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研究报告

Autolysis of *Lactococcus lactis* MG1363 induced by growth inhibitors

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Abstract: [Objective] Autolysis is a physiological accommodation of bacteria to lyse themselves under stress conditions. The aim of this study is to investigate comprehensively the autolysis of the lactic acid bacteria (LAB) strains stressed by various growth inhibitors. **[Methods]** The methods include the autolysis assay of of LAB strains with various origins, as well as the determination of autolysis profiles of *Lactococcus lactis* MG1363 under the conditions specified by different growth media and inhibitors' interference. **[Results]** The results showed that the autolysis of *L. lactis* MG1363 could be induced in glucose strictly-limited medium through the action of ampicillin (Amp), and this phenomenon could only occur at the glucose exhausting point, indicating a growth phase-dependent pattern. And concomitantly, the expression of four predominant autolysins was significantly altered to a variable extent upon the addition of Amp. Furthermore, all the tested inhibitors negatively affected MG1363's autolysis under non-nutrient conditions, probably suggesting a mode of co-regulation between cell wall synthetic and hydrolytic enzymes. **[Conclusion]** Collectively, a significant discrepancy was proposed with respect to the autolysis of LAB strains administered with different inhibitors, and moreover, this peculiar phenomenon was strictly nutrition-and growth phase-dependent.

Keywords: Autolysis, Antibiotics, Growth inhibitor, Lactic acid bacteria, *Lactococcus lactis*

生长抑制剂所诱导的乳酸乳球菌 MG1363 的自溶表型及其生长阶段特异的性质

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摘 要：【目的】自溶是细菌在压力环境下通过自身裂解而获得的一种生理适应现象，研究的目的是全面探讨乳酸菌株在生长抑制剂条件下的自溶表型及机理。【方法】对多种来源的乳酸菌株的自溶能力进行检测，通过在不同生长条件和抑制剂压力条件下乳酸乳球菌 MG1363 的生长检测对其自溶表型进行分析。【结果】在葡萄糖严格受限的培养基中，氨苄青霉素的加入能够显著诱导 MG1363 的自溶，而且该自溶现象只发生在葡萄糖耗尽的时间点，展现出一种狭窄的生长时期依赖的特征。与此同时，因为氨苄青霉素的加入，4 种主要的自溶酶的表达都发生

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了不同程度的显著改变。此外，所有受试的抑制剂都削弱了 MG1363 在非营养条件下的自溶，表明该菌株可能具有一种涉及细胞壁合成和降解酶的共同调控的模式。【结论】乳酸菌株在不同生长抑制剂条件下的自溶表型存在很大差异，且该自溶体现出营养条件和生长时期严格依赖的特征。

关键词：自溶，抗生素，生长抑制剂，乳酸菌，乳酸乳球菌

Lactic acid bacteria (LAB) is a group of gram positive bacteria utilizing sugar to generate lactic acid[1-2]. Due to its probiotic status, LAB held a crucial position in dairy industry. Autolysis is the bacterial physiological behavior to lyse themselves under stress conditions, through the action of peptidoglycan hydrolases (PGHs)[3-7]. Autolysis was generally induced by a range of rigid environmental conditions, including altered medium component, nutrition deficiency and growth inhibition[8-9]. The starter's autolysis during cheese manufacture could efficiently increase the free amino acid concentration in the curd, thus facilitating the development of flavor compounds and acceleration of the ripening process[9-11]. Therefore, it's of industrial and technological importance to screen the highly autolytic lactic acid bacteria strains and study their autolytic mechanisms under various environmental conditions.

The bacterial autolysis was generally involved in the cessation of peptidoglycan synthesis, such as elongated stationary phase or antibiotic treatment[12-14]. The autolytic profiles of *Staphylococcus aureus* strains were profoundly influenced by the antibiotic treatment[15-16]. Recently, it was evidenced that *Bacillus subtilis* and *Enterococcus faecium* could be used as the reporter strains to screen the autolysis-inducing agents[17-18]. The literature unraveled the importance to investigate the antibiotics' roles in the autolysis induction of the studied organism. However, despite the fact that the living environment of most LAB strains was frequently invaded by antibiotics, how antibiotics affected LAB's autolysis and survival still remains unknown.

It's aimed here to investigate the autolytic properties of various LAB strains primarily from Chinese traditional fermented milk, examine the enzymatic activities and transcription levels of various peptidoglycan hydrolases, and then investigate the autolytic profiles of *L. lactis* MG1363 elicited by a series of growth inhibitors.

1 Materials and Methods

1.1 Bacterial strains and growth conditions

The strains involved in this study were listed in Table 1. *L. lactis* was grown in M17 broth[3], (Oxoid, Basingstoke, UK.) supplemented with 0.5% of glucose, and the culture condition was set as 30 °C without agitation. For the glucose-limited media used in the induction experiments, the glucose concentration was adjusted as 0.25% and 0.05% for 1/2 GM17 and 1/10 GM17, respectively. The same conditions were also used for the growth of enterococcal strains. The lactobacillus strains were grown at 37 °C without agitation in MRS broth (Oxoid, Basingstoke, UK.).

1.2 Autolysis of whole cells in buffer solution

The autolytic phenotype of the LAB strains was evaluated as follows: the strains were cultured to the mid-exponential phase (OD_{600}=1.0−1.5) or stationary phase (OD_{600}=2.0) and then collected at 5 000×g for 3 minutes. The cells were washed once and resuspended in 50 mmol/L Tris-HCl (pH 7.0) or other buffer used, making the final OD_{600} reach 0.6−0.8. Subsequently the suspension was incubated at 30 °C for 72 h. The degree of autolysis was expressed as the reducing percentage of OD_{600} after 24 h or 72 h.

1.3 Total mRNA extraction and quantitative PCR

Total mRNA were extracted when MG1363 was grown for 5 h in GM17, in order to ensure the presence of autolysins. In addition, samples were also obtained from 1/10 GM17 culture at 2 h following the Amp's administration at 5 h of growth, in order to examine the alterations of autolysins upon the induced autolysis. The total RNAs were extracted using the RNA Extraction Kit (RNA-Solv Reagent, Omega Biotech, USA), according to the manufacturer's instructions. Subsequently, the random primer was used to carry out the reverse transcription reaction according to the manufacturer's instructions, (PrimeScript™ II 1st strand cDNA Synthesis Kit, TaKaRa Bio, Dalian,

China.), resulting in the total cDNA.

The 25 μL reaction pool of fluorescent quantitative PCR was composed of: 12.5 μL of SYBR premix Ex*Taq* (TransStart Top Green qPCR Super Mix, TransGen Biotech, Beijing, China); 1 μL of 10 μmol/L forward and reverse primer each; 2 μL of 100 ng/μL cDNA template and 8.5 μL of deionized water. The real-time fluorescence PCR device was LC-96 (Light Cycler® 96 Real-Time PCR System, Roche Life Science; Beijing, China). The reaction procedure was set as one cycle of 95 °C for 30 s; 40 cycles of 95 °C 5 s, 50 °C 30 s, 72 °C 1 min, followed by the dissociation stage of 95 °C 15 s, 60 °C 30 s and 95 °C 15 s. The transcription levels were calculated as the amounts relative to that of 16S rRNA gene under the same conditions. The primers used were listed as follows: *AcmA*: Forward primer: 5′-CTTATCAAGGAAAGAGCGTCGTA-3′, Reverse primer: 5′-CGCCATAACTTGGGTCGGTA-3′; *AcmB*: Forward primer: 5′-TATGGGAAATGGTGGAGAA-3′, Reverse primer: 5′-TGGCGTTCGCTGACA ATA-3′; *AcmC*: Forward primer: 5′-ATTCCGTTTCG GCTCATAC-3′, Reverse primer: 5′-TCAGTCGCATA GCCAGAGC-3′; *AcmD*: Forward primer: 5′-TTGGA ATCTAGTGGTGGTCA-3′, Reverse primer: 5′-TTGG GTCAGTAGCATAACG-3′; *YjgB*: Forward primer: 5′-AGGTGTTCCCTATGTTTGG-3′, Reverse primer: 5′- ATTCCCACATGGTCTCCAC-3′.

1.4 Protein extraction and renaturing SDS-PAGE

4 mL of LAB culture were collected when grown to the mid-exponential phase. The cells were washed once and resuspended in 40 μL of deionized water. Subsequently, 10 μL 5×SDS loading buffer (250 mmol/L Tris-HCl (pH 6.8), 10% (*W/V*) SDS, 0.5% (*W/V*) BPB, 50% (*V/V*) Glycerol, 5% (*W/V*) β-mercaptoethanol) was added to the cell suspension and mixed thoroughly before boiling for 3 min. The cell debris was then removed by centrifugation at 12 000 r/min for 10 min, and 5 μL of the supernatant was subjected to the SDS-PAGE gel.

SDS-PAGE was performed with 12% (*W/V*) polyacrylamide separating gels. Renaturing SDS-PAGE was performed as previously described[19] with 0.2% autoclaved *Micrococcus lysodeikticus* cells included in the gel. The gel was then incubated in 25 mmol/L Tris-HCl (pH 7.0) buffer containing 1% Triton X-100 at 37 °C for 2−16 h or till the apparent degrading bands occurred.

1.5 Determination of glucose concentration

Glucose concentration of liquid medium was determined by dinitrosalicylic acid (DNS) method[8]. 3 mL of reagent (1% DNS, 0.2% phenol, 0.05% Na_2SO_3 and 1% NaOH) was added to 3 mL of glucose-containing solution. The mixture was boiled for 5 min and naturally cooled to room temperature. Optical density value was measured at 575 nm.

1.6 Autolysis induction assays

The growth inhibitors used in this study were Cm (A protein synthesis inhibitor with MIC of 8 mg/L), Amp (A cell wall synthesis inhibitor with MIC of 4 mg/L), Km (A protein synthesis inhibitor with MIC of 4 mg/L) and SDS (A detergent and membrane disrupter). In order to examine autolysis of the *L. lactis* strains grown in 1/2 GM17 media, Cm or Amp was added to the culture at different growth points during the exponential phase (OD_{600} 0.25−2.00). And then the bacterial growth curve continued to be depicted, till the diauxic growth ended. The same protocol was utilized in the induction assays pertaining to the strains in 1/10 GM17 media. The final concentration of Cm was 8 μg/mL, 50 μg/mL and 200 μg/mL, and the concentration of Amp was 4 μg/mL and 100 μg/mL, respectively.

To investigate the antibiotic effects on the buffered autolysis capability of *L. lactis* strains, Cm, Amp, Km and SDS with various concentrations were added to the GM17 culture with OD_{600} of 0.3. When the optical density reached 0.7, the cells was collected by centrifugation at 8 000×*g* for 3 min, washed once by sterilized water, and suspended in 25 mmol/L Tris-HCl (pH 7.0) containing 0.25% Triton X-100, making the final concentration reach 1.0. The bacterial suspension was incubated at 30 °C, and the OD_{600} changes were determined after 5 h, which was used to represent the autolysis capability of this strain.

2 Results

2.1 Screening and species identification of LAB strains

To extensively screen functional LAB strains, the selective media (GM17 and MRS) were utilized to isolate lactic acid bacteria from various natural habitats[20], such as chicken intestine, human faces, traditional yogurts from Xinjiang district. The isolates were subsequently subjected to 16S rRNA gene sequencing, leading to the identification of 14 new

LAB strains, consisting of *Lactococcus lactis* (Wa, W56, F8, KJ1002-4, KJ2001-1, KJ1002-9, KJ2007-1, KJ2030-2, KJ2024-2), *Enterococcus faecalis* (KJ9), *Enterococcus faecium* (N9, KJ37, KJ3) and *Lactobacillus casei* (Table 1). Of note, all of the tested strains, as well as a model strain MG1363, were manifested as catalase-negative (Data not shown).

2.2 Autolysis of LAB strains and their peptidoglycan hydrolase activity

Since it was previously shown that different growth phases were responsible for the distinct autolysis capabilities[3], all of the strains were tested for their autolysis rate at either or both growth phases (Exponential and stationary). As shown in Figure 1, the autolysis profiles among strains varied significantly, regardless of whether they belong to the same species or not (*Lactococcus lactis* or *Enterococcus faecium*). Thus lactococcal autolysis could be considered as strain-specific. Among the tested strains, strain KJ9, KJ3 and KJ1002-4 showed the highest degree of autolysis, rendering them candidates for any food industries warranted by the highly autolytic LAB strains. Besides, compared to stationary phase, the autolytic activities were readily released at exponential phase for almost all the strains (Figure 1).

Table 1 Lactic acid bacteria strains, their origins, relative characteristics and species identification
表 1 乳酸菌株的来源、相对特征及种属鉴定

Strains	Species	Origin and relative characteristics
MG1363	*Lactococcus lactis*	Cheese starters
Wa	*Lactococcus lactis*	Chicken intestine, nisin-producing
W56	*Lactococcus lactis*	Chicken intestine
F8	*Lactococcus lactis*	Chicken intestine
KJ1002-4	*Lactococcus lactis*	Fermented milk from Xinjiang
KJ2001-1	*Lactococcus lactis*	Fermented milk from Xinjiang, nisin-producing
KJ1002-9	*Lactococcus lactis*	Fermented milk from Xinjiang, nisin-producing
KJ2007-1	*Lactococcus lactis*	Fermented milk from Xinjiang, nisin-producing
KJ2030-2	*Lactococcus lactis*	Fermented milk from Xinjiang
KJ2024-2	*Lactococcus lactis*	Fermented milk from Xinjiang
KJ9	*Enterococcus faecalis*	Chicken intestine
N9	*Enterococcus faecium*	Chicken intestine
KJ37	*Enterococcus faecium*	Chicken intestine
KJ3	*Enterococcus faecium*	Chicken intestine
S1	*Lactobacillus casei*	Human feces, fast flocculating

To further detect the enzymes attributable to the varied autolytic profiles, renaturing SDS-PAGE was performed using the bacterial proteins collected from the mid-exponential phase. The electrophoresis (Figure 2) revealed that, *L. lactis* MG1363 showed a lysis band corresponding to the running position of *AcmA*, while strain KJ9 showed typical bands corresponding to the size of AtlA, either of which was thought to be the major peptidoglycan hydrolase for *L. lactis* and *E. faecalis*, respectively.

In order to further examine the expression profiles of lactococcal autolysins, the mRNA levels of all five autolysins, namely, *AcmA*, *AcmB*, *AcmC*, *AcmD* and *YjgB*[19-22], were determined at 5 h of growth in the culture of MG1363. It was shown (Figure 3) that, in contrast with control strain *Lactobacillus casei* BL23, all five autolysins were actively transcribed in this early and mid-exponential stage.

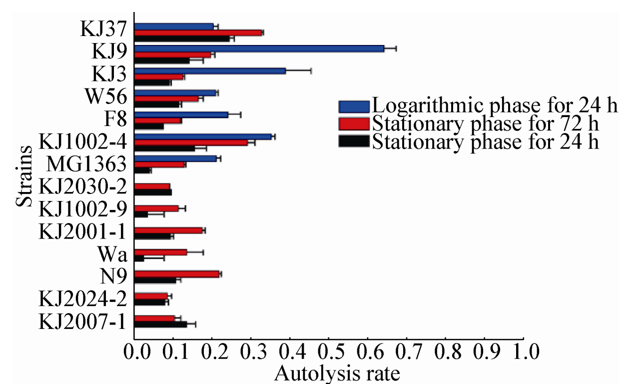

Figure 1 The buffered autolysis of *Lactococcus lactis* and other LAB strains

图 1 乳酸乳球菌和其他乳酸菌株的自溶检测

Note: The strains cultured in GM17 medium were collected at exponential (blue bars) and stationary phases (red and black bars), and then resuspended in Tris-HCl buffer for 24 h (blue and black bars) or 72 h (red bars); the autolysis rate was calculated as the percentage of the decreased OD_{600} values during the incubation.

注：在 GM17 培养基中的菌株在指数生长期(蓝条)和稳定期(红条和黑条)分别进行收集，并重悬在 Tris-HCl 缓冲液中 24 h (蓝黑条)或 72 h (红条)；自溶率计算为在孵育期间 OD_{600} 的减少比率.

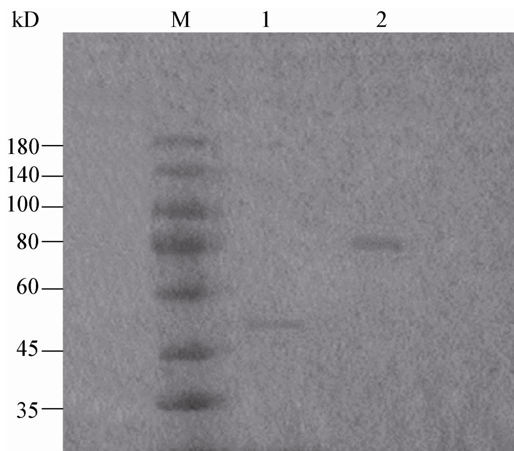

Figure 2 Renaturing SDS-PAGE of *L. lactis* MG1363 and *Enterococcus faecalis* KJ9

图2 乳酸乳球菌MG1363和粪肠球菌KJ9的复性SDS-PAGE电泳

Note: M: Marker; 1: *L. lactis* MG1363; 2: *Enterococcus faecalis* KJ9. Autoclaved *Micrococcus lysodeikticus* cells were incorporated into gels and served as the substrates of enzymatic degradation; the gels were incubated with the renaturing buffer for 24 h.

注：M：Marker；1：乳酸乳球菌 MG1363；2：粪肠球菌 KJ9. 灭活的溶壁微球菌细胞加入到凝胶中并作为酶解底物；凝胶在复性缓冲液中孵育 24 h 完成复性反应.

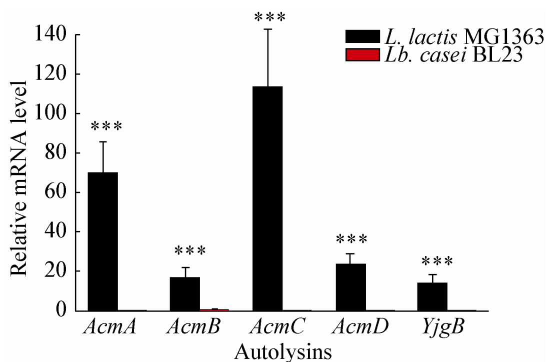

Figure 3 Expression levels of autolysin genes for *L. lactis* and *Lb. casei* strains

图3 乳酸乳球菌和干酪乳杆菌菌株自溶酶基因的表达水平

Note: Total RNAs were prepared from MG1363 cells grown in GM17 media for 5 h and analyzed by real-time fluorescence quantitative PCR with specific primers for each gene; The transcript amount was standardized by the amount of 16S rRNA gene in each sample; *Lb. casei* BL23 is a lactic acid bacteria strain besides *L. lactis*, thus not harboring the autolysins involved here; The same growth periods of this strain were also subjected to expressional analysis as control. *** stands for P<0.001.

注：在 GM17 培养基中培养 5 h 的 MG1363 细胞的总 RNA 提取出来后进行荧光定量 PCR 的检测；定量内参为 16S rRNA 基因；干酪乳杆菌 BL23 是除去乳酸乳球菌之外的一种典型乳酸菌株，因此并不含有本文所述的自溶酶；处于同样生长时期的该菌株用作表达分析的对照使用. ***代表 P 值小于 0.001.

2.3 Autolysis induction by growth inhibitors for *L. lactis* in 1/2 GM17 and 1/10 GM17

Bacillus subtilis was prone to autolysis induced by growth inhibitors when cultured in the carbon source-limited media[8]. Therefore, the glucose-limited (1/2 GM17) and strictly glucose-limited media (1/10 GM17) were used for the autolysis induction assays, respectively. All the antibiotic concentrations used in this study were in reference to their minimum inhibitory concentrations against *L. lactis* and minimum autolysis induction concentrations against *B. subtilis*[8]. Specifically, the growth inhibitors could be classified as protein synthesis inhibitor (Cm and Km), cell wall synthesis inhibitor (Amp) and membrane disrupter (SDS), respectively.

Given that growth conditions could considerably influence the lactococcal physiological behavior, the growth curve and glucose consumption were first determined for *L. lactis* MG1363 grown in 1/2 GM17 (Figure 4A). Glucose was exhausted when OD_{600} arrived at approximately 2.0 following 5 hours' incubation. However, no diauxic growth was observed in this glucose-limited medium. Subsequently, 50 μg/mL of Cm was added to the culture at a range of growth phases to determine the bacterial lysis profiles. Basically, Cm's adminstration triggered a profound bacteriacidal effect whenever the intervention occurred, with the bacterial growth ceased 1 h later in all cases (Figure 5). Following another 1 h's decline, the OD_{600} values remained unchanged, indicating that no sharp autolysis was induced under this condition, probably due to the fact the viable cell population had been reduced to a low number. Besides, the induction at glucose exhausting point (5 h) did not seem to generate any dramatically abnormal consequences.

Subsequently, Cm with changed concentration (8 μg/mL, 200 μg/mL) was subjected to the same assay. No obvious differences were observed (Data not shown). This suggests that Cm-induced autolysis in glucose-limited medium was concentration-independent.

Due to the above-mentioned data, a strictly glucose-limited medium (1/10 GM17) was utilized for the following experiments. Under this condition, *L. lactis* MG1363 exhibited a surprising diauxic growth (Figure 4B): the bacterial growth ceased with OD_{600} reaching 0.7, and then resumed 4 h later. The transition period between the two consecutive growth

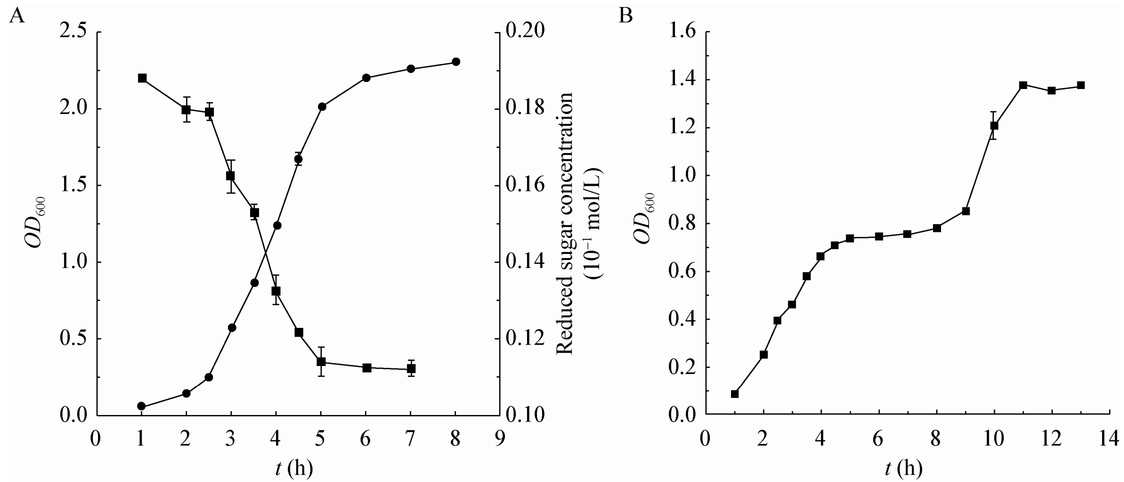

Figure 4 The growth curve and glucose consumption of *L. lactis* MG1363 in 1/2 (A) and 1/10 (B) GM17 broth

图 4 乳酸乳球菌 MG1363 在 1/2 和 1/10 GM17 培养基中的生长曲线及葡萄糖消耗

Note: A: ●Represent the OD_{600} values of the culture; ■Represent the glucose concentration. B: ■Indicated the OD_{600} values of the culture.

注：A：●代表培养液的 OD_{600} 值；■表示葡萄糖的含量. B：■代表培养液的 OD_{600} 值.

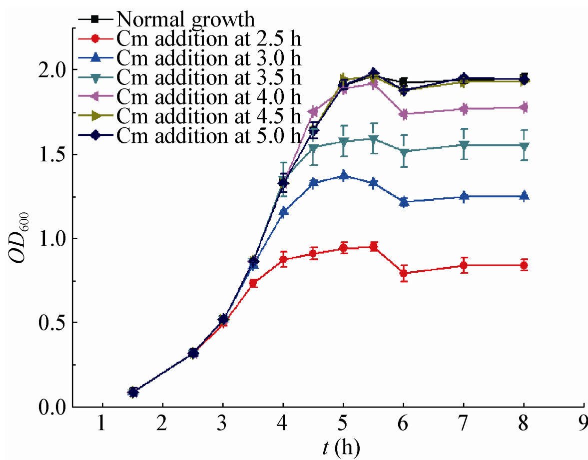

Figure 5 OD_{600} changes of *L. lactis* MG1363 strains caused by the addition of Cm

图 5 氯霉素加入所导致的乳酸乳球菌 MG1363 的 OD_{600} 值的改变

Note: Cm with the concentration of 50 mg/L was added to the culture at various time points; the altered growth curves under these conditions were then determined.

注：50 mg/L 的氯霉素分别加入不同培养时间点的培养液中，并对在上述条件下发生变化的生长曲线进行测定.

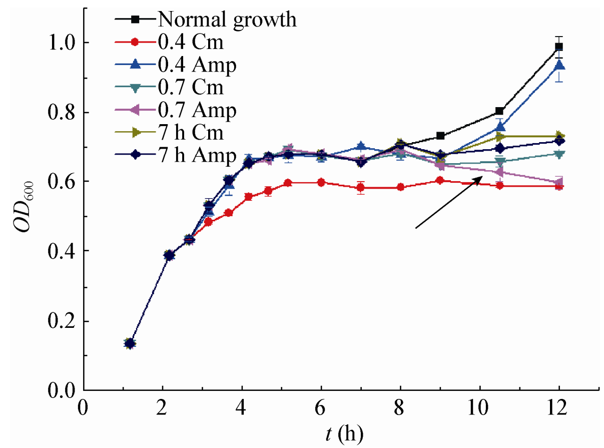

Figure 6 Antibiotic-induced autolysis of *L. lactis* MG1363 propagated in 1/10 GM17 media

图 6 在 1/10 GM17 培养基中乳酸乳球菌 MG1363 受抗生素诱导的自溶

Note: Cm (8 mg/L) and Amp (4 mg/L) were added to the culture at various time points, respectively. 0.4 and 0.7 are adding antibiotics to the culture when the OD_{600} values of the strain reach up to 0.4 and 0.7 respectively; 7 h is adding antibiotics to the culture when the strain was grown for 7 hours; the black arrow indicated the growth curve under the condition of *OD* 0.7 Amp.

注：8 mg/L 的氯霉素和 4 mg/L 的氨苄青霉素分别在不同时间点加入培养液中；0.4 和 0.7 表示抗生素加入的时间点是 OD_{600} 分别到达 0.4 和 0.7 的时间；7 h 表示抗生素加入的时间点是菌株培养到 7 h；黑箭头表示在 *OD* 0.7 时刻加入氨苄青霉素时的生长曲线.

stages was supposed to be autolysis-susceptible, thus this period was chosen to assess the induced autolysis of MG1363 culture.

Figure 6 showed the various growing profiles following the addition of Cm or Amp at early-exponential phase (0.4), the turning point (0.7)

and the diauxic point (7 h). It was revealed that the significant autolysis of MG1363 was induced by Amp, a cell wall synthesis inhibitor, only at the turning point of growth (when OD_{600} reached 0.7). In contrast to it, Amp with the same concentration could not even inhibit the bacterial growth when it was added at other stages (Figure 6). Besides, the concentration used here (4 mg/L) was less than its MIC in *L. lactis*. These data demonstrated that the autolysis caused by Amp was specifically dependent on the growth phases when the intervention occurred, and it could only be triggered when the strains transited from the first growth to the lag phase of the second growth. Additionally, the treatment of Cm did not seem to induce autolysis at any rates (Figure 6).

In order to investigate whether autolysins were involved in this induced process, the transcription levels of *AcmA*, *AcmB*, *AcmC*, *AcmD* and *YjgB*, the five major autolysins in *L. lactis*, were determined in the presence or absence of Amp's administration. Surprisingly, it was shown from Figure 7 that, the mRNA levels of *AcmA*, *AcmB*, *AcmD* and *YjgB* exhibited significant decline with the influence of Amp, implying that autolysins probably did not play pivotal role in the induction of sharp autolysis.

2.4 Autolysis induction by growth inhibitors for *Lactococcus lactis* in non-nutrition buffer

The buffer system was frequently utilized to determine the bacterial autolytic profiles[23], and

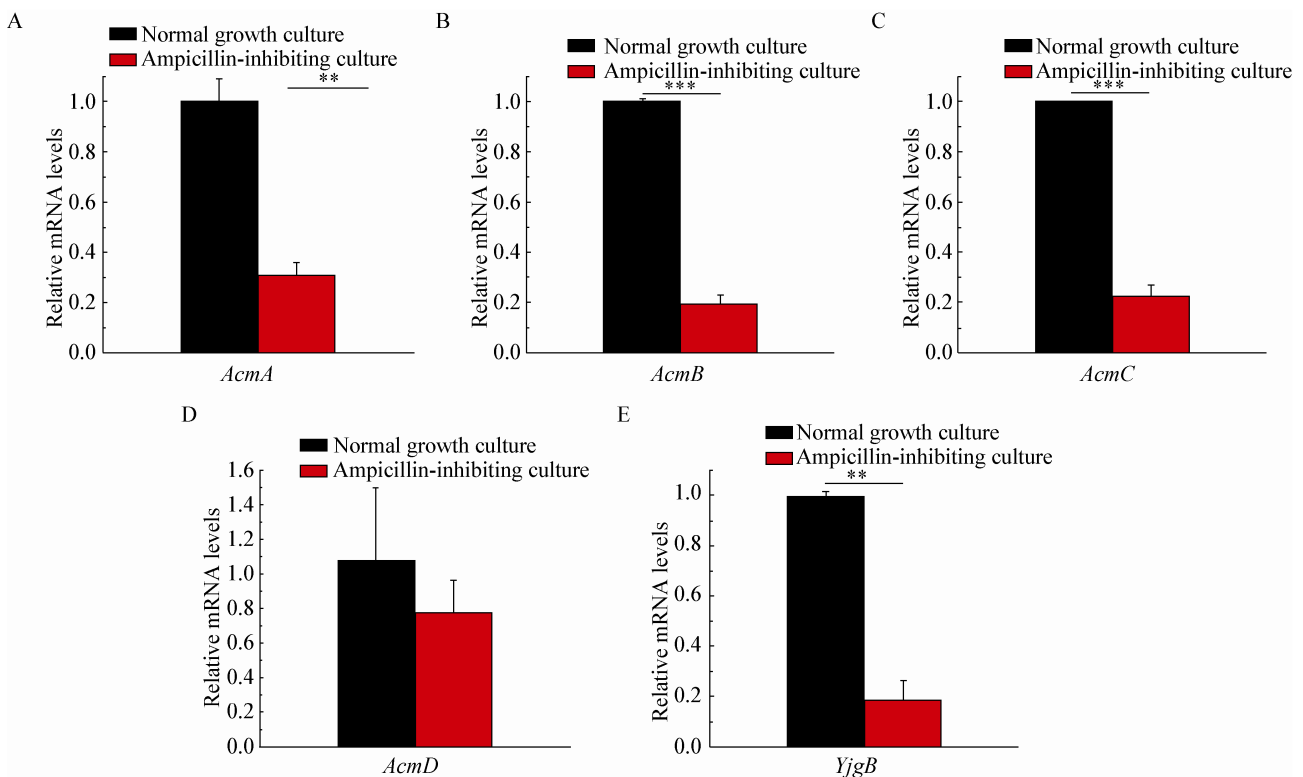

Figure 7 Expression levels of autolysin genes for *L. lactis* MG1363 upon induction of autolysis

图 7 乳酸乳球菌 MG1363 在诱导下其自溶酶基因的表达水平

Note: Total RNAs were prepared from MG1363 cells grown in 1/10 GM17 media at 2 h following Amp's induction at 5 h of growth, and analyzed by real-time fluorescence quantitative PCR with specific primers for each gene (A, B, C, D, E for *AcmA*, *AcmB*, *AcmC*, *AcmD* and *YjgB*, respectively); The transcript amount was standardized by the amount of 16S rRNA gene in each sample. **: Stands for $P<0.01$; ***: Stands for $P<0.001$.

注：MG1363 细胞在培养 5 h 后加入氨苄青霉素进行诱导，之后继续培养 2 h 后收集细胞提取 RNA，并通过荧光定量 PCR 分别检测 *AcmA* (A)，*AcmB* (B)，*AcmC* (C)，*AcmD* (D)和 *YjgB* (E)五个基因的表达水平；转录体的定量以样本中 16S rRNA 基因的水平为内参. **：P 值<0.01；***：P 值<0.001.

Triton X-100 was added to the system to facilitate the release of the autolysins' activities. In the present investigation, the bacterial cells were collected and suspended in Tris-HCl buffer (pH 7.0) containing 0.25% Triton X-100, followed by the addition of Amp (4 μg/mL), Km (8 μg/mL), Cm (8 μg/mL) and SDS (20 μg/mL). The resulting autolysis profiles were presented in Figure 8.

Based on Figure 8, it was found that compared to the control group, the autolysis capabilities of *L. lactis* MG1363 was inhibited to a variable extent upon the addition of various growth inhibitors, and Cm showed the most prominent inhibitory effect. However, the tendencies seemed reversed as incubation time prolonged. This result might be a reflection of a diminished release of enzymatic activities of autolysins, when their cognate partners, cell wall synthetic enzymes, were strongly inhibited.

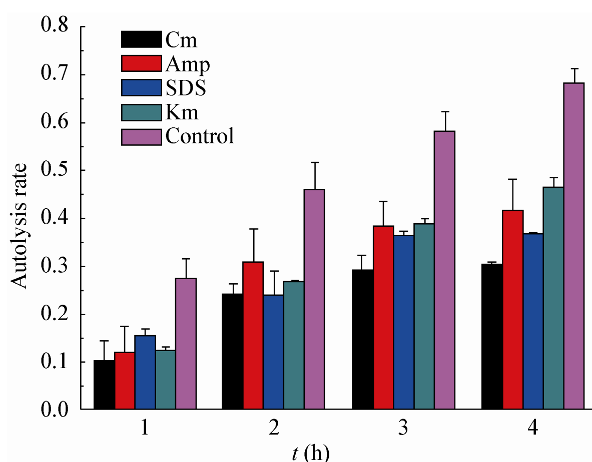

Figure 8 The buffered autolysis of MG1363 by the addition of different growth inhibitors

图8 MG1363在不同生长抑制剂作用下的缓冲液自溶水平

Note: Cm, Amp, Km and SDS were added to the MG1363 culture with OD_{600} value of 0.3; Subsequently the strains were collected when it reached 0.7 and then resuspended in 25 mmol/L Tris-HCl (pH 7.0) buffer with 0.25% Triton X-100, with its final concentration of 1.0; The suspension was incubated at 30 °C, and the decreased percentage of OD_{600} values was used to represent the autolysis extent during 4 hours.

注：在生长到 OD_{600} 值为 0.3 时的 MG1363 培养液中分别加入氯霉素、氨苄青霉素、卡那霉素和 SDS；随后菌株在生长至 0.7 时收集下来并重悬在 25 mmol/L 的 Tris-HCl (pH 7.0)并添加有 0.25%的 Triton X-100 的缓冲液中，使得菌体的吸光度值达到 1.0；悬液在 30 °C 条件下孵育 4 h，期间 OD_{600} 的减少比值被用来表征自溶水平.

3 DISCUSSION

Autolysis was a strain-specific physiological phenomenon (As demonstrated in Figure 1), and its profiles were significantly influenced by the environmental conditions, such as salt concentration, pH, temperature, rennet's induction, etc[24]. Among them, medium compositions, as well as growth inhibitors, were generally regarded to play the predominant roles in autolysis induction[8]. The results here revealed that inhibitors' induction of autolysis could only occur when bacteria was grown in 1/10 GM17, instead of 1/2 GM17. Moreover, the induced autolysis was strictly growth phase-specific, that is, it could only occur at the turning point of its first growth. Based on these findings, it's tempting to speculate that, when bacteria was transited from glucose exhausting point to lag phase of the second growth, the rigid regulation of autolysin activities might be weakened, rendering it susceptible to the inhibitors' adverse effect. Therefore, it's the complex interplay of carbon deficiency and inhibitor's disruption that consequently contributed to the lysis of *L. lactis* strains.

Basically, this study is a proof that the LAB organisms are more prone to the induced autolysis when it's engaging in the remodeling process caused by energy conversion. This phenomenon could be interpreted by the disruption of proton motive forces, or alternatively the abnormal function of two-component regulatory system. Furthermore, it was also shown that autolysis could only be induced in the culture of 1/10 GM17, instead of 1/2 GM17. Therefore, it was reasonable to hypothesize that the timing that carbon source depletion occurred was also the key determinant for the autolysis induction. In other words, the growth inhibitors could trigger autolysis of *L. lactis* strains only in the case of glucose depletion, and only when the depletion was elicited in the early-exponential stage.

A strange observation manifested in this investigation is the down-regulation of lethal autolysins during the narrowly-scoped process of sharp lysis. So what is the leading cause of this bizarre lysis? Of note, the autolysins' expression was often co-regulated with cell wall synthetic enzymes[25], a molecular mechanism for bacteria to keep the balance of these antagonizing enzymes. Thus, the autolysins' decline might be attributed to either the deficiency of

protein synthesis, or the concerted regulation with a diminished cell wall synthesis. In addition, it should not be ignored that the cell wall susceptibility might also be changed to some extent by the administration of growth inhibitors. Particularly, various peptidoglycan modifications, such as O-acetylation or de-N-acetylation, were able to lead to significant changes of autolysis phenotype, with its concrete mechanisms awaiting further investigation.

In summary, this study comprehensively investigated the autolysis profiles of *L. lactis* induced by a variety of growth inhibitors, and the growth phase-specific autolysis was first discovered in lactic acid bacteria. The findings here shed new light on the understanding of cellular mechanisms towards autolysis of lactococcal strains, paving way for the better elucidation and application of this bizarre physiological phenomenon.

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