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Research Note

Preliminary Analysis of the Lipase Gene (*gehM*) Expression of *Staphylococcus xylosus* In Vitro and during Fermentation of Naturally Fermented Sausages (In Situ)

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ABSTRACT

Coagulase-negative catalase-positive cocci (CNCPC) play a very important role during the fermentation of sausages. In particular, they are involved in the aroma formation of the final product, because they release lipases that are able to free short-chain fatty acids that are contributing to the sensory characteristics of the fermented sausage. Few studies have been undertaken to elucidate the regulation of lipase gene expression in *Staphylococcus xylosus* by substrate molecules or products of lipolysis. The aim of this study was to analyze the *gehM* gene expression of *S. xylosus* DSMZ 6179 in vitro with growth media containing different concentrations of lipids and in situ during the maturation of fermented sausages. The results obtained suggest that a concentration that increases in triglycerides in the growth medium suppresses the expression of the lipase gene.

In modern fermentation processes, flavor development and consistent product quality have become as important as preservation. Increased knowledge of both the endogenous and microbial lipolytic enzymes may be useful in the accelerated ripening of dry-fermented sausages, the modified generation of free fatty acids for flavor improvement, and the correction of some flavor defects *(12, 25, 27).* Members of coagulase-negative catalase-positive cocci (CNCPC) (more specifically, *Staphylococcus* spp.) release lipases that are able to free short-chain fatty acids, which are responsible for the aroma of fermented sausage *(4). Staphylococcus xylosus* is frequently isolated as the main *Staphylococcus* species, but others have also been reported: *S. carnosus, S. simulans, S. saprophyticus, S. epidermidis, S. haemolyticus, S. warneri,* and *S. equorum (1–3, 5, 11, 30).* Nevertheless, *Kocuria varians* and *Kocuria kristinae* are identified in fermented sausages, too *(9).* Lipolysis constitutes a biochemical process with an important contribution to flavor development, because free fatty acids with insaturations will act as substrates for further oxidation to form volatile compounds with aroma properties *(27).* Traditionally, microbial species were selected as starter cultures according to their lipolytic activity for guaranteeing adequate flavor development in meat products, especially fermented sausages. But Demeyer et al. *(6)* and Molly et al. *(14)* suggest that there is an increased importance of endogenous muscle and fat lipase in relation to microbial lipase. Fat tissue, which constitutes the major fat fraction of the sausage (28 to 42% in the function of sausage types) *(21),* is mainly composed of triglycerides (27 to 41%) *(21).* Intramuscular fat, although present in minor amounts of the sausage, also contains phospholipids, which are rich in polyunsaturated fatty acids. The act of distinguishing and understanding the different enzymes from different microbial groups is very difficult and complicated. In general, the amount of lipase produced is dependent on the environment, such as composition of the nitrogen, carbon, and lipid sources; inorganic salts; cultivation temperature; and availability of oxygen *(20).* Lipases, which are triacylglycerol acylhydrolase, act best at lipid-water interfaces and under any conditions in which an increase in the water content of the surface area would be expected to increase the activity of the enzyme *(28).* The amount of free fatty acids increases along the process either in traditional or industrial dry-fermented sausages. Increases in the concentrations of free fatty acids may reach up to 2.5 to 5% of the total fatty acids, depending on the type of sausage and the processing conditions *(18).* The majority of free fatty acids are released from triglycerides in the neutral fraction. The rate of release of individual fatty acids decreases in the order of linoleic > oleic > stearic > palmitic and is always valid for the homologous fatty acids with equal carbon atoms *(6, 15, 22).* Because there is a preponderance of unsaturated fatty acids in position 3 of triglycerides, some kind of specificity is indicated *(4).* However, for the isosaturated fatty acids, the order may be different: palmitic > stearic and C16:1 > C18:1 *(15, 22). Staphylococcus* lipases do not show stereospecificity in the hydrolysis of triglycerides. However, they may have different specificities, such as the hydrolysis of

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tri-, di-, and monoglycerides at different rates, preference for types of fatty acids, or positional specificity where the enzyme hydrolyzes the esters at preferential positions *(29).* It has been observed in sausages that lipases prefer to hydrolyze the external positions of the triglyceride molecule *(16).* It has been reported that bacterial lipases are produced during the exponential growth phase, and the production is greatly influenced by growth conditions: the maximum amount is formed at optimum temperatures and pH for growth *(17).* Most of the sugars repressed lipase production. For example, glucose supported good growth but completely repressed lipase production. Organic acids supported good growth and lipase production. However, lipids had a different effect on the regulation in the function of the lipid type *(13).* Elibol and Ozer *(7)* reported that aeration or agitation or perfluorocarbon inclusion resulted in an increase in lipase production.

However, few studies have been undertaken to elucidate the regulation of lipase gene expression in *S. xylosus* by substrate molecules or products of lipolysis.

The ability of *S. xylosus* to produce free fatty acids in fat-containing medium at different environmental conditions has been investigated by Sorensen and Jakobsen *(23)* and Sorensen et al. *(24),* who found that pH, salt concentration, and age of the culture influenced lipase production, whereas production was not significantly affected by temperature.

The aim of this study was to evaluate the lipase gene (*gehM*) expression of *S. xylosus* in different growth media (in vitro) and during a natural fermentation of sausages (in situ).

MATERIALS AND METHODS

Bacterial strains. The strain used in this study was *S. xylosus* DSMZ 6179. For the optimizations of the hybridization conditions, the following CNCPC strains were used as well: *S. xylosus* DSM 6179, *K. varians* DSM 20033, *S. simulans* DSM 20322, and *S. carnosus* subsp. *carnosus* DSM 20501 (Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Braunschweig, Germany). *S. epidermidis* DSA 0CB1 and *S. warneri* DSA 0CB9 were obtained from the collection of the Food Science Department, University of Udine, and they were isolated from a natural fermentation of sausages, identified by species-specific PCR, and then confirmed by sequencing the V3 region of the 16S rDNA *(10).* All the strains were grown in brain heart infusion (BHI) broth (Oxoid, Milan, Italy), at 30°C for 24 h.

Media and growth conditions. *S. xylosus* was grown for about 36 h at 30°C in different culture media. They were as follows: (i) BHI broth (Oxoid); (ii) BHI broth plus 2% (vol/vol) tributyrin (Sigma, Milan, Italy) (BHI2%); (iii) BHI broth plus 10% (vol/vol) tributyrin (BHI10%); (iv) nutrient broth (NUT; Oxoid); (v) NUT broth plus 2% (vol/vol) tributyrin (NUT2%); and (vi) NUT broth plus 10% (vol/vol) tributyrin (NUT10%). The growths were followed by measuring the optical density of the cultures at 600 nm with the SmartSpec 3000 spectrophotometer (Biorad, Milan, Italy) and by the plate count method on BHI agar (Oxoid). Expression studies were performed after 8, 10, 12, 14, 16, and 18 h of incubation. Different volumes of broth, depending on the optical density as a consequence of growth, were centrifuged at 12,100 × *g* for 10 min at 4°C, the supernatant fluid was discarded, and the RNA was extracted from the pellet.

RNA extraction from pure cultures. At each sampling point, cells were centrifuged at 12,100 × *g* for 10 min at 4°C. The pellet was transferred into a 1.5-ml microfuge tube containing 0.3 g of glass beads, 0.5-mm diameter, and 300 μl of breaking buffer (2% Triton X-100, 1% sodium dodecyl sulfate [SDS], 100 mM NaCl, 10 mM Tris, pH 8, and 1 mM EDTA [pH 8]). Three hundred microliters of phenol-chloroform 5:1, pH 4.7 (Sigma), was added, and three 30-s treatments at 12,100 × *g*, with an interval of 10 s, were performed in a bead beater (Fast Prep, Bio 101, Vista, Calif.). Three hundred microliters of TE (10 mM Tris and 1 mM EDTA [pH 8]) was added, and a centrifugation for 5 min at 12,100 × *g* at 4°C was performed. The supernatant fluid was transferred to a 1.5-ml tube containing 1 ml of ice-cold absolute ethanol. After incubation for 5 min at room temperature, RNA was precipitated by centrifugation at 12,100 × *g* for 10 min at 4°C. The pellet was then vacuum dried and resuspended in 50 μl of sterile distilled water. One microliter of DNase RNase-free (Roche Diagnostics, Milan, Italy) was added, and the RNA samples were incubated for one night at 37°C before being stored at −80°C. The RNA solution was checked for the presence of residual amounts of DNA by performing PCR amplification. When positive signals were detected, the DNase treatment was repeated to eliminate the DNA. All the solutions were nuclease free.

Direct extraction of RNA from sausages. For the study of the expression in situ, fermented sausages were prepared by traditional techniques without the use of starter cultures. Sausages, characterized by short-period ripening, were examined at 0, 3, 5, 7, 14, and 28 days. From each sampling point, 10-g samples, in triplicate, were homogenized in a stomacher bag with 20 ml of saline and peptone water for 1 min. After each preparation had settled for 1 min, 1 ml of supernatant fluid was transferred into a screw-cap tube containing 0.3 g of glass beads and centrifuged at 4°C for 10 min at 12,100 × *g*. The resulting pellet was treated with 1 ml of petrol ether–hexane (1:1; Sigma) for 10 min at room temperature to extract lipids. A second centrifugation was performed, as described above, and the pellet was resuspended in 150 μl of proteinase K buffer (50 mM Tris-HCl, 10 mM EDTA, pH 7.5, and 0.5% [wt/vol] SDS). Twenty-five microliters of proteinase K (25 mg/ml; Sigma) was added, and a 1-h treatment at 65°C was performed. After this step, 150 μl of 2× breaking buffer was mixed in the tubes. Three hundred microliters of phenol chloroform–isoamyl alcohol 5:1, pH 4.7 (Sigma), was added and treated in a bead beater (Fast Prep, Bio 101). Three hundred microliters of 10 mM Tris and 1 mM EDTA, pH 8, was added, and a centrifugation for 5 min at 12,100 × *g* at 4°C was performed. The supernatant fluid was transferred to a 1.5-ml tube, and the RNA was precipitated as described above.

***gehM* probe.** Primers geh3 (5′-GTA GAA AAA GCG AAT GAA CAA C-3′) and geh4 (5′-CCT GGT TGC CAA TCT TTA TAT AC-3′) *(18),* targeting the *gehM* gene coding for the lipase of *S. xylosus* (accession number AF208229), were used in the PCR amplification for the synthesis of the probe to use in the hybridization assays. Reactions were carried out in a final volume of 50 μl containing 10 mM Tris-HCl, pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 2 mM (each) deoxynucleoside triphosphate (dNTP); 0.2 μM (each) primer; 1.25 U of *Taq* polymerase (Applied Biosystems, Milan, Italy); and 100 ng/ml of DNA. Digoxigenin-dUTP (Roche) was added to the dNTP mix in a ratio of 1:19 with respect to dTTP. Amplification was performed for 35 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min. PCR products (DNA

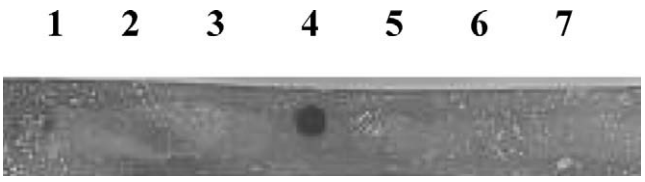

FIGURE 1. *RNA dot-blot performed to specifically detect the* gehM *gene from* S. xylosus. *The probe did not hybridize to any of the strains used as controls. Lane 1,* S. warneri *DSA 0CB9; lane 2,* S. carnosus *subsp.* carnosus *DSM 20501; lane 3,* K. varians *DSM 20033; lane 4,* S. xylosus *DSM 6179; lane 5,* S. simulans *DSMZ 20322; lane 6,* S. epidermidis *DSA 0CB1; lane 7, negative control.*

probes) were checked by electrophoresis in 2% agarose gel with 0.5× Tris-borate-EDTA used as a running buffer. Ethidium bromide (0.5 μg/ml) was added to the gel before solidification. After the run, gels were examined by the BioImaging System GeneGenius (SynGene, Cambridge, UK).

Total RNA hybridization. Twenty micrograms of total RNA was applied to Zeta-Probe GT membranes (Biorad) with a BioDot blotting apparatus (Biorad) and had been previously treated with 10% H_2O_2 for at least 30 min, as recommended by the manufacturer. Hybridization was performed overnight in 20 mM sodium phosphate (pH 7.4) and 7% (wt/vol) SDS at 45°C. The membrane was washed twice in 20 mM sodium phosphate (pH 7.4) and 5% (wt/vol) SDS for 15 min each time and twice in 20 mM sodium phosphate (pH 7.4) and 1% (wt/vol) SDS for 15 min each time. The washes were carried out at 65°C. Detection was performed with a digoxigenin chemiluminescence kit (Roche), used as recommended by the manufacturer. RNA was also purified from the CNCPC standard strains and used to optimize the hybridization conditions as described above.

RESULTS

Optimizing of the hybridization conditions. Two experimental conditions were optimized in order to follow the expression of the *gehM* gene in *S. xylosus* only (Fig. 1) or in all the CNCPC strains used in this study (Fig. 2). This choice was based on the necessity to understand the contributions of *S. xylosus,* which is the principal *Staphylococcus* spp. isolated from naturally fermented sausages, as well as the contributions of other CNCPC members present in these kinds of products, to the lipolytic process. In Figure 1, it can be observed that when the membrane hybridization was performed at 45°C and the washing was done at 65°C,

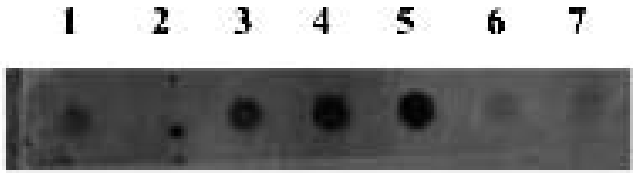

FIGURE 2. *RNA dot-blot under the hybridization conditions allowing the detection of the lipase gene from the CNCPC members used in the study. The probe, with low stringency conditions for the washing, hybridized to all the bacterial strains used as a control. Lane 1,* S. warneri *DSA 0CB9; lane 2, negative control; lane 3,* S. carnosus *subsp.* carnosus *DSM 20501; lane 4,* K. varians *DSM 20033; lane 5,* S. xylosus *DSM 6179; lane 6,* S. simulans *DSM 20322; lane 7,* S. epidermidis *DSA 0CB1.*

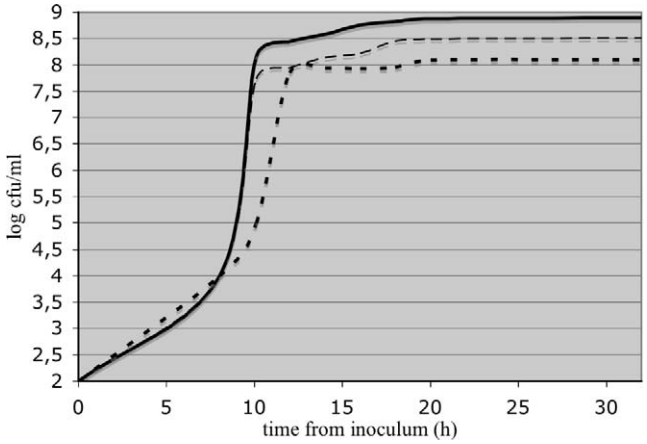

FIGURE 3. S. xylosus *growth curve in BHI media with different concentrations of tributyrin. ————, BHI broth; — — —, BHI broth plus 2% tributyrin; – – – –, BHI broth plus 10% tributyrin.*

only *S. xylosus* was characterized by a signal, whereas all the other species used in the blotting did not produce any visible spot. When the temperature of the hybridization and the washing of the membrane was decreased to 40 and 60°C, respectively, the probe did not hybridize exclusively to *S. xylosus.* Under these conditions, all the lipase genes from the CNCPC strains used in this study could be detected (Fig. 2), even if some of them gave very faint signals.

Expression in vitro. The results of the growth curves of *S. xylosus* DSMZ 6179 in the different media used in this study are shown in Figures 3 and 4. At each sampling point, the bacterial growth was evaluated by (i) measuring the optical density of the cultures at 600 nm in order to understand immediately the trend of the cell growth, (ii) contemporarily plating onto BHI agar (Oxoid), and (iii) counting the CFU per milliliter after an incubation of 48 h at 30°C in order to compare the data obtained. In BHI, it can be observed how the microorganism entered its exponential growth phase after about 8 h from the inoculum, and it reached the stationary growth phase after 16 to 18 h. When tributyrin was added to the medium (BHI2% and

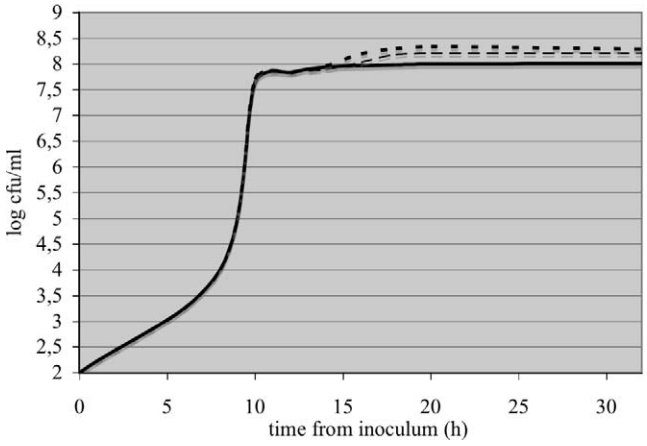

FIGURE 4. S. xylosus *growth curve in NUT media with different concentrations of tributyrin. ————, BHI broth; — — —, BHI broth plus 2% tributyrin; – – – –, BHI broth plus 10% tributyrin.*

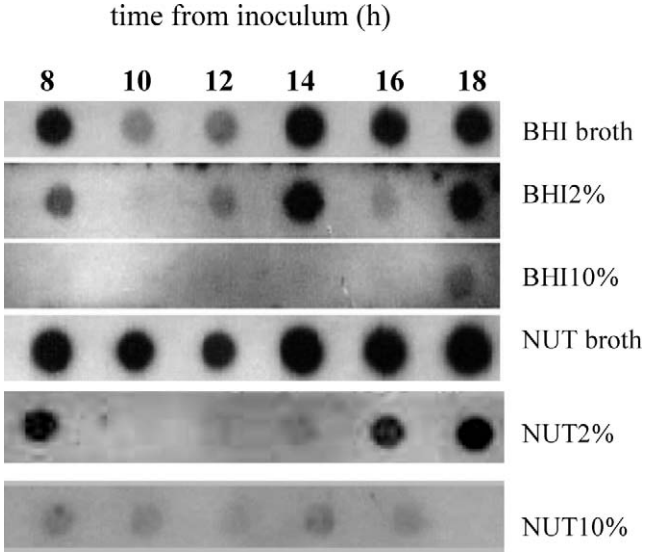

FIGURE 5. *Expression analysis of the* gehM *gene in different growth media. Lane designations represent the sampling hours. BHI, brain heart infusion broth; BHI2%, BHI plus 2% tributyrin; BHI10%, BHI plus 10% tributyrin; NUT, nutrient broth; NUT2%, NUT plus 2% tributyrin; NUT10%, NUT plus 10% tributyrin.*

BHI10%), a decrease in the growth was observed (Fig. 3). For both BHI2% and BHI10%, the CFU per milliliter values were significantly lower when compared with the growth in BHI. However, the length of the growth phases did not change dramatically. Only in BHI10% was a longer lag phase observed. In all cases, the stationary phase was reached 16 to 18 h after the inoculum. When the growths in the NUT media were monitored, the trends shown in Figure 4 were obtained. The three curves were very similar, even if it seemed that an addition of tributyrin slightly stimulated the growth of *S. xylosus.* However, the differences obtained are, in our opinion, too small to be considered significant. In all three NUT media, the growth obtained was much lower than in BHI media.

At each sampling point, RNA was extracted from the culture and blotted onto the membrane to study the *gehM* gene expression. The results obtained are shown in Figure 5. In BHI, the expression of the gene was higher in the early exponential phase (8 h) and in the stationary phase (from 14 h), whereas at the rest of the sampling points, it decreased significantly. This trend was also observed in BHI2%, even though the intensity of the spots at the beginning of the exponential phase was less evident if compared with those found in BHI. In BHI10%, the gene was not expressed at any time. The expression trends in NUT media were completely different. The *S. xylosus gehM* gene was expressed when growth was performed in NUT, whereas its expression decreased with the addition of tributyrin. As shown, in NUT2%, the gene was expressed at the beginning of the stationary growth phase, whereas in NUT10%, the gene was not expressed.

Expression in situ. The expression of the *gehM* gene from *S. xylosus* was also studied in situ during a natural fermentation of sausages. Analyzing the RNA extracted directly from the sausages, no visible spots were obtained at any considered point, under either of the hybridization conditions specific for the *S. xylosus gehM* gene or the conditions allowing the detection of the lipase gene from other CNCPC members.

DISCUSSION

Very little is known about the regulation of lipase synthesis in staphylococci. For some of the staphylococcal lipases, a maximum level of synthesis during the stationary growth phase has been reported *(7, 8, 13, 26).* Therefore, the staphylococcal lipases are likely to be regulated by the global regulatory system *agr* (accessory gene regulator), which is known to regulate the expression of the genes of several exoproteins and cell wall–associated proteins in staphylococci *(7, 19).*

The aim of this preliminary study was the study of the expression of the *gehM* gene, encoding for the lipase in *S. xylosus,* in vitro and in situ. In vitro analyses were conducted with two different growth media (BHI and NUT) with three concentrations of tributyrin (0, 2, and 10%) added to the media. These experimental conditions were chosen in order to understand the influence of triglycerides in the expression of the gene encoding the enzyme that acts as a catalyst for their hydrolysis. BHI is a rich medium that contains not only proteins but also lipids, because it is prepared from brain extracts, while NUT is a poorer medium that does not contain lipids, and its composition is mainly proteic.

Considering the growth curves of *S. xylosus* in the six different media tested in this study, it is possible to observe that the microorganism behaved differently. The growths in BHI media were more satisfactory with respect to NUT media, independently of the tributyrin added. Only in BHI10% were the growth trends comparable to the ones obtained with the NUT media. It is important to point out that the addition of tributyrin had a greater inhibitory effect on the growth of *S. xylosus* when it was used in BHI broth (Fig. 3). No significant differences in CFU per milliliter values were observed in NUT media. Generally, in all the media, the stationary phase was reached after 14 to 16 h, but the maximum cell counts were different.

Interesting results were obtained when the RNA extracted at each sampling point was analyzed with the probe specific for the *gehM* gene. The first clear evidence was the difference in the expression levels of the gene in BHI and NUT without tributyrin. As shown in Figure 5, in NUT, a constant and strong expression of the tested gene was observed, whereas in BHI, strong expression occurred only at 8 h (early exponential phase) and at 14, 16, and 18 h (stationary phase). These results underline the involvement of lipids in the regulation of the *gehM* gene expression. In NUT, where they are absent, the expression was on during the entire period followed. This hypothesis is also sustained by the results obtained when tributyrin was added to the media. The increase in its concentration down-regulated the expression of the *gehM* gene. Although at a 2% concentration the expression occurred mainly at the early exponential phase and in the stationary phase, a concentration of 10% inhibited the expression.

When expression analysis in situ was performed, no visible spots were obtained from the RNA extracted directly from the fermented sausages at any sampling day or for either of the hybridization conditions used. These results can be explained by admitting that the gene is not expressed during production, but most probably, we can explain this fact by taking into consideration the low counts of CNCPC during the monitoring fermentation. They did not reach counts higher than 10^5 CFU/g (plant C) *(10)* and thereby did not provide a sufficient lipase-specific RNA to give a positive hybridization reaction.

It can be concluded that the *gehM* gene encoding for the lipase in *S. xylosus* is regulated by the presence of triglycerides in the culture medium. This thesis is supported not only by the results obtained with both BHI and NUT broths, but also by the addition of tributyrin in these media, underlining the need to test the role of lipolysis products in the expression regulation.

The experimental conditions for the expression analysis in situ should be reconsidered because of the results obtained. In our opinion, a better opportunity to understand how the gene is expressed during the fermentation of sausages would be to follow a production in which inoculation of *S. xylosus* as a starter culture takes place. In this way, the cell counts obtained would be higher than the counts reached in the natural fermentations, giving better chances to have visible spots in the expression assays.

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