

## Antimicrobial Effect of Chitosan on the Growth of Lactic Acid Bacteria Strains Known to Spoil Beer

Pankaj Garg, Yoo Jin Park, Dona Sharma, and Tammy Wang

Department of Microbiology & Immunology, UBC

**Chitosan is a non-toxic compound that is known to possess anti-microbial activity. This study investigated the potential anti-microbial activity of chitosan against select lactic acid bacteria, specifically, *Lactobacillus brevis*, *Lactobacillus casei*, *Pediococcus claussenii* and *Pediococcus damnosus* strains known to cause major beer-spoilage. The anti-microbial activity of chitosan was tested at a concentration of 0.5 g/L. A turbidity assay was used to monitor the growth of lactic acid bacteria strains in the presence and absence of chitosan. The growth of all lactic acid bacteria strains was decreased when chitosan was added. Lactic acid assay and diacetyl assay were used to measure beer spoilage. The presence of chitosan reduced the production of lactic acid. Also, the diacetyl assay showed that chitosan was able to reduce the amount of diacetyl to levels below its flavor threshold in cultures contaminated with *Lactobacillus brevis* BSO31, *Lactobacillus casei* CCC B9657 and *Pediococcus damnosus* Molson b76.**

---

Chitosan is a natural linear biopolysaccharide produced by alkaline N-deacetylation of chitin which is the major component of the shells of crustaceans such as crabs (7). Chitosan is non-toxic, biocompatible and bio-degradable, and has been reported to have antimicrobial and antifungal effects (10). Although unmodified chitosan is insoluble at neutral pH, it is soluble under slightly acidic conditions (pH < 5) (1). Previous studies by Hoover and Knorr have shown that the minimum concentration of chitosan required for antibacterial activity is 0.1 g/L (13). Chitosan is commercially available product used in high concentrations as a hemostatic agent in bandages, soluble dietary fibers, and diet pills. It could also be used as an anti-microbial product, with potentially beneficial properties, in the food industry such as in beer-making.

The process of beer-making involves several stages where microbial contamination from the raw materials such as hops, brewing water, and additives can cause beer spoilage and in turn large economic loss (5). Microbial contaminations can Beer is a hostile environment for most microbes to grow due to high ethanol content (up to 10%) and low pH (between 3.8 - 4.7). However, few strains of lactic acid bacteria (LAB) from the *Lactobacillus* and *Pediococcus* genera are known to have the most damaging effects on beer. For example, *Lactobacillus brevis* is a major beer spoiling LAB that is responsible for more than half of the beer spoilage incidents (5). LAB spoil beer by creating haze or rope, and giving rise to unpleasant flavor changes such as sourness, and atypical odors (13). Moreover, *Lactobacillus casei*, *Pediococcus damnosus*, and

*Pediococcus claussenii* are known to produce high levels of diacetyl in finished beer; diacetyl adds an undesirable buttery flavor to beer (8).

The purpose of this study was to determine if chitosan has antibacterial activity against the LAB that are known to cause beer spoilage. Specifically, we examined whether chitosan can inhibit the growth of *L. brevis*, *L. casei*, *P. damnosus* and *P. claussenii* in beer. Since *L. brevis*, *L. casei*, *P. damnosus* and *P. claussenii* are the most well-known beer-spoiling LAB, they served as a discrete test-set for assessing the antibacterial activity of chitosan. We hypothesized that chitosan can act as an antibacterial agent and therefore can be used as a preservative in acidic beverages like beer. To investigate the effect of chitosan on LAB, growth of each strain was monitored. As all of the selected LAB species produce lactic acid and diacetyl as their major end products of the carbohydrate metabolism (4), the levels of lactic acid and diacetyl were also used to assess bacterial growth and beer spoilage. The results from this study support our hypothesis as the presence of chitosan showed decrease in bacterial growth, lactic acid production and diacetyl production in beer samples contaminated with strains known to spoil beer.

### MATERIALS AND METHODS

**Bacterial strains.** Twelve strains of lactic acid bacteria were provided by Dr. Barry Ziola from the University of Saskatchewan. The strains used were three *L. brevis* strains (BSO31h, CCC 96S1L, CCC B1300), three *L. casei* strains (CCC 92G2L, CCC B9657, CCC B1205), three *P. claussenii* strains (CCC B1098NR, ATCC BAA-

344<sup>T</sup>R, ATCC BAA-344<sup>T</sup>NR), and three *P. damnosus* strains (Molson B48, Molson 49, Molson b76).

**Acclimatization of LAB and growth conditions.** The LAB acclimatization protocol was adopted from Haakensen *et al.* (4). All 12 strains were growing in de Man, Rogosa and Sharpe (MRS, Sigma, #69966) broth as previously described (4) when received. In order to acclimatize the bacterial strains to beer, 500 µL of each bacterial culture was inoculated into 9.5 mL of 50:50 media containing 50% beer (Molson Canadian, Canada) and 50% double-strength modified MRS (2x mMRS, with Tween 20 excluded) broth. After 4 days when the growth in the 50:50 media were visible, 500 µL of each culture from the 50:50 media was inoculated into 9.5 mL of media containing 85% beer and 15% 2x mMRS broth (85:15 media), 85:15 media supplemented with 1% acetic acid (85:15 media+1% acetic acid, 10mL 1% acetic acid added to 140mL 85:15 media) (ACROS, Geel, Belgium, #64197), and 85:15 media containing 0.5 g/L chitosan (Sigma, #448869) dissolved in 1% acetic acid (85:15 media +chitosan, 0.075g chitosan dissolved in 10mL 1% acetic acid added to 140mL 85:15 media). Acetic acid (1%) was used to dissolve chitosan in beer. MRS+1% acetic acid was used as a control to make sure that the observed effect is not due to acetic acid but chitosan. Nine days post-inoculation when the growth was visible in the 85:15 media, 500 µL of each culture was inoculated into 100% beer, 100% beer supplemented with 1% acetic acid and 100% beer containing 0.5 g/L chitosan dissolved in 1% acetic acid. All LAB strains were incubated anaerobically at 30°C.

**Turbidity assay.** Bacterial growth was measured as turbidity at OD<sub>600</sub> in a Beckman DV Series 530 spectrophotometer. The untreated media sample without LAB (85:15 media without LAB), the acetic acid treated sample without LAB (85:15 media+1% acetic acid without LAB), and the chitosan treated sample without LAB (85:15 media+chitosan without LAB) were used as blanks for the untreated media sample with LAB (85:15 media with LAB), the acetic acid treated sample with LAB (85:15 media+1% acetic acid with LAB), and the chitosan treated sample with LAB (85:15 media+chitosan with LAB), respectively.

**Lactic acid assay.** Lactic acid assay was modified from the procedure by Kimberley and Taylor (9). For each LAB culture sample, 500 µL was removed and centrifuged in Eppendorf tubes at 13000 rpm for 2 minutes using the Eppendorf Centrifuge 5415D to separate the culture supernatant from the cell pellet. Next, 10 µL of each culture supernatant was placed in a glass tube. To each test tube, 490 µL of distilled water was added. Then, 3 mL of 93% H<sub>2</sub>SO<sub>4</sub> (J.T. Baker Chemical Co., Phillipsburg, NJ, USA, #96943) solution was added to the tubes and the tubes were vortexed. The samples were placed into a 97°C water bath for 10 minutes and moved to a room temperature water bath to cool down. Once samples were at room temperature, 50 µL of 4% CuSO<sub>4</sub>•5dH<sub>2</sub>O (Sigma, #7758998) solution was added to each tube. Then, 100 µL of 1.5% 4-phenylphenol (Sigma, #134341) solution was added to each sample and the tubes were vortexed. Samples were incubated at room temperature for 30 minutes. After 30 minutes, gradient of blue shades appeared in the tubes containing different concentrations of lactic acid. Relative concentrations of lactic acid were estimated by comparing the colors of each sample to the beer sample containing no lactic acid (light blue, denoted as [-]).

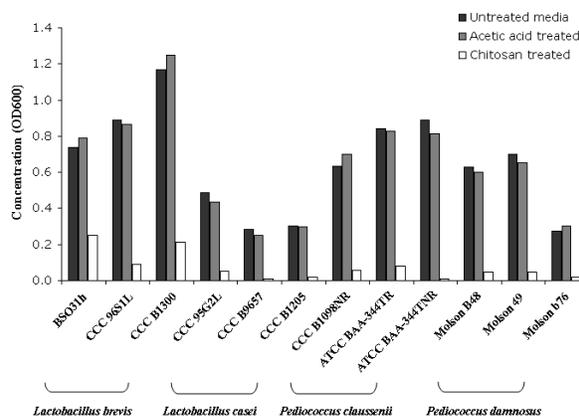
**Diacetyl assay.** Diacetyl assay was modified from the procedure by Eggleton *et al.* (2). Stock alkali solution was prepared containing 60 g/L of NaOH (Fisher Scientific, ON, Canada, #1310732) and 160 g/L of Na<sub>2</sub>CO<sub>3</sub> (Sigma, #5968116) in distilled water. Then, 1% α-naphthol solution was made by diluting the α-naphthol powder (Sigma, #N1000) in the stock alkali solution. As the solution of α-naphthol in alkali does not keep more than 2 to 3 hours, fresh 1% α-naphthol solution was made each time before performing the diacetyl assay. The saturated solution of creatine was made by adding small amounts of creatine (Sigma, #57001) into 75 mL of distilled water. The standard diacetyl solutions were prepared to the final concentration of 0.00, 0.02, 0.04, 0.06, 0.08, 0.10, and 0.12 mg/L by

diluting the 97% diacetyl (Sigma, #B85307) stock solution in distilled water. For each LAB culture sample, 4 mL of the culture was centrifuged at 13000 rpm for 2 minutes to isolate the culture supernatant. To each standard diacetyl solution (mixed with 3.5mL of beer) or each 3.5 mL of culture supernatant sample or 3.5mL of beer only for the control, 4 mL of solution made up of 1 part saturated solution of creatine and 3 parts of 1% α-naphthol solution was added. The flask is shaken lightly to ensure mixing of the reagents. Then, the mixture was made up to 15 mL total with distilled water and allowed to stand for 30 minutes. After 30 minutes, gradient of pink shades appeared in the flasks containing diacetyl compound. Relative concentrations of diacetyl in each sample were estimated by comparing the color of each sample with the diacetyl standards. Each of the colour intensities was given a score. A “-” score indicated no change in colour, “+” score indicated concentrations close to 0.02 mg/L, “++” indicated concentrations around 0.04 to 0.06 mg/L, “+++” indicated concentrations around 0.08 to 0.10 mg/L, and “++++” indicated concentrations around 0.12 mg/L.

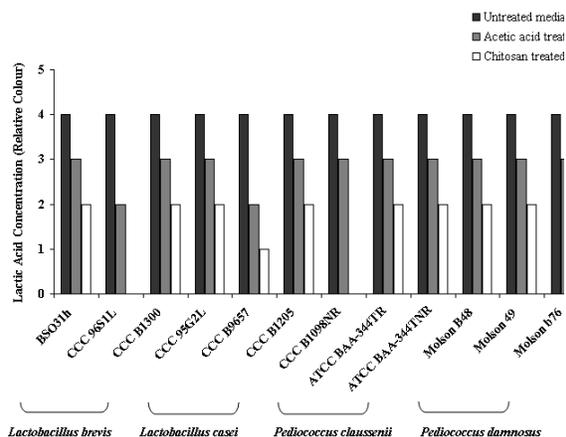
## RESULTS

**Growth of the LAB strains in 85:15 media in the presence and absence of chitosan.** As shown in Figure 1, the relative growth of all the LAB strains in the chitosan treated samples were significantly less than their growth in the other two treatments without chitosan. The turbidity values of the LAB strains in the chitosan treated samples were reduced by at least 66% compared to their turbidity values in the untreated media samples.

In the absence of chitosan, the *L. brevis* strain CCC B1300 had the highest growth in the untreated media sample and the acetic acid treated sample. In the presence of chitosan, it showed a reduction of 82% in the turbidity values. The turbidity levels of all the LAB strains in both the treatments without chitosan (untreated media sample and acetic acid treated sample) were quite similar. The turbidity of cultures inoculated in 100% beer was measured after 22 days of incubation. However, the growth of LAB in 100% beer was too low



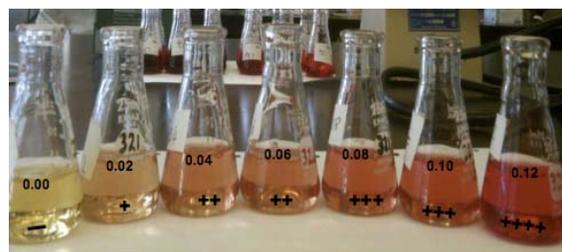
**FIG. 1. Effect of chitosan on the growth of LAB strains after nine days.** Media treated with acetic acid served as a control to assess the effect of the acetic acid present as a solvent in the chitosan treated samples.



**FIG. 2. The effect of chitosan on the presence of lactic acid by various LAB strains after nine days of growth.** Media treated with acetic acid served as a control to assess the effect of the acetic acid present as a solvent in the chitosan treated samples. The colour developed in the lactic acid assays for the samples was compared to the colour of the control lacking lactic acid. The larger numbers correspond to darker colours.

for turbidity assay analysis (data not shown).

**Lactic acid accumulation in 85:15 media in the presence and absence of chitosan.** The production of lactic acid in LAB cultures was detected using a chemical lactic acid assay. Overall, the samples without LAB were a much lighter blue-grey colour than the samples with LAB and the samples with LAB were a much darker navy blue colour than the samples without LAB (data not shown). The three treatments without LAB (untreated media, acetic acid treated and chitosan treated) when visually compared to each other varied only slightly (data not shown). The colour intensity formed was given a numerical value where zero denotes no lactic acid present as compared to the treatments without LAB and values 1 to 4 denotes lactic acid present in increasing quantities. Figure 2 shows the varying degrees of lactic acid in each of the 12 lactic acid bacteria strains in the three treatment samples with LAB when compared to their corresponding treatments without LAB. In general, the untreated media samples had more lactic acid present while the acetic acid treated samples and the chitosan treated samples had decreasingly less lactic acid present (Fig. 2). In the acetic acid treated samples, there were two strains (*L. brevis* CCC 96S1L and *L. casei* CCC B9657) that had less lactic acid present when compared to all the other strains. These two strains (*L. brevis* CCC 96S1L and *L. casei* CCC B9657) along with *P. clausenii* CCC B1098NR also had less lactic acid present in the chitosan treated samples when compared to all the other strains.



**FIG. 3. Diacetyl colour standards.** A positive reaction due to the presence of diacetyl is shown by a colour change to pink. The number of “+” indicate the relative intensity of colour developed.

**Diacetyl accumulation in 85:15 media in the presence and absence of chitosan.** The production of diacetyl in LAB cultures was estimated after nine days of growth using the diacetyl assay. As shown in Figure 3, a gradient of pink colour developed in the diacetyl colour standards containing known concentrations of diacetyl ranging from 0.00 to 0.12 mg/L. For each of the strains grown in the presence and absence of chitosan, diacetyl was estimated by visually comparing the intensity of the solution colour with the control colour standards. Table 1 shows the estimated amount of diacetyl in terms of positive scores in each culture in the presence or absence of chitosan.

As observed by direct vision colorimetry and by comparing the treatment sample solutions to the colour standards in Figure 3, all strains except *L. brevis* CCC 96S1L were capable of producing diacetyl at levels near 0.12 mg/L (Table 1). In the presence of 1% acetic acid, there was a partial reduction in the amount of diacetyl produced by *L. brevis* BSO31 from around 0.12 mg/L to around 0.02 mg/L. All other strains did not show any reduction in the amount of diacetyl produced in the presence of 1% acetic acid. In the presence of 0.5 g/L chitosan, there was a partial reduction in the amount of diacetyl produced by *L. casei* CCC B9657 and *P. damnosus* Molson b76, from around 0.12 mg/L to around 0.02 mg/L, and complete reduction of diacetyl production in *L. brevis* BSO31 culture. All other strains did not show any visual reduction in the amount of diacetyl produced.

## DISCUSSION

Our turbidity data suggested that chitosan is an effective inhibitor of the growth of LAB under our experimental conditions since the turbidity values of the LAB cultures treated with chitosan were significantly lower than the LAB cultures that were devoid of chitosan (Fig. 1). In addition, the turbidity values of the *L. brevis* strain CCC B1300 in particular also support this result because this strain had maximum growth in

**TABLE 1.** The effect of chitosan and acetic acid on the accumulation of diacetyl in 85:15 media cultures after nine days of growth.

Strain	Diacetyl Colour Score		
	Untreated media sample	Acetic acid treated sample	Chitosan treated sample
<i>Lactobacillus brevis</i>			
BSO31	++++	+	-
CCC 96S1L	-	-	-
CCC B1300	++++	++++	++++
<i>Lactobacillus casei</i>			
CCC 95G2L	++++	++++	++++
CCC B9657	++++	++++	+
CCC B1205	++++	++++	++++
<i>Pedicoccus claussenii</i>			
CCC B1098NR	++++	++++	++++
ATCC BAA-344 <sup>T</sup> R	++++	++++	++++
ATCC BAA-344 <sup>T</sup> NR	++++	++++	++++
<i>Pedicoccus damnosus</i>			
ATCC BAA-344 <sup>T</sup> R	++++	++++	++++

the absence of chitosan and showed significantly lower growth in the presence of chitosan. The ability of chitosan to inhibit the growth of the *L. brevis* strain CCC B1300 is highly supportive evidence and indicates that chitosan could be a promising antibacterial preservative for beer because *L. brevis* is the most dominant beer spoiling *Lactobacillus* species which has been reported to be responsible for more than half of the incidents of beer spoilage (13).

The growth of all LAB strains in both treatments without chitosan (untreated media and acetic acid treated samples) was similar at the end of the nine day incubation period. This indicates that the growth conditions of medium and temperature in our experiment were suitable for the growth of these LAB strains. Therefore, we can assume that the decrease in the growth of LAB in the presence of chitosan (chitosan treated samples) was caused by chitosan. However, the results of our experiment were limited to the growth of LAB in 85:15 media rather than in 100% beer.

It is possible that there may be a variation in the turbidity results if the LAB strains were tested for growth in 100% beer instead of 85:15 media. We tried to induce the growth of the LAB strains in 100% beer, 100% beer with acetic acid, and 100% beer with 0.5 g/L chitosan dissolved in 1% acetic acid. However, the time period of our project was not sufficient to induce considerable growth of these strains in 100% beer. In order to overcome this limitation of time and to get a broader perspective of the potential of chitosan as an antibacterial preservative for beer, we focused on other parameters including the accumulation of metabolites like lactic acid and diacetyl in the LAB cultures in the presence and absence of chitosan.

From the lactic acid assay results, the chitosan treated samples had less lactic acid present than the untreated

media and acetic acid treated samples. This shows that chitosan seemed to have an ability to inhibit growth and also the production of lactic acid. As seen in Figure 2, the acetic acid treated samples had more lactic acid present when compared to the chitosan treated samples but they also had less lactic acid present when compared to the untreated media samples. This shows that acetic acid may have some minor role in inhibiting the production of lactic acid too. Therefore, in the chitosan treated samples, it may be the dual action of both chitosan and acetic acid that had an improved effect in inhibiting the production of lactic acid by LAB when compared to acetic acid treated samples or to untreated media samples.

In the acetic acid treated samples, there are two LAB strains (*L. brevis* CCC 96S1L and *L. casei* CCC B9657) which display lower lactic acid when compared to the other ten LAB strains (Figure 2). A similar trend is seen in the chitosan treated samples. In the chitosan treated samples, *L. brevis* CCC 96S1L, *L. casei* CCC B9657 and along with *P. claussenii* CCC B1098NR display lower lactic acid when compared to the other nine LAB strains. In fact, *L. brevis* CCC 96S1L and *P. claussenii* CCC B1098NR showed no lactic acid present in the chitosan treated samples. It is possible that acetic acid is better at inhibiting the production of lactic acid in *L. brevis* CCC 96S1L and *L. casei* CCC B9657 than in the other ten strains and chitosan dissolved in acetic acid is extremely well at inhibiting *L. brevis* CCC 96S1L and *P. claussenii* CCC B1098NR and better at inhibiting *L. casei* CCC B9657 than in the other nine strains. It is also possible that these three strains generally produced less lactic acid when compared to the other LAB strains examined. In this second scenario, the untreated media samples for these three LAB strains would be expected to also be lower in

lactic acid too but our results show that this is not the case. It could either be that the second scenario was unlikely or that the second scenario was possible but that the colour for the assay had saturated and any changes would not have been visibly evident. A third possibility was that the concentrations of the bacterial growth in these three strains were lower when compared to the other strains and therefore the production of lactic acid was also decreased. But when Figure 2 and Figure 1 were compared, no correlation is seen between a lower bacterial concentration and a lower presence of lactic acid. Therefore, this third possibility is also unlikely.

The chemical assay used to determine the presence of lactic acid is not specific to just lactic acid because compounds such as rhamnose, arabinose, fucose, mannosamine, methionine, tryptophan, and formic acid will also affect the colour formation (9). Furthermore, as we have found, even beer will also affect the colour formation. As a result, due to the unspecific nature of this chemical assay, it was hard to determine which of the above three possibilities were the reasons for observing the trends seen in our lactic acid assay.

The flavor threshold of diacetyl is 0.10 mg/L, above which it causes a buttery flavor (11). According to Table 1, the diacetyl amount was reduced from around 0.12 mg/L to less than 0.10 mg/L in the presence of chitosan in the *L. brevis* BSO31, *L. casei* CCC B9657 and *P. damnosus* Molson b76 cultures. For all other strains, the diacetyl levels were around 0.12 mg/L which is more than the flavor threshold even in the presence of chitosan. This observation indicates that 0.5 g/L chitosan in 85:15 media was successful in preventing beer spoilage caused by diacetyl in cultures of *L. brevis* BSO31, *L. casei* CCC B9657 and *P. damnosus* Molson b76. Since our results were limited to nine days of growth in 85:15 media, we would need to extend the incubation period in order to assess if the amount of diacetyl stays below the flavor threshold for these strains. Because our assay could only estimate the amount of diacetyl present in the cultures, a better method of quantifying the amount of diacetyl needs to be carried out in order to determine the fold reduction in the presence of chitosan.

To our knowledge, this is the first report that has demonstrated the reduction of diacetyl in the presence of chitosan in cultures of select *Pediococcus* and *Lactobacillus* strains. In the spoilage process by diacetyl, the precursor of diacetyl,  $\alpha$ -acetolactate, is produced and secreted through the yeast membrane into the beer. Once it gets into the beer, it reacts with oxygen and a chemical reaction results in the production of diacetyl (8). Diacetyl can then pass back into the yeast cell where it is finally reduced to the mono-alcohol called acetoin and di-alcohol butanediol

(12). Although the mechanism of action of chitosan is not known, we can speculate that chitosan acts at either one or more of the above steps involved in the spoilage process and results in the overall reduction of the amount of diacetyl. Since the only step independent of yeast is the chemical conversion step, it can be ruled out as a possible point of action because we did not see a reduction of diacetyl in all of the strains. But all the strain dependent steps (production and secretion of  $\alpha$ -acetolactate, uptake of diacetyl and enzymatic conversion of diacetyl) could be a potential action point for chitosan. Therefore, further experiments need to be conducted to determine the step at which chitosan acted to prevent spoilage by diacetyl.

We had hypothesized that chitosan at pH less than 5 has antibacterial activity against beer-spoiling LAB including *L. brevis*, *L. casei*, *P. damnosus* and *P. claussenii* and hence could be used as a preservative in acidic beverages like beer. The turbidity results showed that chitosan had significant antibacterial activity against all of the tested beer-spoiling LAB strains. The lactic acid assay showed that chitosan, at a concentration of 0.5 g/L, was able to decrease the accumulation of lactic acid in all of the 12 LAB strains examined. However, because the lactic acid assay used was a chemical assay that potentially reacts to many other compounds including beer itself, it is best to confirm the results using another assay that is more specific to lactic acid. Although 0.5 g/L of chitosan was able to decrease the growth and accumulation of lactic acid for all the strains in 85:15 media, it was only able to prevent diacetyl spoilage for cultures of *L. brevis* BSO31, *L. casei* CCC B9657 and *P. damnosus* Molson b76. Thus, chitosan could be used as an alternative antimicrobial agent during beer-making process to prevent contamination and spoilage by *L. brevis* BSO31, *L. casei* CCC B9657 and *P. damnosus* Molson b76. If chitosan is to be used as an antimicrobial agent for the other strains in which we see a reduction in growth and lactic acid accumulation but not diacetyl accumulation, other additional methods of clearing diacetyl from beer must be employed.

Also while the growth of LAB was not observed in the chitosan treated samples, lactic acid and diacetyl was still detected. And for some strains, the diacetyl present was even over the flavour threshold. This could be from the lactic acid and diacetyl that was produced and already present during LAB growth in the acclimatization steps and was transferred when the 85:15 media were inoculated with the LAB. Or it is possible the chitosan was able to inhibit LAB growth but was not able to prevent LAB production of lactic acid and diacetyl. Further experiments would need to be done to determine the reason for this observation.

## FUTURE EXPERIMENTS

In order to better understand the antibacterial potential of chitosan against the tested beer-spoiling LAB strains, the experiment can be repeated by growing these strains in 100% beer and then monitoring their turbidity. This also means that the experiment would need to be carried out over a longer period of time to allow the LAB strains to attain considerable growth in 100% beer. In addition, transferring a greater volume or concentration of initial inoculum to the 100% beer may help improve the growth in beer.

To determine the presence and/or quantity of lactic acid produced by LAB, enzyme based assays such as lactate dehydrogenase assay, which is specific to only lactic acid, are recommended.

For the diacetyl assay, instead of using visual colorimetric approach, which can only be used to estimate the amount of diacetyl in the culture, high performance liquid chromatography (HPLC) can be used to quantify the amount of diacetyl present in each culture.

## ACKNOWLEDGEMENT

This project was supported by the Department of Microbiology & Immunology, University of British Columbia, Vancouver, Canada. We would like to thank the department for their generous contribution of supplies, equipment, and laboratory space. We would like to thank Dr. William Ramey and Jessica Labonté for providing support and insight into improvements on the protocol throughout the experiment and for their guidance throughout the various challenges encountered. We would also like to thank Dr. Barry Ziola, professor at the University of Saskatchewan, for providing us with all the strains used in this study. Special thanks to Ms. Vanessa Pittet, a graduate student in Dr. Barry Ziola's lab, for helping to get the initial cultures growing and for preparing them for us.

## REFERENCES

1. **Amidi, M., and W. E. Hennink.** 2009. Chitosan-based formulations of drugs, imaging agents and biotherapeutics. *Adv. Drug Deliv. Rev.* **62**:1-2.
2. **Eggleton, P., S.R. Elsdon and N. Gough.** 1943. The estimation of creatine and of diacetyl. *Biochem. J.* **37**:526-529.
3. **Gades, M. D., and J. S. Stern.** 2005. Chitosan supplementation and fecal fat excretion in men and women. *J. Am. Diet. Assoc.* **105**:72-77.
4. **Haakensen, M.C., L. Butt, B. Chaban, H. Deneer, and B. Ziola.** 2007. HorA-specific real-time PCR for detection of beer-spoilage lactic acid bacteria. *J. Am. Soc. Brew. Chem.* **65**:157-165.
5. **Hollerová, I. and P. Kubizniaková.** 2001. Monitoring Gram positive bacterial contamination in Czech breweries. *J. Inst. Brew.* **107**:355-358.
6. **Hoover, D. G., and D. Knorr.** 1992. Antibacterial action of chitosan. *Food Biotechnol.* **3**:257-272.
7. **Je, J. Y., and S. K. Kim.** 2006. Chitosan derivatives killed bacteria by disrupting the outer and inner membrane. *J. Agric. Food Chem.* **54**:6629-6633.
8. **Kallmeyer, M.** February 2003. The role of diacetyl in beer. [Online]. Available: <http://www.draymans.com/articles/arts/03.html> (March 8, 2010).
9. **Kimberley, A.C., and C. Taylor.** 1995. A simple colourimetric assay for muramic acid and lactic acid. *Appl. Biochem. Biotechnol.* **56**:49-59.
10. **Park, Y., K. M. Hyun, P. S. Cheol, C. Hyeonsook, J. M. Kyeong, N. J. Woon, and H. K. Soo.** 2008. Investigation of the antifungal activity and mechanism of action of LMWS-chitosan. *J. Microbiol. Biotechnol.* **18**:1729-1734.
11. **Pejin, J.D., O.S. Grujic, N.J. Marjanovic, D.N. Vujic and S.D.K. Tanackov.** 2002. Determination of diacetyl and 2,3-pentanedione in beer by GC/MS using solid-phase extraction comulns. *Acta Periodica Technologica.* **33**:45-54.
12. **Tolls, T.N., J. Shovers, W.E. Sandine and P.R. Elliker.** 1970. Enzymatic removal of diacetyl from beer. *Appl. Microbiol.* **19**:649-657.
13. **Vaughan, A., T. O. Sullivan, and D. Sinderen.** 2005. Enhancing the microbiological stability of malt and beer - a review. *J. Inst. Brew.* **111**:355-371