SURVIVAL OF PROBIOTIC LACTOBACILLUS ACIDOPHILUS IN ACIDIC ENVIRONMENT IS ENHANCED IN THE PRESENCE OF SACCHAROMYCES CEREVISIAE

Le Nguyen Han, Dong Thi Anh Dao

Ho Chi Minh City University of Technology

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SUMMARY

A number of health benefits have been proved for probiotic bacteria by many studies and probiotics are increasingly incorporated into foods. However, these market preparations have shown low viability of probiotics in human digestion. Therefore, providing viable probiotic cells to the colon and maintaining their metabolic activity against severe conditions of human digestion are increasingly interested by many recent scientific researches. In this trend, our research showed that by creating a physical barrier, the presence of Saccharomyces cerevisiae (SC) in Lactobacillus acidophilus (LA) suspension can effectively protect probiotic cells from stresses of digestion. After 150 minutes in simulated gastric juice, the survival of LA is significant improved (p<0.05) by forming cell-cell contact with SC cells. The LA-only cells show that most cells die with viability of 0% due to low pH medium, compared with 11.025 ± 1.127% of LA+SC mixture. Besides, we found that the cell concentration ratio at 1:10 between SC and LA cells performs highest protective effects on the probiotic in the acidic environment with 10.122 ± 1.348% LA viability. This concentration ratio is the critical value because when the SC concentration is increasingly higher (SC÷LA concentration ratios higher than 1÷10), LA viability shows no significantly different increase. We also found that yeast cells with oxidized carbohydrates on cell’s surface have many adverse impacts on co-aggregation (4.003 ± 0.115% after 240-minute treatment) while non-viable yeast cells with damaged and denatured protein on cell’s surface still maintains a high percentage of co-aggregation with LA (26.050 ± 0.259% after 240-minute treatment).

Keywords: Co-aggregation, Lactobacillus acidophilus, Saccharomyces cerevisiae, simulated gastric juice, survival

INTRODUCTION

Probiotics are living microorganisms which once consumed with adequate intake through digestion will bring positive effects on the host’s activity of intestinal microflora and improve its health. Several studies claimed that probiotics contribute to decrease of serum cholesterol and blood pressure, prevention of vaginitis, decreased incidence and duration of diarrhea etc. (Klaenhammer et al., 1999; Lee et al., 2008). Probiotics, most of which belong to lactic acid bacteria (LAB) and bifidobacteria, proved to have most positive effects on maintaining the intestinal ecosystem (Picot et al., 2004).

Several studies employing various techniques such as encapsulation, probiotic training for low pH environment resistant strains, nutrient supplementation, etc. have been conducted and shown positive results at varied degrees in terms of enhancing probiotic viability (Michida et al., 2006; Özer et al., 2005; Picot et al., 2004; Sultana et al., 2000). More recently, another technique called co-aggregation is considered an innovative in this field. Co-aggregation is defined as a process in which genetically-distinct microorganisms adhere to others’ surface via specific molecules or some links, forming complex multispecies biofilms. Aggregation can occur among microbial cells of the same species (auto-aggregation) or different ones (co-aggregation) and this combination has been reported to improve probiotic strength in extreme condition (Collado et al., 2007).

Based on the co-aggregation mentioned above, a number of studies have discussed the roles of some kinds of yeast in maintaining probiotic viability in milk culture in several months (Graham et al., 1943). Torulopsis sp., a type of yeast as Soulides (1955) pointed out the increase of S. thermophilus and L.
**MATERIALS AND METHODS**

**Microorganisms, cultivation conditions, and enumeration**

*Lactobacillus acidophilus*

ATCC 43121 (LA) was used in this study. Freeze-dried cells were rehydrated in 5 mL MRS broth and then incubated in conditions appropriate for their growth (37°C in 18 h). After that, cultures were moved into liquid MRS broth and grown in the same condition above until reaching the concentration of 10^8 CFU mL\(^{-1}\). Collection process was conducted at 5000rpm centrifugation for 5 minutes at low temperature (4°C). Cells collected from MRS broth were washed twice with a solution of sodium chloride 0.9%. The washed cells then were selected for later experiments. The concentration of living cells was determined by pour plate method in MRS agar. Plates were also incubated in the same conditions mentioned above (Chávarri et al., 2010).

*Saccharomyces cerevisiae*

BY 4741 (SC) were also rehydrated in 5 mL of YM broth and adjusted to pH 5.0 with 1 M HCl. Then, the inoculated broths were move to liquid YM broth at 30°C for 24 hours to collect stationary phase with cell concentration of 10^6 CFU mL\(^{-1}\) (Lim et al., 2015). Cells collected from YM broth were washed twice with a solution of sodium chloride 0.9%. The washed cells then were selected for later experiments and cells concentration was determined by pour plating in YM agar. Plates were also incubated in the same conditions mentioned above.

In this study, in order to evaluate the enhancing effects of probiotics in co-aggregation with yeast, natamycin (Natamax, Danisco) was used at final concentration of 50ppm to inactivate yeast growth when pouring plating at 37°C in 48 h (Liu et al., 2009).

The data is reported in the current study are the average values of triplicate determinations (plating) from separate experiments.

**SC and LAB treatment**

SC and LA cells were treated according to the method in Gołowczyz et al., (2009). To denature protein molecules on surface of SC cells, SC suspensions were sterile in autoclave in 121°C in 30 minutes to make all cells die completely (non-viable SC (NSC)). To oxidize carbohydrates on surface of SC cells, after washed with a solution of sodium periodate 0.05M and incubated in 30 minutes to form oxidized SC cells (OSC). Initial SC cells without any treatment were called viable SC cells (VSC).

**Preparation of simulated gastrointestinal juices (SGJ)**

SGJ were used as environment stressing factor on the survival of LA in this study. SGJ was prepared by following method that previously used in Michida et al. (2006). Suspending pepsin (P7000, 1:10,000) was dissolved into a solution of sodium chloride (NaCl 0.5% w/v) so that its concentration reached 3g L\(^{-1}\). Using concentrated HCl or NaOH 0.1 mol L\(^{-1}\) to make a solution having a desirable pH.

**Effect of SC concentration on probiotic’s viability**

LA concentration was initially fixed at 6.5 × 10^6 CFU mL\(^{-1}\). SC concentration was based on this LA concentration and prepared with varied ratios. Two suspensions (20 mL each) were combined to form 40 mL cell mixture which was then incubated at 37°C for 20 minutes. The control sample was prepared with only 20 mL LA suspension at 6.5 × 10^6 CFU mL\(^{-1}\) added with 20 mL of sodium chloride 0.9%. Afterwards, SGJ pH 2.0 previously prepared was used to cause stress on the mixed culture in 150 minutes. After 150-minute treatment, pour plate method was conducted to identify LA viability. The optimal SC and LA ratio found in this examination was used for later ones.
Effect of varied pH values on enhancement effect of SC on LA viability

The optimal SC and LA ratio mentioned above was chosen to conduct this experiment. 20 mL of each suspension at this ratio was combined to form 40 mL cell mixture and then incubated at 37°C for 20 minutes. The control sample was prepared as mentioned above. Afterwards, SGJ with varied pH values: 5.8, 3.5, 3.0, 2.5, 2.0 was used to cause stress on the mixed culture in 150 minutes. Samples were taken each 30 minutes for pour plate method to identify LA viability.

Effect of SC viability on survival of LA

VSC and NSC were prepared as mentioned above. 20 mL of each was combined with 20 mL LA suspension according to the optimal ratio above. After that, the prepared SGJ pH 2.0 was used to cause stress on the mixed culture in 150 minutes. Samples were taken each 30 minutes for pour plate method to identify LA viability.

Effect of SC concentration on probiotic’s viability

As indicated in Fig. 1, SC concentration significantly improves LA viability. Control sample (no added SC) and SC÷LA concentration at 1:50 shows nearly no presence of viable LA cells after 150-minute treatment in SGJ pH 2. From 1:40 to 1:30 SC÷LA ratios, SC presence has higher effect on LA viability at respectively 0.298 ± 0.109% and 2.084 ± 0.511% LA viability. SC÷LA concentration at 1:20 shows a significant improvement on LA protection, at 7.055 ± 0.740% LA viability. However, when the SC concentration is increasingly higher (SC÷LA concentration ratios higher than 1:10), LA viability shows no significantly different increase from 10.122 ± 1.348% to 11.201 ± 1.243% (P>0.05).

Hence, this suggests that each certain LA concentration requires a critical SC concentration which once is surpassed, LA viability shows no significantly different improvement. SC÷LA concentration at 1:10 which proves the most effective ratio for viability enhancing effect of SC was chosen as the optimal ratio for later experiments.

So far, there have been different reports on finding a suitable concentration ratio between yeast and LAB, greatly depending on varying microorganisms examined and also shown dissimilar results. For instance, Phebe et al. (2015) demonstrated that in McIlvaine’s buffer solution pH = 2, 1:1 ratio of L. rhamnosus HN001 to viable SC concentration is needed to effectively protect LAB in acidic environment. In their studies, the initial L. rhamnosus 8.45 ± 0.07 Log CFU mL⁻¹ fell to 7.28 ± 0.31 Log CFU mL⁻¹, equivalent to 6.76% viability. Meanwhile, Ningning et al. (2011) reported that they only needed 5 Log CFU mL⁻¹ SC cells for 8 Log CFU mL⁻¹ L.paracasei, equivalent to 1×1000 of SC and L.paracasei. However, after 60-minute treatment, L.paracasei viability remained only 5.98 Log CFU mL⁻¹ in comparison to 8 Log CFU mL⁻¹ of initial concentration, equivalent to 1% protective effect. All the differences above prove that each species of
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probiotic when in contact with the same SC yeast needs certain amount of SC cells which can be explained by the different in structure’s cell surface and dimension between varied probiotic strains.

Effect of varied pH values on enhancement effect of SC on LA viability

1:10 ratio of SC:LA concentration was chosen to conduct this experiment. Most of the current probiotic preparations are taken in via digestion. With 2 liters of gastric juice daily released and very low pH, humans’ stomach forms a barrier that kills most probiotics. Fig.2A shows that SGJ pH 2 and pH 2.5 eliminates most LA cells (in LA-only sample) and so does SGJ pH 3.0 and pH 3.5 though less seriously. As for controlled pH, cell viability tends to increase due to substrate-rich medium and appropriate-for-growing pH (pH=5.8), consequently cell survival significantly rose after 150 minutes. According to Fig. 2, the LA-only increases to 20.7% compared with 14.09% of LA cells in (LA+SC) at this control pH value. The LA-only cells have more interaction with substrates while LA cells in (SC+LA) have less because of lower nutrition competition of the LA-only sample.

In SGJ pH 2, the LA-only cells with initial viability of 100%, after 30 and 60 minutes of treatment, has lower survival of 38.32 ± 1.745% and 20.408 ± 1.483% respectively. With 90-minute
The experimental results show that the presence of SC cells, despite whether they are viable (VSC) or not (NSC), has positive impact on improving LA viability. Fig. 3 shows that after 150-minute treatment, both VSC and NSC show almost the same degree in enhancing LA viability, at respectively 11.125 ± 1.127% and 10.252 ± 0.687% (P<0.05). This result is quite congruent with those presented by Phebe et al. (2015) and Ningning et al. (2011) as they asserted that there is no statistically significant result between the effect of VSC and NSC.

Effect of SC at various pretreated methods on co-aggregation between SC and LA was examined and results were shown in Table 1. Co-aggregation percentage between LA and OSC shows no significant change. Moreover, LA and OSC combination indicates lower aggregation ability than others. Meanwhile, 2 groups LA+VSC and LA+NSC have obviously higher co-aggregation percentage and there is no significant statistical difference between them. These results also coincide with the results in Fig. 3 in that the survival of LA when combined with VSC and NSC is the same.

The results also show that OSC cells with oxidized carbohydrates on cell’s surface has many adverse impacts on co-aggregation. Meanwhile, NSC with damaged and denatured protein on cell’s surface still maintains a high percentage of co-aggregation with LA. This is quite congruent with the hypothesis of Golowczyc et al. (2009) and Kogan et al. (2007) that the protein on bacteria’s surface will link with polysaccharides on SC’s surface. This kind of polysaccharides also proves their roles in adherent specificity to Caco-2 cell, the continuous cells of heterogeneous human epithelial colorectal adenocarcinoma cells.
Effect of cell component on the co-aggregation between SC and LA

Besides, Golowczyc et al. (2009) affirmed that a lectin-like activity of proteins on bacteria’s surface had an important role in connecting with SC cells to form co-aggregation. In their studies, LA cell’s proteins on their surface were denatured by heat treatment and LA cell’s polysaccharides on their surface were oxidized, but both of them showed no adhering ability to Caco-2 cells. Therefore, it is necessary to protect LA’s proteins on cell’s surface because they have an important role in adhering to Caco-2 cell and forming co-aggregation with SC cells. Co-aggregation between LA cells and SC cells means these important proteins are protected, indicating improvement in of probiotic survival in human digestion.

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>LA</th>
<th>LA+VSC</th>
<th>LA+NSC</th>
<th>LA+OSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>3.797 ± 0.199&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.030 ± 0.362&lt;sup&gt;e&lt;/sup&gt;</td>
<td>11.957 ± 0.332&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.33 ± 0.30&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>60</td>
<td>5.357 ± 0.206&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.287 ± 0.624&lt;sup&gt;e&lt;/sup&gt;</td>
<td>13.283 ± 0.404&lt;sup&gt;g&lt;/sup&gt;</td>
<td>2.10 ± 0.42&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>90</td>
<td>5.200 ± 0.495&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.460 ± 0.417&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.050 ± 0.250&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.077 ± 0.405&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>120</td>
<td>8.443 ± 0.518&lt;sup&gt;d&lt;/sup&gt;</td>
<td>23.190 ± 1.338&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.157 ± 1.246&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.150 ± 0.276&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>180</td>
<td>12.200 ± 0.304&lt;sup&gt;e&lt;/sup&gt;</td>
<td>25.537 ± 0.400&lt;sup&gt;e&lt;/sup&gt;</td>
<td>25.810 ± 0.449&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.870 ± 0.114&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>240</td>
<td>11.943 ± 0.070&lt;sup&gt;d&lt;/sup&gt;</td>
<td>26.737 ± 0.645&lt;sup&gt;g&lt;/sup&gt;</td>
<td>26.050 ± 0.259&lt;sup&gt;j&lt;/sup&gt;</td>
<td>4.003 ± 0.115&lt;sup&gt;lm&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means with the same letter are not significantly different (P>0.05).

CONCLUDING REMARKS

This study confirmed that SC cells in particular and yeast in general have positive effects on improving probiotic’s survival. Our findings suggested the promising effectiveness of co-culture of two strains in enhancing viability of vulnerable microorganisms like probiotic. While other techniques show remarkable limits which prevent their widespread application (such as microencapsulation entailing high cost for materials and many steps which reduce probiotic strength, training low pH tolerant strains consuming time and effort etc), co-aggregation has a lot of potentials in producing functional foods with probiotic.

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REFERENCES

NÂNG CAO KHÁ NÂNG SỐNG CỦA PROBIOTIC LACTOBACILLUS ACIDOPHILUS TRONG MÔI TRƯỜNG ACID BẰNG SỦ HIỆN DIỆN CỦA SACCHAROMYCES CEREVISIAE

Lê Nguyên Hân², Đỗ Thịnh Anh Đạo
Trường Đại học Bách khoa, Đại học Quốc gia Thành phố Hồ Chí Minh

Tóm tắt

Vi sinh vật probiotic từ lâu đã được chứng minh bởi rất nhiều nghiên cứu về tác dụng của chúng đối với sức khỏe của con người. Chính bởi các tác dụng có ích ấy, probiotic đã được nghiên cứu bò sử dụng vào nhiều loại thực phẩm khác nhau. Tuy nhiên, các sản phẩm này có một số đặc điểm liên quan lây lê sống của probiotic rất thấp khi được vào môi trường tiêu hóa. Do đó, việc cung cấp các tế bào probiotic sống và vận duy trì được hoạt tính của chúng khi vào đại tràng trong những điều kiện khắc nghiệt của hệ tiêu hóa ngày càng được nhiều nghiên cứu trên thế giới quan tâm. Trong xử thọ đó, nghiên cứu của chúng tôi chỉ ra rằng vỏ vào việc tạo ra một hàng rào bảo vệ, sự hiện diện của tế bào nền men Saccharomyces cerevisiae (SC) trong hỗn hợp Lactobacillus acidophilus (LA) đã bảo vệ được tế bào probiotic trước những điều kiện khắc nghiệt của hệ tiêu hóa. Sau 150 phút trong môi trường dịch dạ dày giả lập, khẩu năng sống của LA được cải thiện rõ rệt (p<0.05) dưới vòng tác dụng tiếp giáp của các tế bào probiotic và nền men. Mấu chốt có thể là LA cho kết quả hiệu qua như tất cả các tế bào probiotic bị chết với tỉ lệ sống 0% so với 11.025 ± 1.127% tỷ lệ sống của LA trong hỗn hợp LA+SC. Bên cạnh đó, tỷ lệ tế bào 1:10 giữa hai loại tế bào SC và LA cho hiệu quả bảo vệ cao nhất trong môi trường pH thấp với tỷ lệ tế bào LA sống là 10.122 ± 1.348%. Tỷ lệ này được xem là giảm trì hòa nhỉ khi nồng độ tế bào SC tăng lên hơn nữa (tỷ lệ nồng độ SC:LA cao hơn 1:10) thì tỷ lệ sống của LA cũng tăng lên không có ý nghĩa thống kê. Chúng tôi cũng nhận thấy rằng tế bào men với bê mặt carbohydrate bị oxy hóa sẽ ảnh hưởng rất nghiêm trọng đến khả năng kết tụ (tỷ lệ kết tụ là 4.003 ± 0.115% sau 240 phút khai sá) trong khi các tế bào men chết với các thành phần protein trên bề mặt bị phá hủy và biến tính vận duy trì được tỷ lệ kết tụ rát cao với LA (tỷ lệ kết tụ là 26.050 ± 0.259% sau 240 phút khai sá).

Τủ khoa: dịch dạ dày giả lập, kết tụ, khả năng sống, Lactobacillus acidophilus, Saccharomyces cerevisiae

² Author for correspondence: E-mail: lenguyenhan@gmail.com