOH did not weaken the adhesive capacity of wk1 greatly (Fig. 3B). In contrast, AA treatment led to a thicker binding of *L. kefir* around *G. geotrichum* (Fig. 3C). This finding may explain the stable microbiota consortia in the acid environment of water kefir.

Unlike NaOH and AA treatment, LiCl treatment aroused a marked loss of attached bacteria (Fig. 3 D). In many previous studies, LiCl was used to remove the surface layer proteins which are located in the paracrystalline layer outside the microorganism cell wall and involved in the adhesive properties (REN *et al*., 2012). The usage of LiCl did have a significant effect on the removal of the surface layer proteins on the fungal cell wall and a consequent loss of attached bacteria, but a few lactobacilli cells still existed outside the surface of *G. geotrichum* wk1, indicating complex interactions between the fungus and lactobacilli. Components on the cell surface, such as polysaccharides and surface layer proteins, are part of these interactions. Other surface molecules involved in the adhesion of *G. geotrichum* wk1 possibly exist (WAŚKO *et al*., 2014). In addition, the treatment of lactobacilli cells with LiCl will be further performed to evaluate the contribution of fungal or bacterial surface layer proteins on the interaction. The adhesion activity of *G. geotrichum* with *L. kefir* observed in this study may be attributed to the complex adhesin molecules present on the cell surface of *G. geotrichum*, which are tolerant to heating and basic/acid condition.

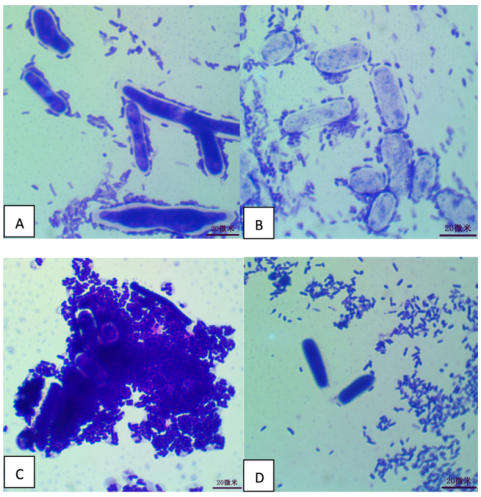

Figure 3: Effect of different treatment on the interaction between *G. geotrichum* and *L. kefir*. (A) Heating at 121°C for 20 min; (B) 1 mol/L NaOH; (C) 0.5 mol/L AA; (D) 5 mol/L LiCl . Scale bar 20 μm.

3.4. Hydrophobicity and aggregation activity

In general, cell wall associated properties, including hydrophobicity, auto-aggregation and coaggregation, were strongly related with interaction between microorganisms (BOONAERT and ROUXHET, 2000; CHEN *et al*., 2010). These properties are attributed to carboxylic groups and Lewis acid-base interactions (REN *et al*., 2012). Solvents with different polarity, xylene, chloroform and ethyl acetate, were used to evaluate the hydrophobic or hydrophilic properties of the adhesin molecules on the cell surface. Strong affinity for apolar xylene indicates good hydrophobicity. High affinity for monopolar and acidic chloroform represents basic and electron donor properties, by contrary, high affinity for monopolar and basic ethyl acetate represents acidic and electron acceptor properties (REN *et al*., 2012; SUN *et al*., 2012). In this study, a strong affinity to xylene (83.49%) and chloroform (87.5%) as well as a low affinity to ethyl acetate (42.22%) indicated the hydrophobic and basic phenotype of the fungus *G. geotrichum* wk1, with strong electron donor property. Different treatments changed the surface properties greatly (Table 1). *G. geotrichum* wk1 even appeared hydrophilic after the treatment of NaOH and AA, with the negative hydrophobicity values.

Table 1: Hydrophobicity, electron donor and acceptor properties of *G. geotrichum* wk1.

	Affinity (%)		
	Xylene	**Chloroform**	**Ethyl acetate**
untreated	83.49±3.18^{a}	87.50±4.67^{a}	42.22±2.76^{b}
Heated	30.09±4.27^{b}	55.77±5.14^{c}	-25.57±2.12^{d}
NaOH-treated	-37.87±3.63^{c}	28.10±5.46^{e}	-50.60±3.81^{e}
AA-treated	-100.28±5.18^{d}	36.65±3.16^{d}	-18.47±4.71^{c}
LiCl-treated	80.81±2.76^{a}	76.57±2.98^{b}	69.19±2.17^{a}

Means (n=3) with different letters in the same column are significantly different (P<0.05).

G. geotrichum wk1 exhibited the extremely strong auto-aggregation ability, reaching 97.48% after 3 h (Fig. 4). All the pretreatments in this study weakened the auto-aggregation abilities of *G. geotrichum* during the first 2 hours, but cells with heating treatment presented an equivalent auto-aggregation capacity with the initial native cells since 3 hours later. Similar tendency existed in the co-aggregation study (Fig. 5), except that cells treated with LiCl had higher co-aggregation ability than cells with NaOH and AA treatment.

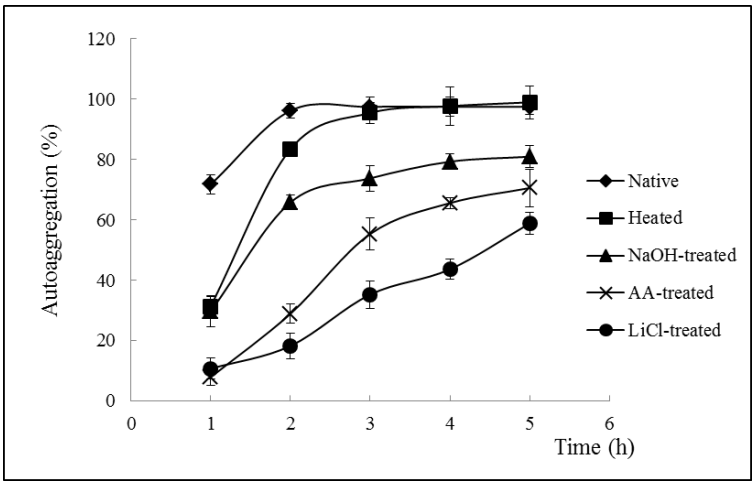

Figure 4: Auto-aggregation abilities of *G. geotrichum* during 5 h incubation.

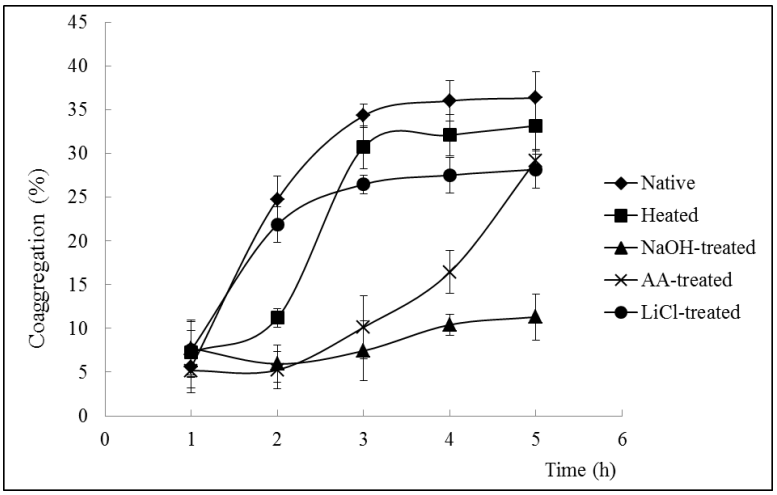

Figure 5: Co-aggregation abilities of *G. geotrichum* with *L. kefir* during 5 h incubation.

The data of cell surface properties obtained here by spectrophotometric method largely correlated with the results of microscopic imaging, except the aggregation characteristics after AA treatment. Spectrophotometric data were affected by complex cell suspension system, reflecting not only the auto-aggregation and co-aggregation properties, but also cell characteristics itself. So in this study, microscopic imaging is a more intuitive method to evaluate of the adhesion capacity.

4. CONCLUSIONS

The interaction between *G. geotrichum* and *L. kefir* on the basis of adhesion pattern was studied in this paper. Previous studies were mostly focused on the contribution of lactic acid bacteria to the kefir grain formation, for example, due to the production of polysaccharides. Researches on the role of yeast or fungi were rarely carried out. The present study showed that *G. geotrichum* wk1 could strongly bind with lactic acid bacteria, suggesting a potential role in the maintaining the symbiotic microbiota of kefir grains. The adhesive capacity decreased slightly after higher temperature and NaOH treatment, and weakened greatly by LiCl treatment, while enhanced by AA treatment. The adhesive activity seemed to be dependent on the cell surface-associated properties, such as hydrophobicity, auto-aggregation and co-aggregation. The findings in this study may be helpful to understand the interactions of participating bacteria and fungi in the kefir grains and the possible mechanism of the grain formation. These results prompted the search for the adhesin molecules on the surfaces of *G. geotrichum* wk1, as well as the quantitation of the adherent LABs, which are currently in progress in our laboratory. In addition, better techniques including electron microscopy will be used to evaluate the morphological changes and the existence of adhesion molecules more clearly.

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