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Characterization of Conjugated Linoleic Acid Production by *Bifidobacterium breve* LMC 520

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This study was performed to characterize the CLA production ability of a bacterial strain, *Bifidobacterium breve* LMC 520, which can actively convert linoleic acid (LA) to *cis*-9,*trans*-11 conjugated linoleic acid (CLA), a major isomer derived from microbial enzymatic conversion. The culture conditions were optimized to improve CLA production under the aerobic conditions. *B. breve* LMC 520 was tested with different amounts of LA in varied culture conditions, such as air, additives, and pH. A maximal level of CLA production (up to 90% of substrate) was obtained after 24 h of incubation in culture medium containing 1 mM LA at pH 5.5 and under anaerobic conditions. There was no decline in the CLA level with prolonged incubation until 48 h. When the effect of pre-incubation with LA on CLA production was tested, there was no significant difference between the CLA-producing activity of pre-incubated and untreated bacteria at the third passage but there was a significant reduction in CLA production by the pre-incubated cells after the fourth passage. These results demonstrate that the CLA-producing activity of *B. breve* LMC 520 could be maximized by numerous environmental factors. The data also indicate its potential for increasing CLA accumulation in dairy products when *B. breve* LMC 520 is used as a functional starter culture.

KEYWORDS: *Bifidobacterium breve*; conjugated linoleic acid; linoleic acid; fermentation; starter

INTRODUCTION

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic acid (LA; $C_{18:2}$, *cis*-9,*cis*-12 octadecadienoic acid) with conjugated double bonds. The double bonds of CLA may be in the positions of 7,9; 8,10; 9,11; 10,12; or 11,13, along with the three-dimensional geometric combinations of *cis* and/or *trans* configurations. CLA is naturally found in a variety of foods derived from ruminants (*1*). Among them, dairy products are the richest sources of natural CLA (*2*, *3*). The major CLA isomers in foods are as follows: *cis*-9,*trans*-11-CLA (also called rumenic acid) > *trans*-7,*cis*-9-CLA > 11,13-CLA (*cis*/*trans*) > 8,10-CLA (*cis*/*trans*) > *trans*-10,*cis*-12-CLA isomer > other minor isomers (*4*–*6*). In recent years, there has been considerable interest in these isomers because of their potential health-promoting properties and proposed positive effects in the prevention of many degenerative diseases (*7*–*12*). Among these beneficial effects, the anti-carcinogenic effects of CLA have been attributed to either mixtures of CLA isomers that contain primarily the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 forms in approximately equal proportions or one of these isomers (*13*). Furthermore, recent studies have shown that each isomer may have different effects on metabolism and cellular function and acts through different cell-signaling pathways (*14*). For the application of CLA for medicinal and nutraceutical purposes, the properties of each isomer should be investigated.

The *cis*-9,*trans*-11 form is the main isomer of CLA and is obtained from ruminants in milk, dairy products, and meat (*2*, *15*). The CLA in dairy products is primarily derived from the enzymatic activity of rumen bacteria, such as *Butyrivibrio fibrisolvens* (*16*). The *cis*-9,*trans*-11 CLA isomer is an intermediate in the biohydrogenation (BH) of LA to stearic acid (SA) by anaerobic rumen bacteria, such as *B. fibrisolvens* (*17*). However, because the natural occurrence of CLA in ruminant tissue is far below the physiologically effective level, CLA is produced as a dietary supplement through organic synthesis, which includes various unidentified isomers (*18*). On the basis of this, extensive trials have been made to enrich CLA in ruminant tissue and dairy products and, thus, to maximize its health-promoting effects (*19*, *20*).

In a previous study, we isolated an active CLA-producing *Bifidobacteria* from breast-fed infants (*21*). The objectives of this study were therefore to identify the optimal conditions for CLA production during fermentation by *Bifidobacterium* species.

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MATERIALS AND METHODS

Chemicals. The lipid standards used in this study were obtained from Sigma Chemical Co. (St. Louis, MO), and MRS broth was purchased from Difco (Detroit, MI). Heptadecanoic acid (Sigma) was chosen as an internal standard (IS). All other chemicals used in the fatty acid analysis were of analytical grade (Fisher, Springfield, NJ).

Microbial Production of CLA. The study employed *Bifidobacterium breve* LMC 520, which has the highest CLA-producing activity among screened bifidobacteria (*21*). For the production of CLA, *B. breve* LMC 520 was subcultured twice in MRS broth with 0.05% L-cysteine·HCl (mMRS; Sigma) at 37 °C for 18 h under O_2-free CO_2, in Bellco tubes (18 × 150 mm; Bellco, Vineland, NJ) that were capped with septum stoppers (Bellco) and aluminum seals (Bellco). The activated cultures were transferred to fresh mMRS and then incubated under different conditions. The growth rate was estimated by a microplate reader at 600 nm for optical density (OD) (Bio-Rad, Hercules, CA). pH was measured with a Mettler-Toledo MP-220K instrument (Mettler-Toledo AG, CH-8603 Schwerzenbach, Switzerland), which was calibrated using a pH buffer (Mettler-Toledo) at pH 4.01 and 7.0, according to the operating manual.

Ethylation for Fatty Acid Analysis. A total of 1 mL of medium, with heptadecanoic acid ($C_{17:0}$) added as an internal standard (IS), was extracted with 12 mL of chloroform/methanol (1:1, v/v). The lower layer was mixed vigorously with 2 mL of 0.88% KCl solution and then evaporated with nitrogen until dry. The extracted lipids were ethylated using 10 mL of 2% H_2SO_4 in ethanol at 80 °C for 60 min (*22*). After 8 mL of saturated NaCl solution and 4 mL of *n*-hexane were added, fatty acid ethyl esters were obtained in the *n*-hexane layer and analyzed for total fatty acids, including CLA isomers, using a 7890A gas chromatograph with a flame ionization detector (Agilent Technologies). The fatty acid ethyl esters were separated using a Supelcowax-10-fused silica capillary column (100 m × 0.25 mm inner diameter, 0.2 μm film thickness; Supelco, Inc., Bellefonte, PA) with 1.2 mL/min of helium flow. The oven temperature was increased from 190 to 240 °C at the rate of 4 °C/min. The temperature of both the injector and detector was 260 °C. A total of 1 μL of sample was injected into the column in the split mode (50:1). The peaks for each CLA isomer and other fatty acids were identified and quantified by comparison to the retention time and peak area of each fatty acid standard, respectively. IS was included as an internal reference before the extraction to determine the recovery of the fatty acids in each sample.

Statistical Analysis. All data were in triplicate and expressed as means ± standard deviations. Analysis of variance was performed by analysis of variation (ANOVA) procedures. Duncan's multiple-range test was used to determine the difference of means, and $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Among the CLA isomers produced by *B. breve* LMC 520, the *cis*-9,*trans*-11 CLA isomer was the main isomer (>90%) and trace amounts of the other CLA isomers were also produced (**Figure 1**). When *B. breve* LMC 520 was incubated in mMRS containing 2 mM LA, its CLA production was highest at the middle of the logarithmic to early stationary growth phase (24 h) and prolonged incubation until 48 h did not appear to further enhance CLA production (**Figure 2**). Bacterial growth was positively correlated wtih CLA production. Therefore, 24 h (0.43 mg/mL) of incubation may be an optimal incubation time for CLA production.

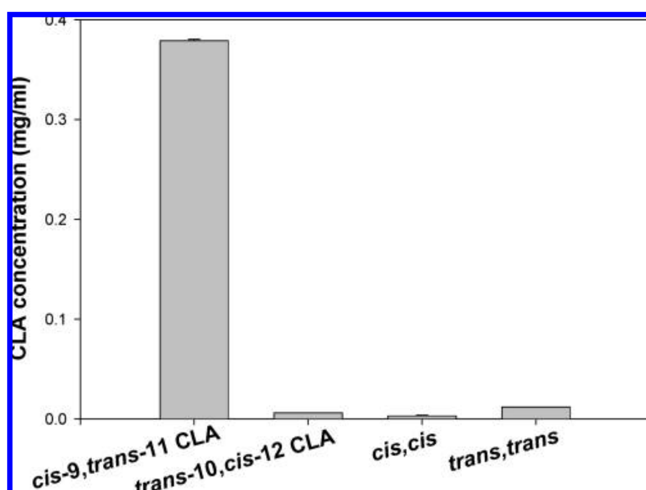

Figure 1. Isomers of total CLA produced by *B. breve* LMC 520.

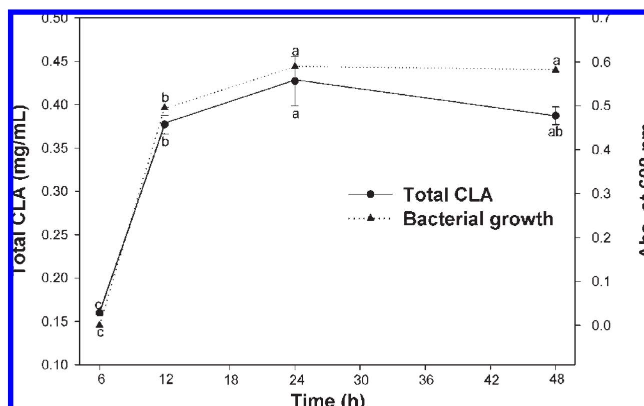

Figure 2. CLA production and growth of *B. breve* LMC 520. A total of 2% (v/v) *B. breve* LMC 520 cultures were inoculated into mMRS containing 2 mM free LA and incubated at 37 °C for 48 h under anaerobic conditions. Values having different letters are significantly different ($p < 0.05$).

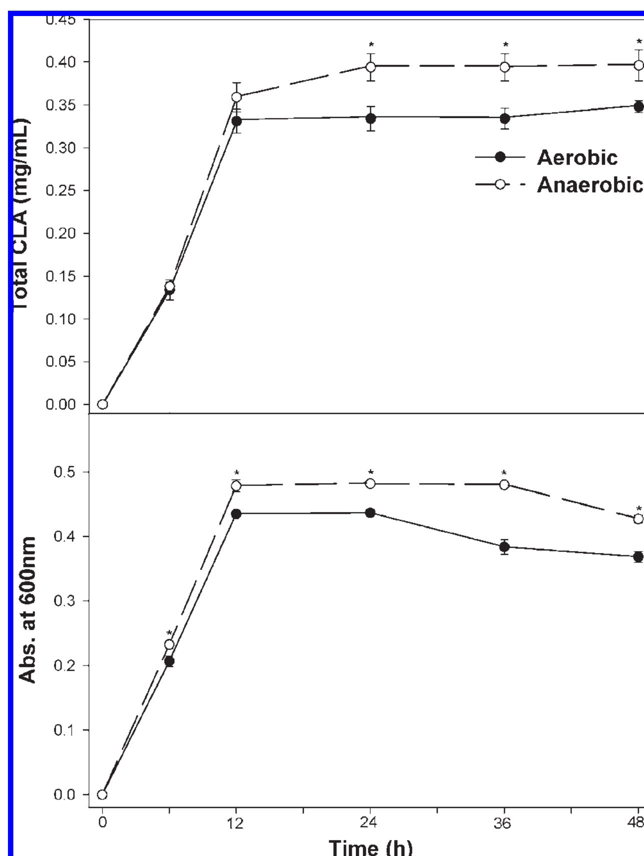

Figure 3. CLA production and growth of *B. breve* LMC 520 at different atmospheric conditions. The culture (2%, v/v) was incubated in mMRS containing 2 mM free LA at 37 °C for 48 h under anaerobic and aerobic conditions. Asterisks indicate that the values at each time point are significantly different ($p < 0.05$).

There was no significant difference in CLA production between the aerobic and anaerobic conditions until 12 h (**Figure 3**). However, CLA production was higher in the anaerobic conditions from 24 to 48 h of incubation compared to the aerobic conditions. Furthermore, the growth of *B. breve* LMC 520 was inhibited under the aerobic conditions, especially after 6 h of incubation, and the degree of inhibition was maintained until 48 h of incubation. Although the CLA-producing activity of *B. breve* LMC 520 was higher in the anaerobic versus aerobic conditions, the strain showed aero-tolerance, which was conformed by a high level of CLA production in the aerobic conditions. In general, the cell growth of *Bifidobacterium* sp. is considerably inhibited under aerobic conditions, but this was not the case for *B. breve* LMC 520. *B. fibrisolvens*, which is known to be a major CLA-producing rumen bacterium, has a higher rate of BH (*23*). However, *B. breve* LMC 520 was shown to have little BH activity. This indicates that *B. breve* LMC 520 is desirable as a CLA-producing starter strain for CLA-enriched dairy products. When *B. breve* LMC 520 was incubated with different concentrations of LA, CLA production proportionally increased nearly 3-fold by the addition of LA up to 8 mM (2.24 mg/mL) (**Figure 4**). Although the highest level of CLA production was observed at 8 mM LA, the conversion rate from LA to CLA proportionally decreased as the LA concentration increased up to 8 mM and the highest conversion rate (more than 90%) was shown at 1 mM (0.28 mg/mL) LA concentration.

To study the effect of pre-incubation with the substrate on CLA production, *B. breve* LMC 520 was pre-incubated with 2 mM LA and without LA for 7 days, with daily passage (**Figure 5**).

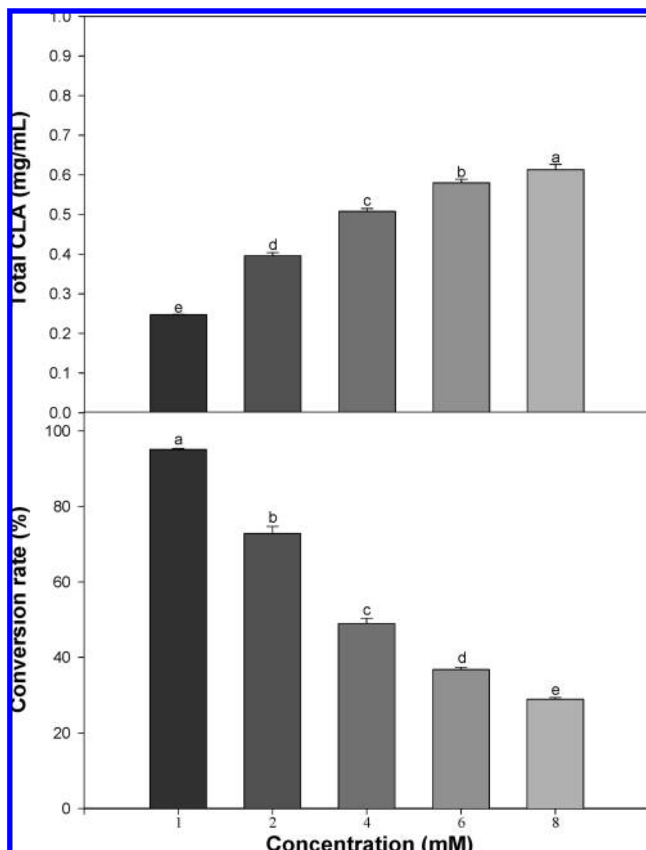

Figure 4. CLA production and conversion rate of *B. breve* LMC 520 at different LA concentrations. The culture was incubated in mMRS containing different concentrations of LA (1−8 mM) at 37 °C for 24 h under anaerobic conditions. Bars with different letters are significantly different ($p < 0.05$).

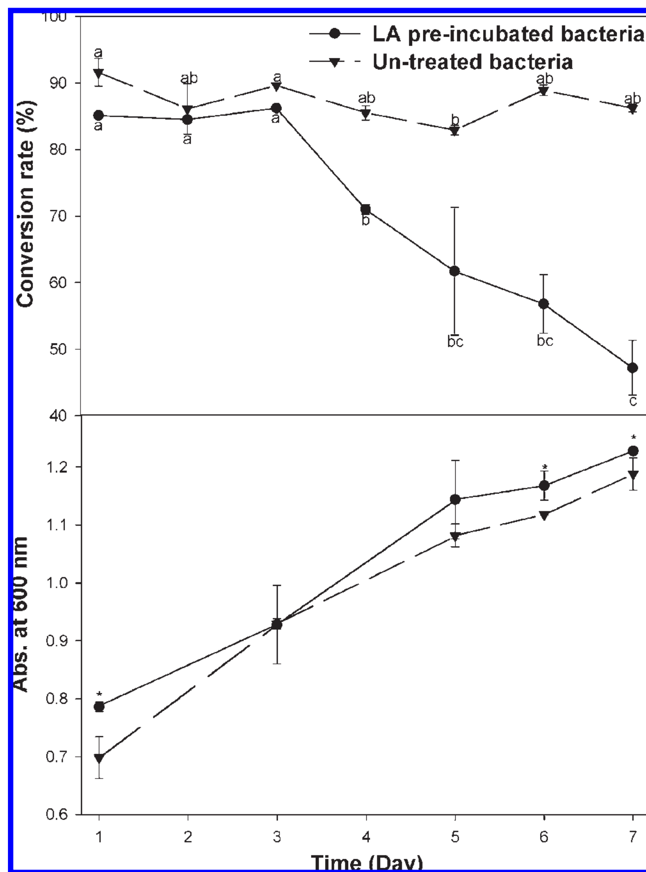

Figure 5. Effects of pre-incubation with LA on CLA production. *B. breve* LMC 520 was pre-incubated with 2 mM LA (pre-incubated bacteria) or without LA (untreated bacteria) for 7 days repeatedly, and then samples from each day were analyzed. Values having different letters are significantly different ($p < 0.05$). Asterisks indicate that the values within each sampling time are significantly different ($p < 0.05$).

Because the CLA production at each time point could vary by substrate concentration, the results are expressed as the conversion rate (%). No significant differences in CLA-producing ability were found between the LA pre-incubated bacteria and the untreated bacteria up to the third passage. However, a significant reduction in CLA accumulation was shown by the LA pre-incubated bacteria from the fourth passage, and the difference in the seventh passage was nearly 2-fold. The bacterial growth between the LA pre-incubated and untreated group was similar. The decrease of growth that affects CLA production was not observed. Although the mechanism of LA pre-incubation is not clearly established, it is probably related to a difference in the adaptability to the inhibitory substrate. Indeed, unsaturated fatty acids including LA are generally toxic to a wide spectrum of bacteria. The effect of LA pre-incubation may not only be related to biochemical metabolism but also to more complex processes, such as expression levels of enzymes involved in CLA production. Further studies should be performed to determine the expression levels of CLA-producing enzymes between LA pre-incubated bacteria and untreated bacteria.

Lactic acid bacteria (LAB) are generally fastidious organisms requiring complex nutrients, such as amino acids and vitamins, for growth and activity. Therefore, we performed tests to demonstrate the effects of media components on CLA production. On the basis of data from a previous study (*24*), yeast extract (YE; 30 mg/mL), Tween 80 (T80; 0.5 mg/mL), glucose (GLU; 10 mg/mL), $MgSO_4$ (MG; 0.5 mg/mL), and phosphate (PO; 4 mg/mL)

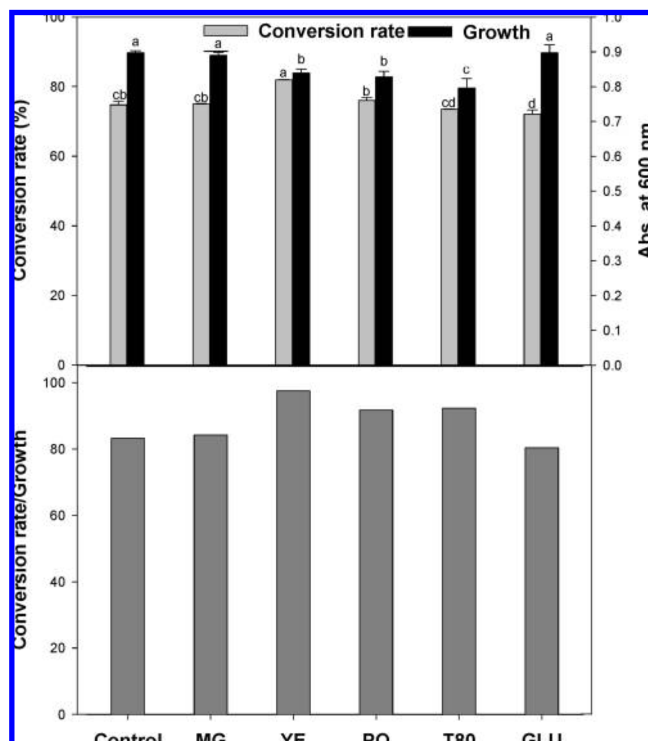

Figure 6. Effects of medium components on CLA production by *B. breve* LMC 520. The culture was incubated in mMRS containing different additives and 2 mM LA at 37 °C for 24 h under anaerobic conditions. Bars with different letters are significantly different ($p < 0.05$). Control, no addition; MG, $MgSO_4$; YE, yeast extract; PO, phosphate; T80, Tween80; GLU, glucose.

were added to mMRS containing 2 mM LA (**Figure 6**). Except for the YE and GLU, all other additives had no significant effect on CLA production. YE is the most commonly used nitrogen source, providing vitamin B complex to LAB, and is used in most fermentation studies as a supplement for minor nutrients (*25*). The conversion rate from LA to CLA was highest among the tested groups when YE was added to the medium. However, the addition of GLU decreased the conversion rate as compared to the control. Furthermore, conversion per growth was highest by the addition of YE when compared to all of the other tested conditions, and it was 1.2 times higher compared to GLU.

As a non-ionic surfactant, T80 has been known to exert a number of positive effects on degradative enzymes in *in vitro* aerobic and anaerobic microbial cultures. In the case of *B. fibrisolvens*, which has energy-requiring BH processes: the reduction of CLA to *trans*-vaccenic acid, the addition of glucose caused CLA reduction by BH. Because *B. breve* LMC 520 is not active in BH, the glucose addition may have had no significant effect on CLA production. The effects of energy sources on CLA production should be further explored, because all of the above results indicate that the energy source may be an important factor for CLA production, and the addition level could be optimized to maximize the production.

pH is an important factor not only for growth but also for enzyme activity in CLA production. The effects of pH on CLA production were tested at different pH levels using acetate buffer (pH 4.0−5.5), potassium phosphate buffer (pH 6.0−7.0), Tris buffer (pH 7.5−8.5), and carbonate buffer (pH 9.0−10.0). *B. breve* LMC 520 was activated, and then 400 mg of washed cells (resting cells) were inoculated into the different pH buffers. The resting cells were incubated at 20 °C for 3 h. The resting cells were used to prevent the inhibitory effect of LA on cell growth. The

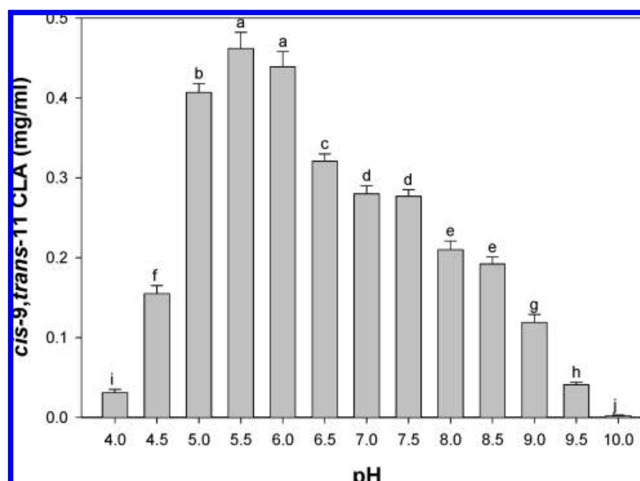

Figure 7. Effects of pH on CLA production by *B. breve* LMC 520. The resting cells were incubated in mMRS containing different pH buffers and 2 mM LA at 20 °C for 3 h under anaerobic conditions. Bars with different letters are significantly different ($p < 0.05$).

highest level of CLA production (0.46 mg/mL) was observed at pH 5.5 and declined thereafter at higher pH (**Figure 7**). This could be an advantage in using this strain as a starter culture for the production of CLA-enriched dairy products. In fact, a pH of 5−5.5 is optimal for the growth of most other starter cultures used for milk fermentation, including *Lactobacillous acidophilus* (*26*).

In conclusion, the CLA production of *B. breve* LMC 520 was principally dependent upon numerous environmental factors, including aeration, incubation time, pH, substrate concentration, and other media components. The pre-exposure of this strain to LA declined CLA production activity. These determined conditions could be applied to increase CLA levels during milk fermentation and dairy production but should be further optimized by exploring other unknown environmental factors.

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