Quorum sensing regulating the productivity and stability of cross-feeding cocultivation

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Abstract

Quorum sensing (QS), a dynamic microbial communication mechanism, has been widely applied to synthetic biology for different functions. The incorporation of genome-scale models (GEMs) provides a powerful tool for understanding the metabolic capabilities and interactions within microbial communities. However, the introduction of the relevant QS devices for the stability and productivity of the microbial community lacks systematic analysis, let alone the GEMs-based rational design and optimization of QS-regulated microbial ecosystems. In this study, we integrated different QS regulation strategies with the metabolic division of labor to establish a combinational GEM for systematically designing and optimizing the process of the two-strain cross-feeding cocultivation. Specifically, taking the production of salidroside within two cross-feeding fermentation as an example, the GEMs were developed for the static and QS-based dynamic regulation strategies, and the corresponding simulation and optimization were performed. Then, we further constructed the QS-based self-regulating, cross-regulating, and hybrid-regulating QS communication network (QSCN) by using synthetic biology toolkits to investigate the productivity and stability of cross-feeding cocultivation. Results showed that the hybrid QSCN can better realize the cell density control and the improvement of productivity (562.57 mg/L). This study offers insights for developing more comprehensive model and regulation strategies for QS-based control in diverse applications.

Keywords

Quorum sensing; dynamic metabolic control; synthetic microbial consortia; genome-scale metabolic models; cross-feeding fermentation; systems biology.

1. Introduction

In the field of synthetic biology, cells are used as biological factories for the large-scale production of high-value chemicals [1], such as pharmaceuticals [2], nutritional products [3], fuels [4], and commercial chemicals [5]. During the production process, chassis cells will undergo unpredictable physiological changes due to the burden of heterologous gene expression and the consumption of intracellular resources, which typically lead to decreased growth and lower productivity [6]. In recent years, with the development of synthetic microbial consortia (SMC), it has been found that interactions between different strains within the community enable them to have better adaptability to environmental changes, substrate utilization, and feed tolerance than a single strain [7, 8]. SMC can also achieve more complex engineering applications through the division of labor in metabolic networks [9, 10]. However, due to the interactions among multiple species, the complexity of SMC is greatly heightened, and it is crucial to regulate the microbial community through communication and coordination to achieve the desired engineering objectives [11, 12]. Therefore, to enhance the understanding of microbial interactions and facilitate rational design, it is essential to integrate phenotype experiments of diverse microbial communities with different quantitative models [13, 14], such as the ordinary differential equations (ODEs) [15-17] and genome-scale metabolic models (GEMs) [11]. GEMs have become the mainstream analytical tool for deciphering microbial interactions, enabling quantitative prediction of intracellular metabolic fluxes, analyzing metabolite uptake and secretion, as well as calculating biomass accumulation [18, 19]. In recent years, the incorporation of microbial metabolic network models into the modeling process of SMC has given rise to various static and dynamic microbial community modeling methods, such as SteadyCom [20], OptCom [21], COMETS [22], and FLYCOP[23]. Note that, utilizing microbial community modeling techniques to build metabolic models in co-culture systems, thus conducting detailed analysis and meticulous optimization of the fermentation process has become a critical strategy for enhancing the yield of target products [24].

Recently, quorum sensing (QS) has been acknowledged as an effective means of regulating microbial populations [25-27]. The QS system can promote metabolic regulation in response to specific cell density thresholds by producing, releasing, and sensing signaling molecules, also termed autoinducers (AI) [28-30]. Specifically,

some QS systems can promote the formation of bacterial biofilms for adapting to toxicity or generating the required biological products [31, 32]. When QS is coupled with transcription factor-based biosensors [33], QS-dependent circuits are able to dynamically distribute carbon flux based on perception to optimize cellular metabolism intermediates or end products [34, 35], offering a novel approach for dynamic control of bacterial populations and metabolic production [36]. Specifically, Gupta et al.[37] utilized the EsaI/EsaR QS system in *Pantoea stewartii* to establish a density-dependent knockout circuit, which was then employed to fine-tune the distribution of carbon flux between biomass and production time, aiming to enhance the yield of gluconic acid. Dinh et al.[38] developed a QS-regulated tool based on the *lux* and *esa* system to dynamically adjust the transcription levels of promoters in a cell density-dependent manner, successfully overcoming challenges in the synthesis pathways of naringenin and salicylic acid. Onjo et al.[39] designed and constructed a synthetic microbiota, including an enzyme-producing strain and a target chemical-producing strain, to improve the production efficiency of isopropyl alcohol. Previously, our research group has developed a novel mixed-fermentation system with both signal transduction and metabolic division for producing salidroside, which was a typical glycoside and has been used for treating or preventing diseases, such as fatigue, cerebral ischemia, hypoxia, and cancer cachexia [3, 40]. We established circuits incorporating control modules such as a QS-based synchronized killing circuit (QS-SKC) and a QS-based metabolic toggle switch (QS-MTS). Finally, we achieved the division of metabolic burden, regulation of cell growth competition, and salidroside production yield increase by 75.97% [16].

Notably, AI molecules in QS system can bind to receptors not only within the same strain but also across different strains, causing signal crosstalk between strains and leading to a complex regulation network [41, 42]. Most QS systems exhibit varying degrees of crosstalk, with some crosstalk even interfering with the function of gene circuits [43, 44]. Thus, most researches on QS were focused on developing strictly orthogonal channels to ensure the predictability of gene circuit outputs [45, 46]. Specifically, to explore the orthogonality and crosstalk of QS signaling molecules, Tekel et al.[47] tested five receptors from different QS systems, such as LuxR, LasR, TraR, BjaR, and AubR, for their response to a range of signaling molecule synthases. Final results revealed that there were two sets of orthogonal AHL synthase and regulator protein pairs (BjaI/BjaR + EsaI/TraR; LasI/LasR + EsaI/TraR)

with minimal crosstalk. Based on six commonly used QS systems, such as *lux*, *las*, *tra*, *rpa*, *rhl* and *cin* QS systems, Kylilis et al. developed a software to identify orthogonal combinations of different receptors and signaling molecules within a given range of signaling molecule concentrations[48]. Wu et al.[49] have also selected *lux* and *las* systems from Gram-positive bacterium along with *agr* and *progX* systems from Gram-negative bacterium, and explored the orthogonality among different QS components. Scott et al.[50] combined the orthogonal QS system with gene circuits having different self-limiting growth dynamics to design two "orthogonal" systems, *lux* and *rpa* systems, maintaining stable co-cultures of *Salmonella typhimurium*. Miano et al.[51] developed a derivable orthogonal QS system, which controls the population composition and dynamic regulation by changing the inducer concentration, combining the inducibility, tunability, population-level coordination, inducer safety, and orthogonality into a single genetic circuit.

Although there are studies demonstrating that QS plays a promising prospect for consortia-based applications, most of them are focused on screening and development of orthogonal QS systems, and the researches and applications concerning crosstalk systems are relatively scarce. Previous researches pertaining to crosstalk are merely limited to exploring the dynamic characteristics of crosstalk systems composed of different QS components, including QS signal crosstalk, QS promoter crosstalk, and QS signal & promoter crosstalk. Previously, using the reported production of isopropanol (IPA) from cellobiose as a case study, our research group has employed mathematical models to investigate the performance of QS-based metabolic control in co-cultivations with or without QS crosstalk [17]. Results showed that QS crosstalk would indeed affect the performances and characteristics of the combined QS devices in population control and IPA production. Considering that productivity and stability are two important characteristics in the metabolic production of cocultivations, how can QS systems affect these two factors should be systematically and deeply investigated. Furthermore, more comprehensive modeling, such as GEMs, and validating experiments should be combined together to promote the understanding of the assembly principle of QS-based SMC to expand their applications in different fields.

Therefore, in this study, we integrated different QS regulation strategies with a metabolic division of labor, conducted the systemic analysis of the productivity and stability of two-strain cross-feeding cocultivation by QS regulation strategies. Firstly,

taking the previously reported cross-feeding cocultivation for producing salidroside as the case study, GEMs were developed for the cocultivations with static control strategies based on nutritional complementarity of amino acids, and COMETS [22] was utilized to realize the simulation and optimization of the coculture system. Then, by inserting QS-related metabolic reactions, we established a community model based on the genome-scale metabolic networks to predict the dynamic changes and population control of QS-regulated co-culture metabolism. Finally, inspired by the model-based analysis, we designed and constructed self-, cross-, and hybrid-regulating QSCNs to realize the population coordination and metabolic dynamic control, and investigated their productivity and stability of cross-feeding cocultivation.

2. Material and methods

2.1 The construction of the GEMs

Our previously reported cross-feeding cocultivation for producing salidroside with nutritional complementarity of amino acids [3] and QS devices [16] were taken as the case studies. It aims to develop the GEMs for the *Escherichia coli* co-culture fermentation system to realize the analysis and optimization of microbial interaction, through reforming and optimizing the biosynthetic pathway of salidroside in different strains. The initial *E. coli* model for tyrosol production is K-12 MG1655. The detailed data for this model is available through the BIGG database (http://bigg.ucsd.edu), with the model file being iML_1515. The initial *E. coli* model for the production of salidroside and UDP-glucose is *BL21* strain, which corresponds to the iB21_1397 model in the BIGG database. Based on the two essential *E. coli* models obtained, we added metabolic details (Table S1) and reaction information (Table S2), as well as performed genetic modifications. In order to enhance a more comprehensive analysis of microbial interactions and provide rational guidance for the design of QS-regulated communities consisting of two *E. coli* strains, we integrated the metabolic information of QS systems, such as AI-2, indole, AI-1, and AI-3, into the existing *E. coli* metabolic network model (Specific details shown in Table S3-S4). The operations and computations of the GEMs were conducted on the software COBRA Toolbox 3.0 (https://opencobra.github.io/cobratoolbox) [52], COMETS 2.10.0, and COMETS MATLAB toolbox (https://github.com/segrelab/comets-toolbox) [22].

2.2 Materials, plasmids and strains

We have listed the reagents and instruments for the construction and validation of plasmids and strains in this study in Table S5. Details about the plasmids used in this study are presented in Table S6. The p15ASLC1 plasmid was designed to use *rpa* QS system to regulate and control gene expression (Figure S1A), achieving the effect of an auto-inducible regulating switch. The signal molecule N-(p-Coumaroyl)-L-homoserine lactone (*pc*-HSL) produced by *rpa* QS system could bind to the RpaR protein, activating the expression of the killing gene *ccdb*, which gradually leads to cell death, thereby reducing the growth advantage of the aglycone strain (AG strain) in producing tyrosol. The p15ASLC2 plasmid consisted of the synthase gene *EsaI*, receptor protein gene RpaR, and promoter Prpa (Figure S1B). When the exogenous inducer IPTG is added, the promoter P_{lac} would activate the expression of the detoxification gene *ccda* and gene EsaR. The p15AMTS1 plasmid was designed to apply *esa* QS system to regulate and control the expression of *citA*, encoding citrate synthase to enhance the TCA cycle and accelerate cell growth of glycoside (GD) strain, achieving the on/off switch for the cell growth promotion control at different stages (Figure S1C). The p15AMTS2 plasmid consisted of synthase gene *rpaI*, receptor protein gene EsaR, Promoter PesaS, and green fluorescent protein (GFP) gene (Figure S1D). The compound of signaling molecule N-(3-oxo-hexanoyl)-homoserine lactone (3OC6-HSL), secreted by the AG strain, and EsaR was essential for regulating the cell growth of GD strain and production of salidroside. The pLX2tyr1plasmid was designed to mainly express gene RpaI, gene *bgl*, which hydrolyzes disaccharide into glucose, red fluorescent protein (RFP) gene and tyrosol-production gene *kdc4* (Figure S1E). The pLX2tyr2 plasmid consisted of gene BGL, RFP gene and tyrosol-production gene *kdc4* (Figure S1F).

The construction of the corresponding strains was achieved by combining the plasmids mentioned above (Table S7). In the self-regulating QSCN system, the AG strain BMT23SLC1 could be obtained by combining p15ASLC1 and pLX2tyr1 plasmids through a series of molecular biology operators. While the GD strain, BMS24MTS1, could be obtained by combing p15AMTS1 and pLX3sal1 plasmids. In the cross-regulating QSCN system, the AG strain, BMT23SLC2, could be obtained by combing p15ASLC2 and pLX2tyr2 plasmids, and the GD strain, BMS24MTS2, could be obtained by combing p15AMTS2 and pLX3sal1 plasmids. In the hybrid-regulating

QSCN system, the AG strain, BMT23SLC3, consisted of p15AT2 and pLX2tyr2 plasmids, and the GD strain was BMS24MTS1 in the self-regulating QSCN system. Primers involved in the construction of plasmids and strains are listed in Table S8.

2.3 Culture media and conditions

The correctly constructed and accurately sequenced plasmids were electroporated into the target strains, respectively. All engineered *E. coli* strains were cultivated at 37◦C and 1.23 xg (220 rpm) shaking. In the monoculture of the strain designed for tyrosol production, incorporate 5 g/L of glucose and 5 g/L of xylose into the M9 medium (17.1 g/L $Na_2HPO_4 \cdot 12H_2O$, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 5 mM $MgSO_4$, 0.1 mM $CaCl_2$). For the salidroside-producing monoculture, add 10 g/L of glucose into the M9 medium. Within the mixed microbial culture system, supplement the medium with 8 g/L of glucose, 2 g/L of xylose, and 5 g/L of cellobiose to support the metabolic requirements of the co-cultivated strains. In addition to the base M9 medium, incorporate the appropriate amino acid solution, and maintain the overall pH at a stable 7.0 throughout the culture process. Throughout the fermentation process, it is essential to conduct periodic sampling every 12 hours and monitor the OD_{600} value of the bacterial suspension.

2.4 Cocultivation analysis

Centrifuged the fermentation broth at 3413.15 xg (6000 rpm) for a duration of 5 minutes, discarded the supernatant, and resuspended the pellet in an equivalent volume of 0.01 M Phosphate Buffered Saline (PBS) solution. Proceed to centrifuge the sample at 3413.15 xg (6000 rpm) for 2 minutes, followed by the removal of the supernatant. This step should be repeated twice to ensure thorough washing of the cellular material. Ultimately, the cell pellet should be resuspended in an equivalent volume of PBS solution. Dispensed 200 μL of the bacterial suspension into 96-well plates, and employed an equal volume of PBS solution as a control. Then, the fluorescence microplate was utilized to carry out the detection at room temperature, ensuring the assay was conducted in triplicate to enhance data reliability, with the numerical readings recorded. For each system, standard curve was generated to delineate the correlation between fluorescence protein expression and optical density (OD) values for monocultures. Then employ the fluorescence microplate to monitor and quantify the fluorescence intensities of RFP and GFP throughout the co-culture fermentation process. According to the standard curves derived from monoculture

experiments, we calculated the population ratio of AG and GD strains within different cocultivations.

2.5 Biomass and metabolite analysis

Growth curves, quantified by OD_{600}, for *E. coli* strains can be measured by the ultraviolet spectrophotometer. Following dilution to a specified concentration, the OD600 for bacterial samples was conducted in triplicate with the average value taken as the final reading. Product detection was conducted by the high-performance liquid chromatography (HPLC) with the conditions set as follows. The assay was performed on a 4.6×150 mm column. UV-detector was set as the wavelength calibrated at 280 nm. The mobile phase was composed of 20% methanol, 80% water, and 0.1% formic acid, with the flow rate set to 1 mL/min. The column temperature was maintained at 25 ℃. HPLC vials were utilized for detection, with inserts added to the vials, 200 to 300 μL of samples added to the inserts, and the injection volume set at 20 μL. Before initiating HPLC analysis, subjected the samples to centrifugation at 6826.3 xg (12000 rpm for 5 min, collecting the supernatant, and filtering it through a hydrophilic filter membrane. Initially, it is imperative to scrutinize the chromatograms of tyrosol and salidroside standards to ascertain their respective retention times. Standard curves for both tyrosol and salidroside should be constructed using at least five concentration points, with the correlation coefficient (R^2) for both curves exceeding 0.99. Subsequently, the yields of tyrosol and salidroside were detected, ensuring the peak times matched those of the respective standards. The ultimate yields of tyrosol and salidroside in the samples were calculated based on their peak areas in conjunction with the established standard curves.

3. Results

3.1 GEMs-based analysis for cocultivations with the static control strategy

In pursuit of simulating and optimizing subsequent bioprocessing stages, we initially performed parameter fitting centered around our reported co-culture system [3] based on the mutual cross-feeding of phenylalanine and tyrosine (Figure 1A). Among the pivotal parameters are the uptake rates of xylose and glucose in the AG strain, the uptake rate of glucose and the secretion rate of salidroside of the GD strain. After optimization, there is a well fit between the experimental outcomes (scattering

dots) and GEM-based calculations (lines) on the consumption of glucose and xylose as carbon sources (as shown in Figure 1B), as well as the production of salidroside (Figure 1C). Furthermore, utilizing the previously constructed and validated co-culture fermentation model, four aspects were selected in this study, including cultivation time, carbon source ratio, initial total cell density, and inoculum ratio of the AG and GD strains, to conduct the optimization of fermentation conditions by orthogonal experiments. After the corresponding analysis, the optimal conditions for the shake-flask fermentation production of salidroside by co-culture system could be determined, i.e., the total carbon source concentration being 10 g/L, comprising glucose of 8 g/L and xylose of 2 g/L, the initial total OD_{600} being 1.0, and the initial cell density of AG strain should be twice that of the GD strain.

Considering that it takes a certain amount of time to accumulate the tyrosol as a precursor of salidroside, we tried to use the inoculation strategy with different times for AG and GD strains to optimize the fermentation process. Specifically, the AG strain was inoculated firstly, then at the 20th hour of the fermentation process, the GD strain was inoculated correspondingly to gradually convert the accumulated tyrosol to salidroside (Figure 1D). Results demonstrated that glucose was fully consumed around 10 hours of fermentation, while xylose was completely consumed at approximately 50 hours (Figure 1E). Ultimately, the simulated yield of salidroside reached 0.1352 mmol (equivalent to 812.55 mg/L), a mere 20% elevation over the experimental outcome, which was not obvious (Figure 1F). By calculating the ratio of the AG strain throughout the entire fermentation process, we found that the proportion of the AG strain would progressively decrease over time, eventually approaching zero (Figure 1G). Therefore, the static control strategy of stabilizing the microbial community exclusively through amino acid auxotroph necessitates additional refinement, which calls for some other dynamic control strategies, such as QS-based regulations.

Figure 1 here

3.2 GEMs-based analysis for cocultivations with QS-based dynamic regulations

Recently, accumulating evidence has verified that QS control mechanisms could be utilized to dynamically regulate microbial cell density and facilitate metabolic production. Previous experimental research conducted by our research group has verified that the implementation of QS devices significantly improves the control

efficiency for salidroside production within two-strain cross-feeding cocultivations [16]. On this basis, this study advanced to develop the QS-based GEM to give a more comprehensive analysis of the productivity and stability of the QS-based dynamic metabolic control. As illustrated in Figure 2A, the simulation results obtained from QS-based GEM exhibited a notable congruence with our reported experimental data. Results demonstrated that the cell density of AG strain could reach a dynamic equilibrium as a consequence of integrating a QS-based synchronized killing circuit (QS-SKC). Simultaneously, as depicted in Figure 2B. the GD strain equipped with QS-MTS devices primarily focuses on increasing its cellular density during the early phases. Once the biomass threshold governed by QS is attained, the aggregate biomass remains stable, diverting more glucose towards metabolic production of salidroside.

Similar to the static control strategy based on amino acid auxotrophic interdependency, we conducted an optimization for the QS-based co-culture system. Specifically, we conducted a systematic simulation to investigate the impacts of different conditions on the titers of salidroside, such as adjusting the carbon source ratio, initial cell density, and inoculation time, thereby achieving the optimization of the fermentation process. Results showed that the increase of the initial ratio of the GD and AG strain indeed significantly potentiates its capacity for tyrosol uptake, thereby improving the production yield of salidroside. It was noteworthy that as the initial ratio of GD and AG strain increases, the rate of increase in salidroside titer gradually slows down (Figure 2C). Regarding the ratio of glucose to xylose, a smaller glucose proportion could inhibit the complete utilization of the precursor molecule tyrosol. Conversely, an excessively high glucose ratio may lead to a scenario where the GD strain exhausts the entire tyrosol supply, with glucose remaining unutilized, thus signifying a waste of carbon resources. Therefore, it is necessary to balance the carbon source allocation between the production of the precursor molecule, tyrosol, and the biosynthesis of salidroside. Ultimately, through optimization and analysis, it was found that the most advantageous ratio of glucose to xylose was 5:1, at which the production titer of salidroside could reach a peak (0.384 mM) (Figure 2D).

In contrast to the instability of the microbial consortia controlled by amino acid auxotrophic, we have also conducted a flux variability analysis on the QS-based cross-feeding cocultivation (Figure 2E). Results demonstrated that as the community growth rate increases, a significant modification occurs in the cell density ratio

between AG and GD strains, specifically showing a decreasing proportion of GD strain within the community. This observed phenomenon can potentially be attributed to the increase of the biomass for microbial community, which effectively distributed the metabolic load of salidroside production, consequently reducing the relative abundance of GD strain, which beard the primary responsibility for metabolic production. As time goes on, AG and GD strains ultimately reached a state of convergence, arriving at a stable and equilibrium community composition. Results showed that strains AG and GD ultimately converged to a stable community composition (AG, 36%; GD, 64%). Therefore, the results mentioned above have confirmed the effectiveness of QS-based dynamic regulations in the metabolic production of salidroside.

However, current experiments and simulations in this section are only focused on the orthogonal self-regulating microbial system, there is a lack of relevant research on the effectiveness of cross-talk regulation strategies for dynamic metabolic control in co-culture systems. Considering that the “promiscuous” QS crosstalk includes diverse combinations of signals, receptors, and promoters, which could function based on self-regulating, cross-regulating, and hybrid-regulating QS-based communication networks (QSCN). Therefore, inspired by the above results, we would construct the cross-regulating and hybrid-regulating QSCN based on different QS gene circuits to optimize the regulation structure of the cross-feeding cocultivation for achieving more stable community and higher salidroside titer.

Figure 2 here.

3.3 Programming QS circuits for individual strains

In pursuit of implementing diverse QS-based dynamic regulation strategies, we constructed several QS circuits by rearranging different *rpa*, *esa*, and *lux* QS modules in five *E. coli* strains. Among them, *rpa* QS system is orthogonal to the *esa* and *lux* QS systems because of the difference in signal molecules, while *esa* system and the *lux* system have a high degree of crosstalk due to the use of the same QS signals, 3OC6HSL (more details in Table 1). Note that the former strains 1, 2, and 3 were designed as AG strains, while the latter strains 4 and 5 were GD strains.

Table 1. Details of the components in three QS systems used in this study

Systems	Synthesis	Signals	Receptor	Promoter	Crosstalk
rpa	RpaI	pc-HSL	RpaR	Plux	Orthogonal with *esa* and *lux* QS system
eas	EsaI	3OC6HSL	EsaR	PeasS	Crosstalk with *lux* QS system
lux	LuxI	3OC6HSL	LuxR	Plux	Crosstalk with *esa* QS system

Specifically, the QS-regulated plasmid design in Strain 1 employed the *rpa* system (as depicted in Figure 3A). In this strain, the *rpaI* synthase catalyzed the synthesis of the signaling molecule pc-HSL, which was capable of accumulating and diffusing in the cultivation. Subsequently, when the concentration of the pc-HSL reached the threshold, the signal would diffuse back into the strain cells. pc-HSL would bind to the intracellular RpaR protein to form a complex, which in turn activated the Prpa promoter, initiating the expression of the killing protein CcdB. This process results in cell death, thereby reducing the competitive growth advantage of the AG strain. Results showed that the coordinated regulation by *rpaI-rpaR-Prpa* and *ccda-ccdb* effectively controlled the growth of AG strain 1 (BMT23SLC1), preventing overgrowth and resource monopolization (Figure 3A, right).

In strain 2, the QS circuit was composed of *esaI*, *rpaR*, and P_{rpa} (see Figure 3B). The signaling molecule 3OC6HSL, produced by the synthase gene *esaI*, diffuses into the extracellular culture and, as cell density increased, bound to the EsaR protein in the corresponding GD strain, thereby facilitating density-dependent dynamic metabolic regulation of the strain growth and production. Meanwhile, pc-HSL produced from other strains could be also accepted by Strain 2 and bound to *rpaR*, thereby regulating its cell killing process.

The QS-regulated plasmid designed in Strain 3 employed the *lux* system (as depicted in Figure 3C). In this strain, the *luxI* synthase catalyzed the synthesis of the signaling molecule 3OC6HSL, which, through self-sensing of its density, bound to the receptor protein LuxR to form a complex, thereby activating the promoter Plux and regulating the killing of the AG strain.

As illustrated in Figure 3D, Strain 4 for salidroside producing employed the *esa* system in the QS circuit. This strain secreted the signaling molecule 3OC6HSL and expressed the receptor protein EsaR. At lower population density, the protein EsaR

could bind to the promoter P_{esaS}, activating the expression of the protein CitA, thereby facilitating the growth of the GD strain. While at higher population density, the protein EsaR could bind to the signaling molecule 3OC6HSL to form a complex that repressed the growth-promoting activity of the promoter P_{esaS}, thereby redirecting the metabolic pathway towards production of salidroside. Therefore, the coordinated regulation by *EsaI-EsaR-PesaS* and *cita* effectively enhanced the cell growth of Strain 4.

With regard to Strain 5, the signaling molecule pc-HSL, produced by the synthase gene *rpaI*, could bind to the protein RpaR in AG strains, effectively regulating their behavior and tyrosol production. In addition, the signaling molecule 3OC6HSL secreted by AG strains could also bind to the EsaR protein in Strain 5 to regulate the metabolic production of the strains. Thus, the cross-regulating QSCN by different QS signals was realized by inter-strain communications.

Certainly, before we combine different single strains for the construction of different control structures, we also need to carry out single bacteria fermentation on AG and GD strains separately to make sure that individual strain can indeed produce corresponding product as we designed. The aforementioned five strains 1-5 were cultured for 48 hours, and the corresponding production was measured every 12 hours. The results showed that the Strain 1, 2, 3, 4, and 5 could produce about 300 mg/L tyrosol or salidroside at 48h (right column in Figure 3). To sum up, the experimental results on the cell growths and production of these five strains were consistent with the expected results based on their designs.

Figure 3 here.

3.4 Two-strain cocultivations with three different QSCNs

In this section, we have designed three different QSCNs for the dynamic metabolic control of the two-strain cross-feeding cocultivations, namely self-regulating, cross-regulating, and hybrid-regulating QSCN, thus investigating the effects of different regulation modes on the productivity and stability in microbial consortia-based applications (Figure 4). Specifically, the self-regulating QSCN was established based on *rpa* and *esa* system, utilizing the QS signaling molecules pc-HSL and 3OC6HSL. Given their completely different chemical structures, these molecules facilitate orthogonal communications (Table 1 and Figure 4A). The cross-regulating QSCN was constructed by exchanging the synthase genes (*rpaI* and *luxI*)

between the two QS plasmids derived from the self-regulating QSCN, and then respectively introducing the modified plasmids into the AG and GD strains enabling their crosstalk and communication through the corresponding signaling molecules (see Figure 4D). The hybrid-regulating QSCN was engineered by utilizing the *lux* and *esa* QS systems to construct two genetic circuits, which were respectively integrated into AG strains and GD strains. Due to the homologous nature of the signaling molecules produced by these two strains, during the co-culture process, the signal molecules would serve a dual purpose, i.e., they could be utilized not only by the generating strains, but also by the other one (shown in Figure 4G).

For better research on co-culture dynamics, we selected the AG strain (Strain 1) and GD strain (Strain 4) from single cultures for the co-cultivation production, due to their robust growth and high productivity during monoculture fermentations. We proceeded to monitor their cell growth ratios and fermentation productivity over a 48-hour period. For the self-regulating QSCN, observing the dynamics of strain proportion changes, the initial inoculation ratio for co-culture was set at 1:1, while throughout the 48 hours, the GD strain maintained a growth dominance, leading to a final composition where Strain 4 accounted for approximately 70% and Strain 1 for 30%. This result indicated that the self-regulating QSCN could modulate the growth equilibrium between the AG and GD strains (as shown in Figure 4B), preventing the exhaustion of any single strain (as observed in Figure 1G). With respect to productivity, during the 48-hour fermentation process, tyrosol, an intermediate product, reached a final accumulation of 127.11 mg/L, while the salidroside production ultimately reached 359.95 mg/L, which was 2.28 times than the control group (157.64 mg/L, see Figure S2). Compared with the control group, the self-regulating QSCN strategy could better coordinate the cell growth balance between the two strains, decrease the accumulation of the intermediate product tyrosol, and increase the final yield of the target product, salidroside. This result further verified that the employment of a relatively independent QS system, as illustrated in Figure 2, indeed exerted a modulatory effect on the cell growth and production dynamics of microbial communities.

For the cross-regulating QSCN, the selected Strain 2 and Strain 5 were co-cultivated and regulated by communicable signaling molecules pc-HSL and 3OC6HSL. Similarly, the initial inoculation ratio of the two strains was set at 1:1, throughout the 48-hour cultivation process, the GD strain maintained an overall

growth advantage over the AG strain. During the prior 24 hours, the proportion of the two strains was nearly 1:1. This was possible because the cell growth and the accumulating and secreting of signaling molecules necessitated a specific duration, resulting in a certain lag in the regulation of AG and GD strains. During the posterior 24 hours, the cell population ratio began to diverge and eventually stabilized at a corresponding level (AG strain, 34%; GD, 66%) (Figure 4E). Regarding the production results, the amount of the intermediate metabolite, tyrosol, in the cross-regulating QSCN system during the co-culture process, ultimately reached 135.11 mg/L, with the yield of salidroside at 245.16 mg/L. In this system, the yield of salidroside showed a certain improvement compared with the control group (157.64 mg/L, see Figure S2), albeit not as significant as that in the self-regulating QSCN system (359.95 mg/L). Note that, the cross-regulating QSCN could lead to better density coordination between the AG and GD strains, this might be offset by a loss in the yield of salidroside, potentially due to the low diffusion efficiency of the QS signaling molecules.

For the hybrid-regulating QSCN, we selected the *lux* and *esa* systems, both capable of generating the signaling molecule 3OC6HSL, to modulate Strains 3 and 4 respectively (as shown in Figure 4G). During the co-culture process, the signaling molecule performs a dual role, i.e., it can be employed not only as the intra-strain signaling within the generating strains, but also for the inter-strain communication between different strains, thereby embodying both self-regulating and cross-regulating signaling interaction mechanisms. As illustrated in Figure 4H, from an overall perspective, the strain ratio tends towards oscillatory convergence, ultimately reaching a balanced state (AG strain, 44%; GD, 56%). This result suggested that within the synthetic microbial consortia, the integration of self-regulating and cross-regulating mechanisms enables a more timely and precise coordination of cellular growth between the two strains, leading to a harmonized balance in their cell densities. The salidroside production ultimately reached 562.57 mg/L, which is 3.57 times that of the control group (157.64 mg/L, see Figure S2), demonstrating a higher performance than both the self-regulating and cross-regulating QSCNs. Furthermore, we noticed that within the hybrid-regulating QSCN strategy, the accumulation of tyrosol was minimal observed (112.15 mg/L). To sum up, the results from this section indicated that the hybrid-regulating QSCN, integrating self-regulating and cross-regulating strategies, could expedite the coordination of growth between strains,

thereby enhancing the production of metabolites.

Figure 4 here.

4. Discussion

As stated above, we have conducted a systematic analysis for the stability and productivity for the two-strain cross-feeding cocultivation with or without QS regulations. Note that we have introduced three common QS systems（*rpa*, *esa*, and *lux*）to construct three different QSCNs in two-strain consortia to regulate their cell growths and metabolic production. And both of them showed significant discrepancies.

As illustrated in Figure S3, the initial inoculation ratios of the self-regulating, cross-regulating, and the hybrid-regulating coculture system are 1:1. There was a large difference in the ratio of GD and AG strains in the self-regulating system, and the growth advantage of GD strain was very obvious, almost twice that of AG strain. In the cross-regulating system, the ratio of the two strains still tended to be stable (around 1:1), and fluctuated significantly after 24h, gradually approaching 2:1. The hybrid-regulating system has a certain fluctuation before 24h, and tends to stabilize around 1:1 after 24h. We used a baseline of 1:1 to calculate the total deviation (TD) for the absolute value of ratios to analyze the quantitative differences brought by the three QSCNs. The results show that the TDs of the self-regulating, cross-regulating, and the hybrid-regulating QSCNs are 4.61, 1.91 and 1.51, respectively. Therefore, it was apparent that the hybrid-regulating QSCN showed the best capability in coordinating the microbial community, followed by cross-regulating QSCN, with self-regulating QSCN being the least effective (see Figures 4B, 4E, 4H, and Figure S3). This may because in the self-regulating QSCN, signals are only effective and totally orthogonal within the generating strains, while in the cross-regulating QSCN, signal communication occurs across strains, albeit with a delay, leading to interdependence of the two strains. The hybrid-regulating QSCN combined the strategies of the aforementioned two systems, enabling not only relatively independent control of strain growth and production, but also facilitating inter-strain communication to effectively coordinate their respective growth rates.

Interestingly, the hybrid-regulating QSCN system proved to be the most effective in maintaining the stability of the strains, concurrently achieving the highest production yields of salidroside (562.57 mg/L) (as shown in Figures 4C, 4F, and 4I).

This phenomenon may be due to the introduction of QS cross-talk, which enables more responsive regulation between the two strains, allowing for a quicker response to the demands of strain killing and metabolic switching, thereby facilitating the conversion of the intermediate metabolite, tyrosol, to the target compound, salidroside. Therefore, the cross-interference between signaling molecules proves to be more beneficial for the co-cultivation of strains, enhancing stability and metabolic production, which was in line with our previous simulation and calculations [17]. Certainly, the cross-talk mechanisms of signaling molecules were different across various biological systems, leading to disparities in the utilization efficiency of these molecules, as well as the utilization degrees of the entire QS elements, thereby affecting the expression of associated activated genes. Therefore, before adopting different QS systems and their corresponding components for the design of specific functions, it is essential to conduct careful analysis, including the qualitative and quantitative analyses of the specific cross-talk strengths, which is necessary for the rational design and utilization of consortia-based applications. Certainly, the purpose of synthetic biology is to build a system that is more certain and easier to operate with the orthogonal QS-based circuits. However, the study of QS crosstalk to be more quantified, thereby expanding the toolbox of synthetic biology, increase the understanding of microbial interactions, and promoting the wider application of QS-based circuit.

Certainly, as we proposed earlier, QSCNs can be applied to the understand and control the natural microbial systems and synthetic microbial consortia, respectively. on the one hand, QSCN holds huge potential for improving our understanding of the dynamics and resilience for different microbial communities, such as human gut microbiota and marine microecosystem. On the other hand, QSCN will do much help in providing new regulation strategies to manipulate synthetic microbiota and developing new QS-based synthetic gene circuits for medical and metabolic engineering. Therefore, it is crucial to understand the intensity of crosstalk among multiple QS systems within the microbial community to make the meaningful QSCN more comprehensive. For the optimization of QS components, the most crucial aspect is to investigate the potential effective or negative cross-talk combinations, in order to construct a more optimized QS system genetic circuit. QS crosstalk can also enhance intracellular communications, thus promoting microbial evolvability to adapt to different environmental conditions [53, 54]. The evolution and function of hybrid

signal receptors provide a molecular basis for the interactions across heterogeneities in complex microbial communities [55]. For example, the crosstalk based on quorum sensing peptides (QSPs) among *Streptococcus pneumoniae* may enable an individual strain to predict antibiotic production by neighboring strains [42]. Miller et al.[56] found that sharing of QS signals among three species can enhance the infectivity of *pseudomonas savastanoi*. Furthermore, in our previous work [17], we also found that some combined QS-based devices with crosstalk could outperform the natural ones. In this study, we found that crosstalk-based QSCN could better stabilize the two-strain cross-feeding cocultivations and improve metabolic productivity than orthogonal control QSCNs.

It is worth noting that the co-culture salidroside-production system employed in this paper is presented as a typical case study for the investigation on characteristics and performances of QS-regulated microbial communities. Thus, the comprehensive optimization of fermentation conditions for maximizing the productivity of the final product salidroside was not conducted as our final target. Certainly, to quantitatively characterize the strain ratios while concurrently maintaining metabolic production, the fluorescent proteins and metabolic pathways were co-expressed in the AG and GD strains, which consequently affected the final productivity of the target product. In the future, we should develop some other strategies that do not affect the metabolic capacity of strains and measure their proportion in a more accurate way to investigate the potential to promote microbial metabolic production.

Currently, the analysis of microbial interactions and productivity based on QS communications lacked the construction and analysis of the corresponding GEMs. Herein, in this work, with the help of GEMs, we have performed a thorough investigation into the productivity and stability of a two-strain cross-feeding coculture system based on amino acid syntrophy and QS communications. The QS-based GEMs could be expanded to assess the cell growth competition in synthetic microbial consortia regulated by different QS-based devices, as well as the final productivity of salidroside. However, the QS-based GEMs in this study were currently limited to the investigation of dynamics of the self-regulating QSCN (more details in Section 3.2), due to the absence of parameters governing the production and unknown kinetics of QS signaling molecules in cross-regulating and hybrid-regulating QSCNs. Furthermore, the development of QS-based GEMs is limited by the incomplete QS-related reaction information, the uneven distribution of QS synthases and receptors,

and the unclear mechanism of signal diffusion and acceptance. Therefore, in our future research, we will focus on the analysis and construction of more comprehensive QS-based GEMs. We also believe that with the enrichment of multi-omics information and the advancement of microbial community models, the construction, analysis and applications of more comprehensive QS-based GEMs, will be achieved at a faster pace.

5. Conclusion

To sum up, this work has optimized the synthetic microbial consortia, with the objective of improving the stability as well as the productivity of the QS-regulated cross-feeding cocultivations. Taking our previously reported salidroside-producing co-culture system as the case study, we constructed and analyzed the GEMs based on amino acid auxotrophic interdependency and QS communications. Results showed that the QS-based regulations could indeed enhance the stability of the microbial community while increasing the productivity of salidroside. We have further designed three different QS-based regulation topological structures, i.e., self-regulating, cross-regulating, and hybrid-regulating QSCNs, and conducted the corresponding molecular biology experiments to investigate the impact of different QS regulations on this process. Although all three QSCNs were capable of regulating the killing of AG strain and the dynamic metabolic switching in GD strain to a certain extent, the hybrid-regulating QSCN showed better performances in stability and cooperative production. AG and GD strains in the hybrid-regulating QS-based co-culture system collaborate and adapt more closely with the cross-talk communications by sharing the same signaling molecule. Finally, this study not only contributes to constructing more comprehensive models of microbial communities that incorporate QS communication regulations, facilitating the analysis and reconstruction of microbial interactions, but also holds significant implications for the development of QS-related applications.

CRediT authorship contribution statement

Danlei Chen: Writing-review & editing, Writing-original draft, Software, Methodology. Zeping Qu: Validation, Methodology, Data curation. Shujuan Yang: Validation, Methodology, Data curation. Yujie Li: Validation, Methodology. Shuxuan Yu: Validation, Methodology. Xin Li: Software, Data curation. Yutong Lu: Writing-review & editing, Formal analysis. Aidong Yang: Writing-review & editing, Project

administration. Chunjiang Liu: Writing-review & editing, Conceptualization. Shengbo Wu: Writing-review & editing, Funding acquisition, Conceptualization. Jianjun Qiao: Supervision, Conceptualization, Project administration

Declaration of competing interest

We declare we have no competing interests

Data accessibility

This article has no additional data.

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Appendix A. Supplementary material

Supplementary information to this article can be found online.

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