INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
15–538/2017/19–1–171–176
DOI: 10.17957/IJAB/15.0259
http://www.fspublishers.org

Full Length Article

Control of *Lactobacillus plantarum* Contamination in Bioethanol Fermentation by Adding Plantaricins

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Abstract

Bacteriocin is considered as a potential biological method for controlling bacterial contamination. Plantaricins can inhibit the growth of *Lactobacillus* species closely related to the producer. In this study, *Lactobacillus plantarum* ATCC 8014 was co-cultivated with *Saccharomyces cerevisiae* S288C to mimic the bacterial contamination in industrial bioethanol fermentation. Plantaricins produced by *L. plantarum* ATCC BAA-793 was added into the co-cultivation system to control *L. plantarum* 8014 contamination. The final ethanol content and cell number of *S. cerevisiae* and *L. plantarum* 8014 were determined to assess the controlling effect. Results showed that plantaricins could effectively control *L. plantarum* contamination and remarkably reduce the inhibition effect on *S. cerevisiae*. Furthermore, plantaricins did no harm to the *S. cerevisiae* growth and bioethanol yield. These results suggested the potential of plantaricins as a novel antibacterial agent for controlling *L. plantarum* contamination during the bioethanol fermentation. © 2017 Friends Science Publishers

Keywords: Plantaricins; *Saccharomyces cerevisiae*; Bioethanol; *Lactobacillus plantarum*; Bacterial contamination

Introduction

With the continuous development of economy, the world falls into the midst of energy crisis and environmental pollution (Giampietro *et al*., 2012). Therefore, environment-friendly and sustainable alternative energy resources, such as bioenergy are urgently needed (Fukuda *et al.*, 2009). As a renewable clean bioenergy, bioethanol would facilitate the reform of energy proportion, relieve energy crisis, and lighten global warming to a certain extent (Katakura *et al*., 2011).

Bioethanol can be produced by many kinds of microorganisms, of which *Saccharomyces cerevisiae* is the most employed species for industrial production (Widiastuti *et al*., 2011). However, industrial-scale bioethanol fermentation is frequently stressed by bacterial contaminants (Muthaiyan *et al*., 2011). Bacterial contamination can inhibit the growth of *S. cerevisiae* and result in decreased bioethanol yield, then eventually lead to economic losses (Thomas *et al*., 2001; Narendranath and Power, 2005). *Lactobacillus* is the major bacteria contaminant in bioethanol fermentation because of its rapid proliferation and tolerance to ethanol and low pH (Narendranath *et al*., 1997). Skinner's study on bacterial contaminants of three fuel ethanol facilities has also shown that *Lactobacillus* species were the most abundant isolates, averaging 51, 38, and 77% of total isolates, respectively (Skinner and Leathers, 2004). In addition, it has been reported while the final ethanol concentrations were approximately 100 g/L (12.7%, vol/vol), the presence of lactobacilli at various concentrations would cause the loss in produced ethanol ranged from 0 to 7.5% (Narendranath *et al*., 1997).

Lactobacillus inhibits the growth of *S. cerevisiae* mainly through generating lactate and competing for nutrients and living space. Firstly, *Lactobacillus* can compete against *S. cerevisiae* for saccharides and other micronutrients in fermentation broth (Narendranath and Power, 2005). Secondly, lactate generated by *Lactobacillus* can decrease the fermentation pH value, thus inhibiting yeast biomass and bioethanol yield (Watanabe *et al*., 2008; Katakura *et al*., 2011). Addition of 4% (w/v) exogenous lactic acid can significantly decrease the bioethanol yield (Graves *et al*., 2006). Thirdly, *Lactobacillus* would also contend with *S. cerevisiae* for subsisting space. This kind of competition is common among microorganisms living in an enclosed environment.

Various methods have been attempted to prevent the adverse effects of bacterial contamination, such as adding antibiotics (Narendranath and Power, 2005; Bischoff *et al*., 2009), exogenous ethanol (Katakura *et al*., 2011), lactate (Watanabe *et al*., 2008), and acetate (Saithong *et al*., 2009). Antibiotics are widely used to eliminate bacterial contamination. However, overuse of antibiotics can result in increased antibiotic-resistant bacteria, which might make antibiotics ineffective, and drug residue, which might be fed

To cite this paper: Liu, B., H. Liu, Y. Zhang and H. Li, 2017. Control of *Lactobacillus plantarum* contamination in bioethanol fermentation by adding plantaricins. *Int. J. Agric. Biol*., 19: 171–176

to livestock and eventually threaten the safety of foodstuff (Narendranath *et al.*, 2000). Moreover, overuse of antibiotics would also lead to emerging and spreading of antibiotic-resistant genes (Zhu *et al*., 2013). Exogenous lactate, which is safer and more acceptable for public, could also inhibit *Lactobacillus* effectively; but the exogenous lactate can also inhibit the fermentation capability of *S. cerevisiae*. In addition, high temperature can reduce the incidence of bacterial contamination. However, most industrial-scale *S. cerevisiae* strains cannot grow or ferment at temperature higher than 35°C (Limtong *et al*., 2007; Watanabe *et al*., 2010). Moreover, other methods have also been attempted to control *Lactobacillus* contamination, such as subjoining sulfite and hydrogen peroxide (Chang *et al*., 1997), adding peptides derived from bovine lactoferrin (Enrique *et al*., 2009), chitosan (Gil et *al*., 2004), and sulfuric acid (Pant and Adholeya, 2007; Tang *et al*., 2010). Sulfuric acid would cause high levels of sulfate ions in the wastewater and heighten the osmotic stress and change pH value of fermentation broth (Narendranath and Power, 2005). Consequently it is meaningful to find alternative and applicable antibacterial methods to manage bacterial contamination in industrial-scale bioethanol production.

Bacterial contamination can be inhibited by biological control, among which bacteriocins produced by lactic acid bacteria (LAB) might be a potential method to safely and economically control *Lactobacillus* contamination occurred in bioethanol fermentation. Over the past decades, LAB-bacteriocins including nisin, pediocin produced by *Pediococcus acidilactici*, and plantaricin produced by *Lactobacillus plantarum* have gained comprehensive attention for their antibacterial activity and have been widely used in food preservation and pharmaceutical industries (Nishie *et al*., 2012; Balciunas *et al*., 2013). Plantaricins produced by *L. plantarum* ATCC BAA-793, are proved heat-stable, degradable by proteases and exhibit strain-specific antimicrobial activity. They could inhibit the growth of species closely related to the producing strain, and do not harm to other organisms (Daeschel *et al*., 1990). This character provides the possible use of plantaricins as novel anti-microbial agent to control LAB contamination occurred in bioethanol fermentation.

In this study, *L. plantarum* ATCC 8014 was co-cultivated with *S. cerevisiae* in YPD broth at the beginning of the culture to simulate the bacterial contamination in industrial bioethanol fermentation. Plantaricins produced by *L. plantarum* ATCC BAA-793 were added into the co-cultivation system to evaluate the controlling effect of bacteriocins on the *L. plantarum* ATCC 8014 contamination, and the side effect of plantaricins on bioethanol fermentation was also examined.

Materials and Methods

Strains, Media and Culture Conditions

The *S. cerevisiae* strain used in this study was S288C and the ATCC No. for this strain was 204508 (http://www.atcc.org/Products/All/204508.aspx). It was cultured in YPD broth (2% glucose, 1% yeast extract, and 2% peptone) at 30°C and shaken at 150 rpm. Both the bacteriocins producer *L. plantarum* ATCC BAA-793 (http://www.atcc.org/Products/All/BAA-793.aspx) and the contaminating *L. plantarum* was ATCC 8014 (http://www.atcc.org/Products/All/8014.aspx) were grown at 30°C in MRS medium (1% peptone, 1% beef extract, 1% yeast extract, 2% glucose, 0.5% sodium acetate, 0.2% diammonium hydrogen citrate, 0.2% dipotassium hydrogen phosphate, 0.058% magnesium sulfate, 0.025% manganese sulfate, and 0.1% (v/v) tween 80, pH 6.8).

Plantaricins Preparation

The plantaricins were prepared as described by Nissen-Meyer *et al.* (1993). After growing at 30°C for 12~16 h to early stationary phase, *L. plantarum* ATCC BAA-793 culture was centrifugalized at 8,000 × g for 20 min, and the supernatant was collected. With addition of ammonium sulfate (75% w/v, final concentration), the plantaricins were precipitated. After separating by centrifugation (12,000 g, 4°C, 15 min), the precipitated plantaricins were resuspended in 20 mM-sodium phosphate buffer (pH 7.0). The pellet was stored (4°C) or to be quantified by Bradford method (Bradford, 1976).

Plantaricins Antimicrobial Activity Optimization

The antimicrobial activity of plantaricins produced by *L. plantarum* ATCC BAA-793 was evaluated by the Oxford cup method with *L. plantarum* ATCC 8014 used as indicator strain and *S. cerevisiae* S288C as negative control; penicillin and clindamycin (1 mg/mL) were used as contrasting agents (Vincent *et al.,* 1944). Two hundred microlitres of prepared plantaricins, penicillin solution, and clindamycin solution were respectively added into Oxford cup, on MRS agar plate (1.5% w/v agar) seeded with for *L. plantarum* ATCC 8014 in exponential phase or YPD agar plate for *S. cerevisiae* S288C in exponential phase, respectively. Plates were incubated at 30°C for 16 h, and then inhibitory zone was measured.

To further optimize the concentration of plantaricins for inhibiting *L. plantarum* ATCC 8014, different concentrations (i.e., 0, 2, 5, 10, 20 and 50 μg/mL) of plantaricins were added into *L. plantarum* ATCC 8014 incubation system at the beginning of culture. *L. plantarum* cell number was determined by serial dilution and plate counting method. The experiment was conducted in triplicate.

Inhibition of *L. plantarum* Contamination using Plantaricins during *S. cerevisiae* Fermentation

L. plantarum ATCC 8014 was co-cultivated with *S. cerevisiae* S288C in YPD broth at the beginning of

fermentation to mimic the *L. plantarum* contamination in industrial bioethanol fermentation. The final cell densities of inoculated *S. cerevisiae* and *L. plantarum* were 4×10^5 and $1.6\text{-}2.3\times10^6$ cells/mL, respectively. To assess the effect of plantaricins against *L. plantarum* contamination, 50 μg/mL (final concentration) plantaricins was added into the *S. cerevisiae* and *L. plantarum* co-cultivation system. Altogether, three groups including control group (only *S. cerevisiae* in YPD mediumbroth), contamination group (*S. cerevisiae* and *L. plantarum* were co-cultured in YPD mediumbroth), and plantaricins treatment group (plantaricins were added into *S. cerevisiae* and *L. plantarum* co-cultivation system), were cultured at 30°C and shaken at 170 rpm. *S. cerevisiae* cell number was counted with a haemocytometer, while *L. plantarum* cell number was determined by serial dilution and plate counting method. The final ethanol content was measured by gas chromatography (GC) analysis. The experiment was conducted in triplicate.

Side Effect Evaluation of Plantaricins on *S. cerevisiae* Fermentation

To monitor the side effect of plantaricins on *S. cerevisiae* fermentation, two groups, that were control group (only *S. cerevisiae* in YPD broth), and side effect evaluation group (plantaricins were added into *S. cerevisiae* fermentation system, the final concentration of plantaricins was 50 μg/mL), were cultivated at 30°C and shaken at 170 rpm. *S. cerevisiae* cell number was counted with a haemocytometer. The final ethanol content was measured by GC analysis. The experiment was conducted in triplicate.

Statistical Analysis

Data were analyzed by IBM SPSS statistics 20 and differences exhibiting $P<0.05$ were considered statistically significant.

Results

Plantaricins Antimicrobial Activity Optimization

It could be clearly observed that Oxford cup with penicillin or clindamycin showed a clear and distinct zone of sterilization, while Oxford cup with plantaricins showed a cloudy zone of inhibition. Although the inhibitory effect was less than that of penicillin or clindamycin, plantaricins still showed antimicrobial activity against *L. plantarum* ATCC 8014 (Fig. 1a). Moreover, both plantaricins and tested antibiotics showed no antimicrobial activity against *S. cerevisiae* S288C (Fig. 1b).

In the *L. plantarum* ATCC 8014 cultures, the addition of different concentrations of plantaricins inhibited cell growth. The growth of *L. plantarum* ATCC 8014 was suppressed from 9 h after culture under low (2 and 5 μg/mL) and intermediate (10 and 20 μg/mL) plantaricins concentrations, while the growth was inhibited from 3 h after culture under high (50 μg/mL) plantaricins concentration ($P<0.05$) (Fig. 2, Supplementary Table S1). According to these results, 50 μg/mL was chosen as the optimized concentration of plantaricins for subsequent experiments.

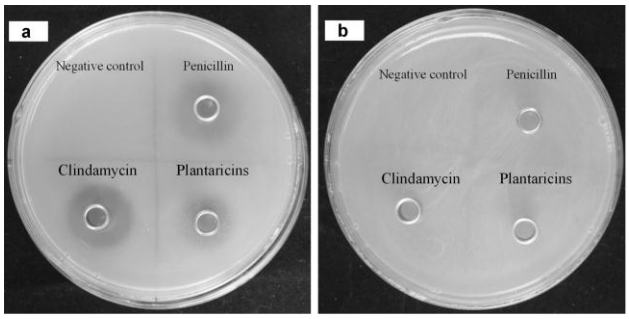

Fig. 1: Antimicrobial activity of plantaricins, penicillin, and clindamycin on (a) *Lactobacillus plantarum* ATCC 8014 on MRS medium and (b) *Saccharomyces cerevisiae* S288C on YPD medium

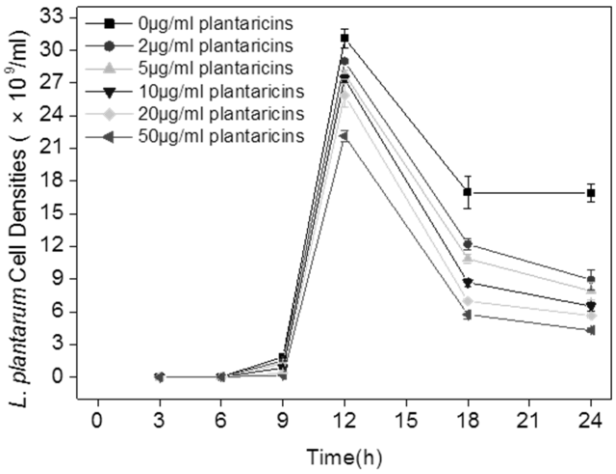

Fig. 2: Inhibitory effect on the growth of *Lactobacillus plantarum* ATCC 8014 by plantaricins with final concentration at 0 (■), 2 (●), 5 (▲), 10 (▼), 20 (◆), and 50 (◄) μg/mL, respectively. Values are the mean of three determinations

Inhibition of *L. plantarum* Contamination using Plantaricins during *S. cerevisiae* Fermentation

To mimic the bacterial contamination in *S. cerevisiae* fermentation, *L. plantarum* ATCC 8014 was co-cultivated with *S. cerevisiae* S288C in YPD medium, and plantaricins were added to assess the efficacy of plantaricins treatment. Compared to the control group, the growth of *S. cerevisiae* in the contamination group was inhibited from 3 h after co-culture ($P<0.05$) (Fig. 3a).

However, addition of plantaricins reversed the inhibitory effect of *L. plantarum* on the growth of *S. cerevisiae*. The yeast cell number in the plantaricins treatment group increased remarkably from 3 h to 18 h after co-culture in comparison with the contamination group (P<0.05) (Fig. 3a).

The final ethanol production in the presence of *L. plantarum* also decreased about 31% in comparison with the control group (P<0.01) (Fig. 3b). However, the addition of plantaricins reversed the inhibition of *L. plantarum* on the final ethanol production (P<0.01) (Fig. 3b), and plantaricins caused about a 37% increase in ethanol yield compared to the contamination group. Furthermore, compared to the control group, plantaricins treatment group showed undiminished ethanol production (P>0.05) (Fig. 3b).

Moreover, the cell number of *L. plantarum* ATCC 8014 was determined to evaluate the antimicrobial activity of plantaricins on *L. plantarum*. Compared to the contamination group, the cell number of *L. plantarum* in the plantaricins treatment group decreased remarkably during early exponential phase (P<0.05) (Fig. 3c). These results about ethanol production and cell number suggested that plantaricins are potentially promising in inhibiting *L. plantarum* contamination during *S. cerevisiae* fermentation.

Side Effect Evaluation of Plantaricins on *S. cerevisiae* Fermentation

The side effect of plantaricins on *S. cerevisiae* fermentation was evaluated without inoculation of *L. plantarum*. Addition of plantaricins did not decrease the cell number of *S. cerevisiae* at each time point (P>0.05) (Fig. 4a). Interestingly, the final ethanol production in the plantaricins treatment group increased 4.6% than that in the control group (P<0.01) (Fig. 4b). These results suggested that addition of plantaricins into *S. cerevisiae* ferementation system is harmless.

Discussion

With the continuous development of economy, energy crisis and environmental pollution are increasingly prominent. Bioethanol is considered as an alternative fuel for reducing consumption of crude oil and then facilitate alleviating environmental pollution (Balat and Balat, 2009). During the bioethanol fermentation, the ethanol yield and the growth of *S. cerevisiae* cells are often inhibited by bacterial contamination, among which LAB such as *L. plantarum*, are the major bacterial contaminants (Skinner and Leathers, 2004). LAB is tolerant to ethanol and low pH and can quickly outnumber the *S. cerevisiae* cells (Bayrock *et al*., 2003). Therefore, they can greatly influence yeast growth. Results shown here also indicated the inhibitory effect of *L. plantarum* on the growth of *S.*

Supplementary Table S1: The statistical P values about *L. plantarum* ATCC 8014 cell number of plantaricins treated groups in comparison with the control group (no plantaricins)

Plantaricins concentrations (μg/mL)	Time (h)					
	3	6	9	12	18	24
2	0.311	0.287	0.006	0.085	0.037	0.003
5	0.175	0.190	0.003	0.029	0.015	0.001
10	0.116	0.153	0.000	0.018	0.005	0.000
20	0.081	0.100	0.000	0.021	0.002	0.000
50	0.032	0.049	0.001	0.001	0.002	0.000

Note: The statistical P values in this table were corresponded to Figure 2

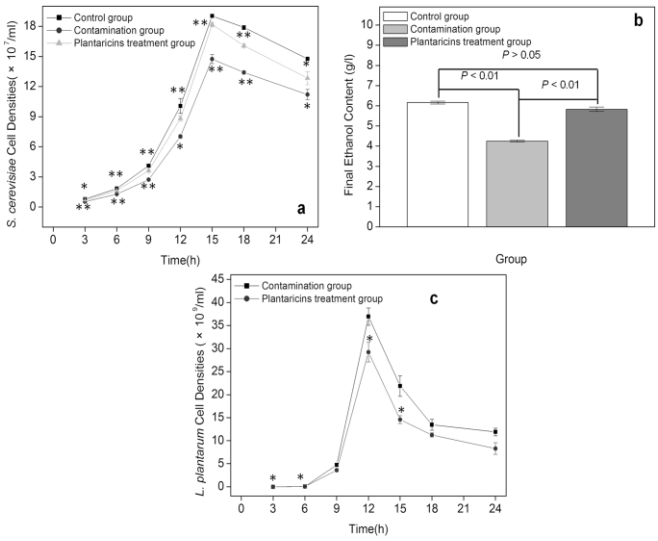

Fig. 3: Effect of plantarcins treatment on *Lactobacillus plantarum* contamination during the culture of *Saccharomyces cerevisiae*. (a) *S. cerevisiae* cell number. In contamination group, **P*<0.05 compared with the control group, ***P*<0.01 compared with the control group. In plantaricins treatment group, **P*<0.05 compared with the contamination group, ***P*<0.01 compared with the contamination group. (b) Ethanol content. Pair-wise compared between the three groups. (c) *L. plantarum* 8014 cell number. **P*<0.05 compared with the contamination group, ***P*<0.01 compared with the contamination group. Values are the mean of three determinations

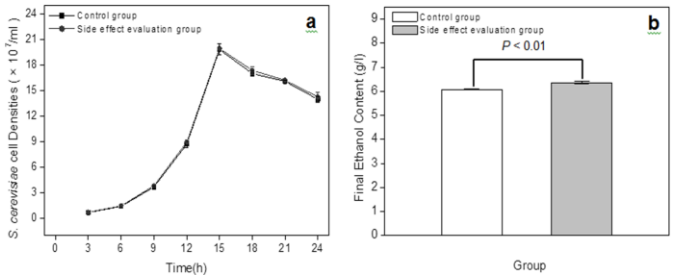

Fig. 4: Side effect of *plantaricins* on fermentation of *Saccharomyces cerevisiae*. (a) *S. cerevisiae* cell number. (b) Ethanol content. Values are the mean of three determinations

cerevisiae and the final ethanol production ($P<0.05$) (Fig. 3).

Bacterial contaminants controlling method should be taken seriously for the purpose of enhancing the bioethanol yield and simultaneously decreasing the cost of production. Biological control has gained much attention for its non-hazardous characteristic and is considered alternative to traditional chemical agents for inhibiting bacterial contamination (Santos *et al*., 2011). Among the biological controlling methods, plantaricins offer an environment–friendly treatment of *L. plantarum* contamination with no harm to *S. cerevisiae*. In the present study, the addition of plantaricins reversed the inhibition of *L. plantarum* on the growth of *S. cerevisiae* to the control group level and removed the negative impact of *L. plantarum* on final bioethanol production (Fig. 3a, b). Moreover, the addition of plantaricins did markedly decrease the cell number of *L. plantarum* at early exponential phase (Fig. 3c). At the beginning of co-culture, resources are relatively abundant. So compared with other phases, hyper proliferative of *L. plantarum* at early exponential phase makes use of the resources originally belonging to *S. cerevisiae* and makes great damage to *S. cerevisiae*. Thus, inhibition of *L. plantarum* contamination at early fermentation is more effective. This laid the foundation for *L. plantarum* control during the whole ethanol fermentation process. These results suggested that plantaricins were effective to control *L. plantarum* contamination during *S. cerevisiae* fermentation. Although these results were acquired from laboratory scale experiments, which containing ethanol at low concentrations, they were still significant for potential application of plantaricins for controlling of *L. plantarum* contamination in industrial bioethanol production containing higher ethanol yield and in which *L. plantarum* contamination was still serious (Skinner and Leathers, 2004).

Compared with antibiotics, natural bacteriocins including plantaricins are safe to environment. In particular, antimicrobial activity of plantaricins has strain-specificity (Daeschel *et al*., 1990), which means they might have no side effect on *S. cerevisiae* growth and fermentation. Additionally, plantaricins not only did no harm to the growth of *S. cerevisiae* (Fig. 4a), but also slightly promoted the production of bioethanol (Fig. 4b). *S. cerevisiae* can secrete several kinds of proteases (Ogrydziak, 1993). As *L. plantarum* contamination was controlled, plantaricins might be degraded by proteases secreted by *S. cerevisiae*. The degraded plantaricins might be utilized by *S. cerevisiae* as nitrogen source. Moreover, it has been reported that supplementation of exogenous tryptophan and proline into culture medium could increase the ethanol tolerance of *S. cerevisiae* (Takagi *et al*., 2005; Ma and Liu, 2010). Some free amino acids might be produced through degradation of plantarcins and conferred ethanol tolerance to *S. cerevisiae* to some extent. Of course, such hypothesis needs further verification. Overall, these results illustrated the biosafety of plantaricins for controlling of *L. plantarum* contamination occurred during bioethanol fermentation.

In summary, the present work illustrated the utility of plantaricins to control *L. plantarum* contamination occurred in bioethanol fermentation. Results showed that plantaricins treatment can effectively control *L. plantarum* contamination in bioethanol fermentation. Moreover, plantaricins treatment did no harm to the growth of *S. cerevisiae*, and to some extent, enhanced bioethanol production. This research implied the potential of plantaricins as a novel bactericide for controlling of bacterial contamination in bioethanol production. Although these results were acquired from laboratory scale experiments, results shown here can still lay the theoretical basis for utilization of plantaricins to control contaminants in industrial bioethanol production, thereby lowering the consumption of antibiotics. In the future, purified plantaricins might be produced as liquid or powder or other forms for application. Finally, there are still other contaminating microbes in the bioethanol fermentation, and use of bacteriocins acting on these microbes will make bacteriocins treatment more efficient.

Acknowledgments

This work was supported by the National Nature Science Foundation of China (No. 31201413), the Fundamental Research Funds for the Central Universities (No. YS1407) and the Higher Education and High-quality and World-class Universities (No. PY201617).

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(Received 21 May 2015; Accepted 23 July 2015)