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Production of Bacteriocin(s) by Four Lactic Acid Bacteria Isolated from Raw Milk on Organic Waste

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Abstract: Four isolates of lactic acid bacteria belonged to *Lactococcus lactis* ssp. *lactis* which isolated from goat's and camel's milk in a previously study were used to produce bacteriocin(s) on organic wastes. Wast products of some date industry (Alsucharia and Alkhlas) and whey were used as broth media for bacteriocin(s) production in comparison with synthetic medium MRS. The whey and dates waste products promote the production and alkhlas was the best. But the addition of yeast extract to whey decreased production when compared with control, on the other hand, addition of 20 g glucose only or with 2.5 g yeast extract increased the production of bacteriocins but stil low in compared with that produced in whey only. Bacteriocins were produced by using a mixture of whey and date broth medium at 1:1. The mixture were increased bacteriocins production in compared with whey and date broth medium separately and the highest production were found by using mixture of whey and Alkhlas if compared with control (whey). Bacteriocin(s) which were produced by the four isolates have an antagonistic effect in a range of pH (2.5, 4 and 4.5) and temperatures 4°C for 72 h, 30, 80 and 100°C for 30 min and finally 121°C for 15 min. Effect of some proteolytic enzymes on bacteriocins were studied, the results showed that proteinase K affected on bacteriocins which produced by G1, G2, C11 and C14 isolates, trypsin affected on bacteriocin(s) which produced by G1, G2, C11 isolates and finally pepsin affected on bacteriocin(s) which produced by G1, G2, C11 and C14 isolates. Also addition of different concentrations of NaCl (40-65 g l^{-1}) have adifferent effects on bacteriocins activities, it were decreased by increase in NaCl concentrations.

Key words: Bacteriocin · organic wast · whey · lactic acid bacteria

INTRODUCTION

Lactic Acid Bacteria (LAB) occur naturally as indigenous microflora in raw milk. Numerous of LAB associated with food systems produce bacteriocins, defined as proteinaceous compounds with activity against related species. Raw milk represents a source of new strains of LAB with the potential to inhibit undesirable microflora for use in biopresrevation of dairy products. Bacteriocins are proteinaceous antibacterial compounds that may be produced by lactic acid bacteria commonly present in foods [1, 2]. Most of bacteriocins produced by gram-positive bacteria are from lactic acid bacteria [3, 4]. They are bactericidal to many Gram-positive bacteria associated with food spoilage and food born illnesses [5-9]. They are degraded by the proteolytic enzymes of the gastrointestinal tract and seem to be non-toxic and non-antigenic to animals. Thus, they can be used to enhance the safety and shelflife of many foods [5-7].

Bacteriocin production is wide spread among LAB present in milk or dairy products [10-13]. The production of bacteriocins is normally performed in complex growth media: de Man Rogosa and Sharpe [14] (MRS), All Purpose with Tween (APT), Alliker, Brain Heart Infusion (BHI), Tryptone Glucose Extract (TGE) and Trypticase Soy Broth medium Yeast Extract (TSBYE) which promote exuberant growths and relatively high bacteriocin levels, their high cost make them unsuitable for a large-scale production. Furthermore, some medium components (e.g. large amounts of proteins, which are not totally consumed by the producer strains at the end of fermentation) may interfere with the subsequent bacteriocin purification [15]. In order to reduce the cost, wastes from the food industry seams to be adequate for asheep culture media to produce bacteriocins in a large amounts, e.g. nisin production on sugar molasses [16], mesenterocin 5 [17], pediocin PO2 [18, 19], lactocin 705 [20], milk whey or mussel processing wasts [19, 21-25], nisin/pediocin mixture production in whey medium was investigated in batch fermentation [26].

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These researchers indicated that whey supported the growth and bacteriocin production, but biomass and bacteriocin levels were lower than those obtained in MRS broth medium.

Studies on complex media and on wastes have demonstrated that bacteriocin production depends on the medium composition [17, 27-31]. This dependence is dueto both the qualitative and quantitative nature of the nutrient sources mainly those of C and N. Additionally, the composition of the media it self provokes different final pH values that also determine the bacteriocin titres [20, 31, 32].

In the present work, we studied the bacteriocin production in synthetic medium (MRS and in wasts of foods industry (whey and dates in Saudi Arabia) by 4 lactic acid bacteria isolates, which were isolated from goat's and camel's milk in a previously study.

We also studied the influence of pH, temperature and proteolytic enzymes on the stability of the bacteriocin(s) extracts which were produced by the four isolates of lactic acid bacteria.

MATERIALS AND METHODS

Bacterial strains and media: Bacteriocin(s)-producing strain which were used in this study were isolated by the direct plating method [3], lactic acid bacteria isolates (G1 and G2) were isolated from goat's milk and isolates (C11 and C14) were isolated from camel's milk in aprevious study all isolates belonged to coccal-shape, *Staphylococcus aureus* were used as the bacteriocin(s) sensitivity indicator. It was grown in nutrient broth medium medium (Difco).

Fermentations were carried out in MRS broth medium for the fermentations components were prepared from single ingredients [14]. All ingredients were purchased from Merck (Darmstadt, Germany) with the exception of peptone and lablemco powder (Oxoid). Solid medium was prepared by adding 1.5% agar (Oxoid) to the broth medium medium. Yeast extraxt (Difco) 2.5 g l^{-1} and glucose 20 g l^{-1} were used as supplements to whey. The energy source (glucose) was sterilised separately and aseptically added to the flasks.

It seems more adequate to use raw materials like some wastes from the food industry as a basis of the culture media. In this study whey and two kinds of dates were used.

Whey preparation and inoculation: Whey were obtained from a local dairy was used as concentrated whey CW. Whey media was prepared as follows: after adjusting the pH to 4.5 with 5 N HCl, they were heated at 121°C for 15 min to denature the proteins and the precipitates were removed by centrifugation at 10,000 = g for 15 min. The supernatants were adjusted to pH 6.3, sterilised at 121°C for 15 min and used as culture media [26]. Yeast extract or glucose was used to supplement the whey. The effect of supplement on the bacteriocins production by LAB isolates were studied. 2.5 g l^{-1} Yeast extract was added to whey and glucose 20 g l^{-1}. Date used with whey 1:1 (V/V). MRS was used as a control medium.

Dates preparation: Wasts of industry two kinds of dates (Alkhlas and Alsuccharia) in Saudi Arabia were used in this study for the production of bacteriocin(s) by LAB isolated from milk. 100 g of date added to 300 ml distelled water, after filtrate the volume completed to 1 l and this solution used as date media.

In vitro fermentations, using 250 ml Erlenmeyer flasks contained 50 ml of MRS broth medium and was sterilised at 121°C for 20 min.

Fermentation experiments: For the preparation of the fermentor inoculum, 10 ml MRS broth medium was inoculated with 0.1 ml of a freshly prepared culture of lactic acid bacteria isolate and was incubated for 12 h at 30°C. From this preculture, 1 ml was added to 100 ml of MRS broth medium medium which, after 12 h of incubation at 30°C, was used to inoculate the fermentation media.

Batch cultures were performed in 250 ml Erlenmeyer flasks containing 50 ml of (MRS, whey and date). The inoculum consisted of 1% (V/V) of a 12-h culture on MRS broth medium. Samples were withdrawn at intervals during incubation periods to measure growth and antibacterial activity. For comparative purposes, 18-h cultures of LAB isolate on MRS broth medium were also performed in the same conditions as the cultures on whey and dates. The flasks were incubated at 30°C for 24 h without pH control. Initial pH of flask cultures was adjusted to pH 6.5.

Production studies: All analytical determinations of these cultures were carried out at the end of the fermentation. For the determination of the bacteriocin activity, cells were removed by centrifugation (5000-g, 15 min). Cell-free supernatant was used to determine the antimicrobial spectrum of activity. The antagonistic activities of the samples were determined for each isolate by the agar well diffusion assay (AWDA) [15]. Twenty

five microliter of each sampels were placed in triplicate into wells (5 mm diameter) made in plates seeded with overnight culture of indicator *Staphylococcus aureus* and examined after incubation at 35°C for 24 h for the presence of growth inhibition.

Growth of lactic acid bacteria isolates on the MRS broth medium medium were assessed by measuring the optical density were determined from the optical density at 660 nm using a spectrophotometer (Hitachi U-1100, Tokyo, Japan) and the calibration curve for the relationship between dry weight and optical density. All measurements were performed in triplicate.

Sensitivity of bacteriocins to temperature, pH, proteolytic enzymes and NaCl: Sensetivity to heat of antibacterial compounds was investigated by treating the culture supernatants at 4°C for 72 h, in water bath at 80 and be 100°C for 30 min and in autoclave at 121°C for 15 min. Immediately after samples were cooled.

To check the pH stability of the non-identified bacteriocins activities were tested by adjusting the pH-values ranging from 2.5-6.5 by adding the appropriate volumes of 4 N HCl or 4 N NaOH. Aliquots of sterile MRS broth medium medium with the pH adjusted to the pH-values were used as controls.

Sensetivity to proteolytic enzymes of antibacterial compounds was investigated by the addition of trypsin, pepsin and proteinase K at final concentration of 1 mg ml^{-1} to the culture supernatants. The samples were incubated at 37°C for 3 h, then the samples boiled for 3 min, finaly samples were cooled. The activities of the treated bacteriocins were determined by (AWDA) as describe above against the indicator strain.

Effect of different concentrations of sodium chloride on the activity of bacteriocin(s) which produced by LAB against *S. aureus* were determined on nutrient agar with NaCl added at concentrations of 40, 45, 50, 55, 60 and 65 g l^{-1} by well diffusion technique [33, 34]. After the adding each concentration of salt to the nutrient agar, the media were autoclaved at 121°C for 15 min [35]. Then cooling to 45°C. One microliter of *S. aureus* activated in nutrient broth medium was pipetted into the medium (100 ml) and the inoculated medium was poured into Petri dishes. When the plates had solidified, wells (5 mm diameter) were cut out of the medium and 50 µl of LAB isolate suspention was pipetted into the wells. The plates were then kept at 4°C for 2 h to allow the diffusion of the suspention into the agar. After the end of overnight incubation (at 35°C), the diameters of inhibition zones were measured.

RESULTS AND DISCUSSION

Production studies: Several complex culture media of high cost have been used for bacteriocin(s) production. In the current study, we have used an effluent from the food industry (whey and dat) as culture media for bacteriocin(s) production of at low costs. These media were used for bacteriocin production by four lactic acid bacteria isolates (G1, G2, C11 and C14) (Fig. 1). Production of bacteriocin(s) at 30°C and at a pH 6.5 were carried out in different media MRS, whey and dates media (Fig. 1). A maximum growth rate (mg/100 ml) were shown in MRS for all isolates and a maximum bacteriocin activity (inhibition zone in mm of *S. aureus*) was appeared by isolate G1 in MRS, whey, date Alkhlas respectively, but

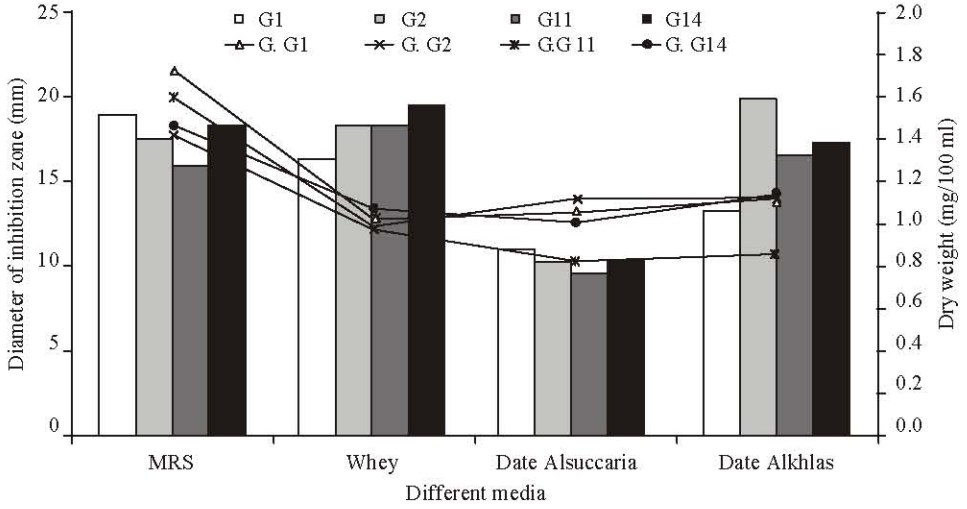

Fig. 1: Influence of different media on the growth and production of bacteriocin(s) by four LAB isolated from goat's and Camel's milk, G: growth

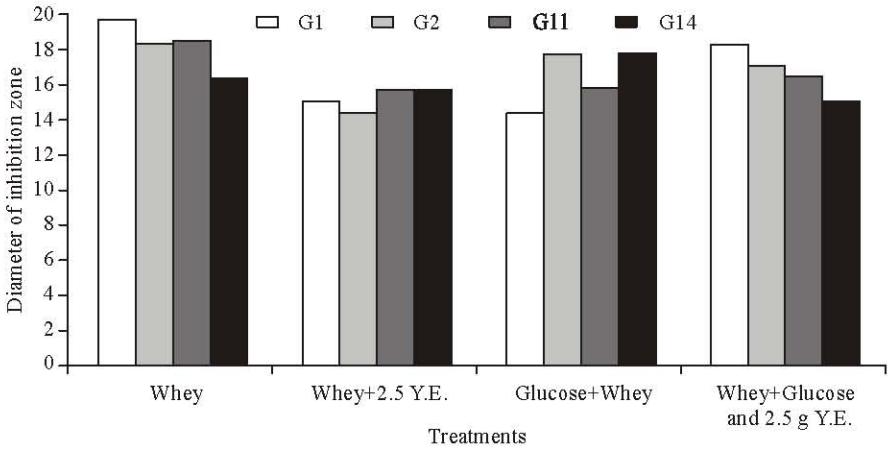

Fig. 2: Influence of supplementation whey medium with YE and glucose on the production of bacteriocin(s) by four LAB isolated from goat's and Camel's milk

the maximum bacteriocin activity by isolate G2 was shown in date Alkhlas, whey, MRS. On the other hand isolates C11 and C14 were given the maximum bacteriocin(s) activity in whey, MRS, date Alkhlas respectively. The lowest amounts of bacteriocins activity were produced in date Alsuccharia by all isolates. Although this fact suggests the possible effect of substrate inhibition. It could also be related to the control that the supplied sugar substrate exerts on the bacteriocin biosynthesis [36]. Biswas *et al.* [27] reported that MRS medium is abetter medium for cell growth and bacteriocin production than other media. Generally, maximum production corresponds to maximum cell concentration [21, 30, 37, 38]. Therefore increased cell concentrations in a high cell-density reactor is expected to increase bacteriocin production. In general bactericin production by lactic acid bacteria occurs during the active growth phase [30, 39, 40]. Conditions favouring bacterial growth and high cell densities are frequently beneficial to bacteriocin production as well [31]. However, a high cell yield does not necessarily result in a high bacteriocin activity since the latter may be limited by a low specific bacteriocin production, i.e. a low bacteriocin production per gram of cells [41, 42]. Hence, there exists a rather complex relationship between environmental conditions and bacteriocin activity levels and no generalisation about the optimum conditions for bacteriocin production can readily be made. The kinetics of both cell growth and bacteriocin production in function of the environmental situation have to be studied to obtain a better understanding of the production mechanism. Earlier detailed studies deal for instance with the production kinetics of amylovorin L471 [40, 43, 44] and sakacin K [41, 42].

In order to investigate the effect of yeast extract or glucose to whey medium on bacteriocin production, 2.5 g l^{-1} yeast extract added to whey (treatment 1), 20 g l^{-1} glucose added to whey (treatment 2), 2.5 g l^{-1} YE and supplied with 20 g l^{-1} glucose added to whey (treatment 3), dat (Alsuccharia or Alkhlas) was added to whey 1:1 (v/v) (treatment 4). Results (Fig. 2) showed that addition of YE, glucose and YE with glucose to whey had negative effects on the production of bacteriocin by LAB isolates, bacteriocin production was increased by isolate G1 in whey medium supplemented with glucose in compared with whey media. De Vuyst and Vandamme [39] also it was found that a high concentration of sucrose increased biomass while bacteriocin production was decreased.

The production of bacteriocin(s) were increased by the addition of Alkhlas date and Alsuccharia date to whey medium when compared with whey medium only.

The results that showed that bacteriocin(s) production were variable, but it was highest in whey with dates. Apparently there would be a dependence between bacteriocin production and presence of different succharides in date as fructose and sucrose and glucose and its concentration (Fig. 3). These observations are in agreement with Pitt and Gaston [45], who told that the type and amount of carbon source may affect bacteriocin production.

Glucose is considered the main carbon source by all microorganisms due to its size, rapid uptake, utilization and cellular energy conversion. However, some bacteria have a complete enzymatic machine that allows them to use complex carbohydrates; for example, *Ent. faecium* shows a variable sucrose fermentation pattern [46]. Brown sugar, molasses and other complex carbohydrates can be

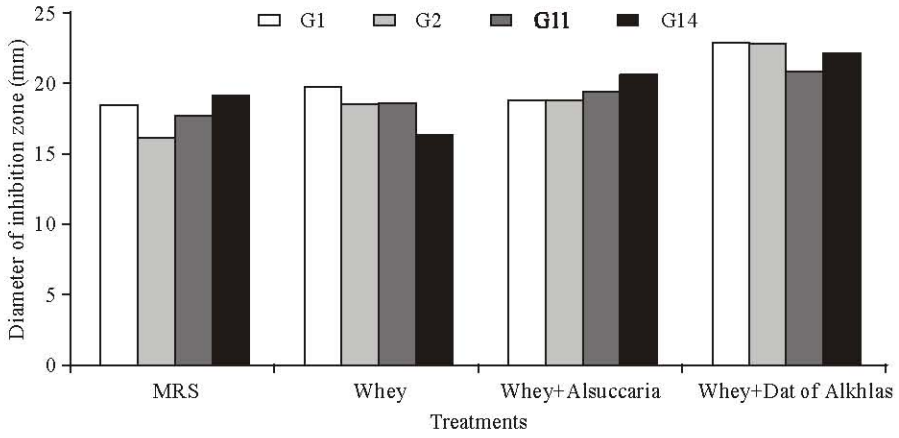

Fig. 3: Influence of whey and date media on the production of bacteriocin(s) by four LAB isolated from goat's and Camel's milk

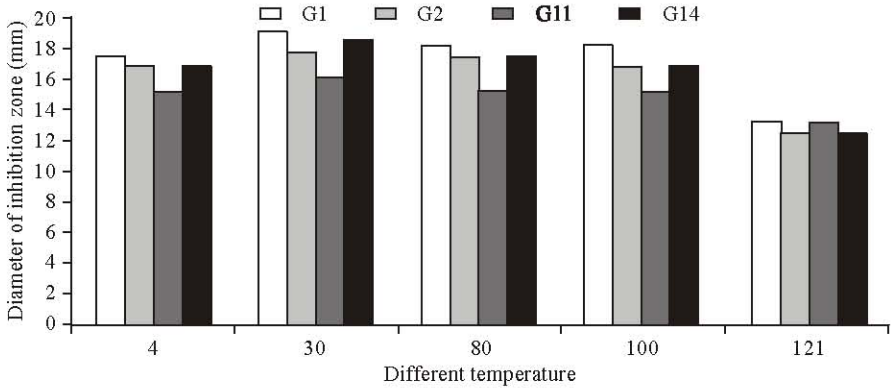

Fig. 4: Sensitivity of bacteriocins produced by LAB isolated from goat's and Camel's milk to differeent tempretures

considered as prebiotics [47]. Several mechanisms can be responsible for the decrease of bacteriocin activity, such as protein aggregation, proteolytic degradation by specific or non-specific enzymes and bacteriocin adsorption to the producer cells [30, 39]. These results could be related to the chemical composition of date that proved to have not only the highest percentage of free monosaccharides such as glucose and fructose, but also more complex sugar.

Sensitivity of bacteriocins to temperature, pH, proteolytic enzymes and NaCl: The temperature ranges and pH tested were chosen based on their usual levels in foods and in their processing operations. Results in Fig. 4 showes that all bacteriocins remained active after 72 h at 4°C, at 80, 100 for 30 min and at 121°C for 15 min, in comparison with its activity at 30°C at the highest tempreture degree the activity became lower but it did not destroyed after heat treatment. These results suggested that the inhibitory compounds produced by these isolates of LAB were a heat-stable proteinaceous compounds.

Bacteriocins produce by isolates G1, was stable at pH 2.5-4.5 but bacteriocin produced by isolate G2, C11 and C14 were stable at pH 2.5-5.

The effect of enzymes, pH values on the antimicrobial activities of cell-free cultures supernatants of LAB isolates summarized in Table 1. Application the bacteriocins to proteinase K at final concentration of 1 mg ml^{-1} led to inactivation of the antagonistic activity of bacteriocin produced by isolate G2, C11 and C14, while activity of bacteriocins produced by isolates G1, G2 and C14 sensetive to pepsin and trypsin partially destroyed the activity of bacteriocins produced by isolate C14 and led inactivation of the antagonistic activities of culture supernatant of isolate G1, G2 and isolate C11.

Table 1: Sensitivity of bacteriocins produced by LAB isolated from goat's and Camel's milk to pH and proteolytic enzymes

Bacteriocin produced by LAB isolate	pH values						Sensitivity to proteolytic enzymes		
	2.5	3.0	3.5	4.0	4.5	5.0	Preinase K	Pepsin	Trypsin
G1	+	+	+	+	+	-	+	-	-
G2	+	+	+	+	+	+	-	-	-
C11	+	+	+	+	+	+	-	+	-
C14	+	+	+	+	+	+	-	-	+

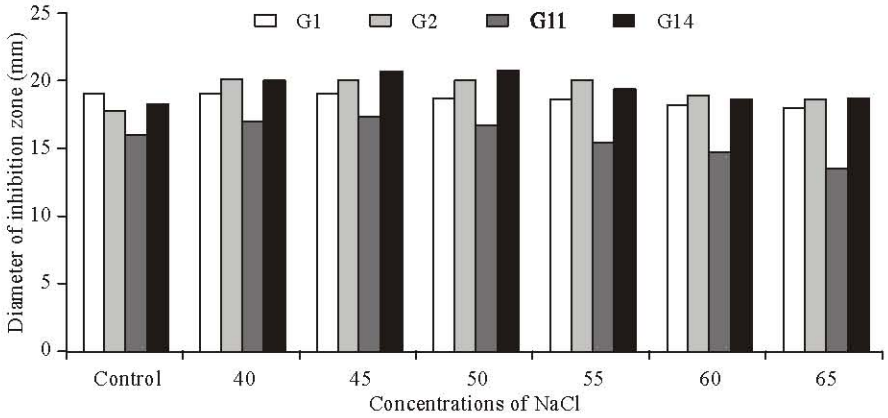

Fig. 5: Sensitivity of bacteriocins produced by LAB isolated from goat's and Camel's milk to differeent concentrations of NaCl

The results showed that bacteriocins which produced by isolates G1 stable at different pH values from 2.5-4.5 and that produced by isolate G2, C11 and C14 were stable to pH 5. It is well known that bacteriocins produced by lactic acid bacteria are sensitivity to other enzymes varies [48]. The antimicrobial activities in the supernatant cell-free cultures of the four isolates of lactic acid bacteria were lost by proteinase K treatment but with stood heat treatments and exposition to range of pH values and high concentrations of NaCl like most LAB-bacteriocins [49]. These observations suggest that this antimicrobial activities are truly due peptide bacteriocin(s).

The activity of bacteriocin(s) did not destroyed by the addition of different concentrstions (40-65%) of NaCl but it became lower and this depened on the isolates and the concentrations of NaCl as shown in Fig. 5.

The antilisterial activity of nisin and the bacteriocin produced by *Carnobacterium piscicola* A9b was lower in the presence of 2-4% (w/v) NaCl [48, 50, 51]. *Lactobacillus casei* and *Lactobacillus helveticum* inhibited of *Bacillus* strains in presence of NaCl depened on the concentrations of salt and the indicator of bacteria and the LAB strain [52]. The protective effect of sodium chloride on *L. monocytogenes* may be due to the presence of Cl-anions that inhibit the binding of bacteriocin molecules to the surface of the cell membraine as proposed by Bhunia *et al.* [53].

CONCLUSIONS

The specific production of bacteriocin by LAB isolates in whey and dates media, reached levels that were equal to higher than production in an synthetic medium. Further optimization of bacteriocins production by these isolates requires more studies.

Data are means of triplicates. Standard errors were less than 5.0% of the means.

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