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Preparation Technology and Antitumor Analysis of the Composite Jiaosu from *Lycium ruthenicum* Murr and *Coix lacryma-jobi*

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Abstract *Lycium ruthenicum* Murr. (*L. ruthenicum*) and *Coix lacryma-jobi* (*C. lacryma-jobi*) were utilized as the composite fermentation raw materials with probiotics to conduct fermentation experiments. Through single-factor tests (fermentation time, inoculation amount, strain ratio, and substrate concentration) and orthogonal tests, the preparation process of the composite Jiaosu was optimized for fermentation time, inoculation amount, strain ratio, and substrate concentration. The total flavonoid content and the antitumor ability of the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu were also determined by spectrophotometric method and MTT assay. The results showed that the optimal preparation process of the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu was as follows: the strain ratio was 1:1:1:2 (*Streptococcus thermophilus*: *Lactobacillus bulgaricus*: *Bifidobacterium adolescentis*: *Lactobacillus acidophilus*, mass ratio), the inoculation amount was 8%, the fermentation time was 6 days, and the substrate concentration was 9%. The measured extracellular polysaccharide content was 0.67 mg/mL, and the flavonoid content was 0.10 mg/mL. The best inhibitory effect on A-549 tumor cells was achieved by Jiaosu at a concentration of 10^{-3} mg/mL, with an inhibition rate of 12.17%. It shows that *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu can hinder the growth, proliferation and other related activities of the tumor cells to a certain extent, which also provides an important theoretical basis for the development of related anti-tumor products.

Keywords: *Lycium ruthenicum Murr, Coix lacryma-jobi, Jiaosu, Antitumor, Fermentation process*

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1. Introduction

Lycium ruthenicum Murr. (*L. ruthenicum*) belongs to the genus *Lycium* of the Solanaceae family and is one of the common varieties of *L. ruthenicum* [1]. It contains abundant bioactive components, such as polysaccharides [2], polyphenols [3], amides [4] and many others, especially rich in anthocyanin polyphenols [5], which have antioxidation, anti-inflammatory, anti-tumor, and preventive effects on cardiovascular diseases, and possess both edible and medicinal values, thus being known as "soft gold" [6].

Coix lacryma-jobi (*C. lacryma-jobi*), as a traditional dual-purpose resource for food and medicine, is widely cultivated in many regions. It has a slightly cold nature and the function of clearing heat and promoting diuresis [7]. Due to its high practical and medicinal values, it has earned the title of "the king of the world's gramineous plants" [8]. It is rich in carbohydrates, proteins, amino acids, and other basic substances that are crucial for maintaining life activities [9]. Recent studies have shown that *C. lacryma-jobi* has various physiological activities such as antioxidant, hypoglycemic and antitumor effects [10,11,12].

Jiaosu is a microbial-fermented product derived from fruits, vegetables or cereals as the main raw materials [13], differing from single-strain ferments (e.g., koji) or purified enzymes. Though the Chinese term Jiaosu literally translates to "enzyme," it denotes a complex fermented matrix rather than isolated enzymes. Unlike single-strain ferments (e.g., koji), Jiaosu demonstrates greater metabolic diversity owing to its mixed microbial consortium and multi-substrate fermentation system. It contains a variety of bioactive substances, such as active enzymes, probiotics, organic acids, polyphenols, amino acids, esters and alcohols [14], which can eliminate free radicals [15] and have functions such as enhancing the body's immunity and regulating endocrine imbalance [16].

In China, there are relatively abundant research achievements regarding the *L. ruthenicum*-based Jiaosu [17,18,19]. Comparatively, research on the *C. lacryma-*

jobi-based Jiaosu is relatively scarce. Most of the studies focus on single components of *C. lacryma-jobi*. For instance, Sun Hui et al. [20] explored the polysaccharides in fermented *C. lacryma-jobi* and found that they significantly improved the fermentation characteristics and quality of low-fat yogurt.

An in-depth and systematic research on both *L. ruthenicum*-based Jiaosu and *C. lacryma-jobi*-based Jiaosu is scarce. Nevertheless, there are some explorations in related fields. Raj K. et al. [21] conducted a comparative analysis of the phytochemicals, antioxidant activities, and chromatographic characteristics of different parts of *L. ruthenicum*, providing insights for the antioxidant research of *L. ruthenicum*. Chandra I. A. et al. [22] evaluated the shelf-life of biscuits formulated with *C. lacryma-jobi* and Moringa leaf flour, delving into the exploration of *C. lacryma* - *jobi* in the development of functional foods.

Research indicates that the anti-tumor mechanisms of polysaccharides are diverse, mainly related to their occurrence sites, structural characteristics, and synergistic effects with other active ingredients [23]. According to the occurrence sites and characteristics, polysaccharides can be classified into exopolysaccharides and intracellular polysaccharides. Exopolysaccharides are polysaccharides secreted outside the cell by microorganisms during their growth and metabolism processes, possessing unique chemical structures and biological activities. In contrast, intracellular polysaccharides exist inside the cell and participate in regulating intracellular physiological processes [24]. The two types of polysaccharides have differences as well as synergistic interactions in terms of their origins, structures, and functions.

In addition, the anti-tumor effect of polysaccharides is often closely associated with flavonoids. Flavonoids are polyphenolic compounds widely existing in plants and often coexist with polysaccharides in natural products. During the anti-tumor process, flavonoids, through their antioxidant [25], anti-inflammatory [26] and other properties, can regulate the intracellular redox state, creating a favorable microenvironment for polysaccharides to regulate the immune system, thus enhancing the immune response stimulated by polysaccharides through macrophages and lymphocytes [27]. Additionally, flavonoids may also improve the targeting of polysaccharides to tumor cells, assisting polysaccharides to directly act on tumor cells, inducing differentiation and apoptosis, inhibiting proliferation and changing the cell cycle distribution, thus exerting an anti-tumor effect [28].

To optimize the dual-substrate fermentation process of *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu, an orthogonal experimental design was employed, establishing the optimal preparation parameters. Anti-cancer activity was evaluated through the extraction and analysis of anti-tumor bioactive components. This method breaks through the limitations of conventional single-substrate fermentation and improves functional Jiaosu production via plant synergy, offering both theoretical and technical foundations for developing plant-based functional foods with tumor-suppressive effects.

2. Materials and Methods

2.1. Materials

L. ruthenicum and *C. lacryma* - *jobi* were purchased from a farmer's house in Zhongwei, Ningxia Hui Autonomous Region (China). Human A-549 non-small cell lung cancer cells were bought from Zhongqiaoxinzhou Biotechnology Co., Ltd. (Shanghai, China).

Streptococcus thermophilus (*S. thermophilus*), *Lactobacillus bulgaricus* (*L. bulgaricus*), *Bifidobacterium adolescentis* (*B. adolescentis*), and *Lactobacillus acidophilus* (*L. acidophilus*) were from Zhongkejiayi Bioengineering Co., Ltd. (Shandong, China). Aluminum nitrate, sodium hydroxide and concentrated sulfuric acid were from Xilong Scientific Co., Ltd. (Shantou, China). Pectinase was purchased from Pangbo Biotechnology Co., Ltd. (Nanning, China). Cellulase was from NCM Biotech Co., Ltd. (Ningxia, China). Anhydrous glucose and sodium nitrite were from Kelong Chemicals Co., Ltd. (Chengdu, China). Rutin was from Ku'er Bioengineering Co., Ltd. (Anhui, China). Phenol was from Kelong Chemical Reagent Factory (Chengdu, China). Dimethyl Sulfoxide (DMSO) was from Solarbio Science & Technology Co., Ltd. (Beijing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Dingguo Changsheng Co., Ltd. (Beijing, China). All other reagents were analytical grades.

2.2. Methods

2.2.1. Preparation of the *L. ruthenicum* and *C. lacryma-jobi* Composite Jiaosu

According to Ge R. et al [29], accurately weigh 10.00 g each of *L. ruthenicum* and *C. lacryma-jobi*. Add distilled water at a material-to-water ratio of 1:20 (mass ratio), soak in an HSY-24 constant temperature water bath at 100°C for 30 min, and then homogenize using a JM-LB50A colloid mill for 5 min. Next, weigh cellulase and pectinase (enzyme vigor: 1000 U/g), add them to the slurry, and incubate at 55°C for 2 h for enzymatic hydrolysis. Afterwards, inactivate the enzymes at 90°C for 10 min, and filter the solution through a 400-mesh sieve to obtain the fermentation broth. Sterilize the fermentation broth in a G154DWS autoclave at 95°C for 20 min, inoculate for probiotic fermentation in a 28EV fermenter, and determine the optimal conditions (inoculum amount, substrate concentration, strain ratio, fermentation time) via orthogonal experiment. Finally, centrifuge, filter, and can the mixture to obtain the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu.

2.2.2. The Single-factor Experiment of the *L. ruthenicum* and *C. lacryma-jobi* Composite Jiaosu

The design of this experiment follows the methodology in 2.2.1 of the text. This study systematically investigated the effects of inoculation amount, substrate concentration, strain ratio, and fermentation time on extracellular

polysaccharide content and residual sugar content during the preparation of the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu, using a single-factor experimental design. For the inoculation amount factor, fermentation was performed for 7 days with a fixed substrate concentration (5% TSS) and a strain ratio (*S. thermophilus*: *L. bulgaricus*: *B. adolescentis*: *L. acidophilus* = 1:1:1:1, mass ratio), testing inoculation amounts of 4%, 6%, 8%, 10%, and 12% (v/v, probiotics/fermentation broth). The second factor was the substrate concentration. Under fixed conditions of 8% (v/v) inoculum amount, a strain ratio (*S. thermophilus*: *L. bulgaricus*: *B. adolescentis*: *L. acidophilus* = 1:1:1:1, mass ratio), and a fermentation time of 7 days, the effects of substrate concentrations (4%, 6%, 8%, 10%, and 12% TSS) were evaluated. As the third factor, the study maintained 5% TSS substrate concentration, 8% (v/v) inoculation amount, and a 7-days fermentation time, while testing different strain ratios (*S. thermophilus*: *L. bulgaricus*: *B. adolescentis*: *L. acidophilus* = 1:1:1:1, 2:1:1:1, 1:2:1:1, 1:1:2:1, 1:1:1:2, mass ratio). For the fourth factor, fermentation time (5, 6, 7, 8, and 9 days) was examined under fixed conditions: substrate concentration (5% TSS), inoculation amount (8% v/v), and strain ratio (*S. thermophilus*: *L. bulgaricus*: *B. adolescentis*: *L. acidophilus* = 1:1:1:1, mass ratio). All experiments were conducted at 37°C using *L. ruthenicum* and *C. lacryma-jobi* fermentation broth as the base medium, with triplicate independent replicates per experimental group.

2.2.3. The Orthogonal Experiment of the *L. ruthenicum* and *C. lacryma-jobi* Composite Jiaosu

Based on the results of the single-factor experiments, an orthogonal test was designed. Four factors, namely inoculation amount, strain ratio, fermentation time, and substrate concentration, were selected. Three levels were chosen for each of these factors, and the $L_9(3^4)$ orthogonal test was conducted, as shown in Table 1.

Table 1. Orthogonal test $L_9(3^4)$

level	factor			
	Inoculation amount/%	Substrate concentration /%	Strain ratio (mass ratio)	Fermentation time/days
	A	B	C	D
1	6	7	2:1:1:1	6
2	7	8	1:1:1:2	7
3	8	9	1:1:2:1	8

2.2.4. Determination of Extracellular Polysaccharide Contents in Composite Jiaosu

The standard curve was established using the phenol-sulfuric acid method [30], where Jin et al. developed steam explosion-assisted extraction for polysaccharide quantification in *Brasenia schreberi*. A glucose standard solution (0.1 mg/mL) was prepared by dissolving accurately weighed 5.0 mg anhydrous glucose in distilled water within a 50 mL volumetric flask, followed by volume adjustment to the calibration mark.

Pipette 0, 0.2, 0.4, 0.6, 0.8, 1.0 mL of the solution accurately into stoppered test tubes, and then add water to each test tube to the 2 mL mark. Added 1 mL of 5% phenol solution accurately and shaken well, followed by 5 mL of sulfuric acid and shaken well. After 10 minutes of resting, the test tube was placed in a water bath at 40□ for 18 min, at the end of which, the test tube was quickly removed and cooled to room temperature. The absorbance at 490 nm was measured using a UV-1100D ultraviolet spectrophotometer to establish the standard curve of glucose standard solution concentration versus absorbance and obtain the regression equation [31]. A glucose-free reagent mixture was set as a blank control: 2 mL of distilled water was measured, 1 mL of 5% phenol solution and 5 mL of sulfuric acid were added sequentially, and the background absorbance was corrected according to the above experimental steps to ensure the accuracy and reliability of the experimental data.

For the determination of the extracellular polysaccharide content of the samples, the fermented *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu was centrifuged at 252 ×g for 15 min, 0.5 mL of the supernatant was mixed with 2 mL of anhydrous ethanol and shaken well, and the mixture was placed at -4°C for 24 h to ensure that the polysaccharides were fully precipitated.

On the next day, the mixture was taken out and centrifuged with the TG16-WS centrifuge at 402 ×g for 15 min to collect the precipitate. Add 1mL of distilled water to dissolve the precipitate, then centrifuge at 402 ×g for 15min, discard the insoluble precipitate at the bottom and collect the supernatant. Pipette 1 mL of the sample accurately and measure the absorbance according to the phenol-sulfuric acid method described above. The extracellular polysaccharide content of the sample was then calculated based on the standard curve.

2.2.5. Determination of Residual Sugar Contents in Composite Jiaosu

The residual sugar content was determined using an LB10T handheld refractometer. The scale value at the boundary line was converted to sugar concentration (mg/mL) based on refractive index measurements calibrated with standard sucrose solutions.

2.2.6. Determination of Total Flavonoid Content

The total flavonoid content of the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu was determined using a modified protocol based on Yu et al. [32], which established the method for health food analysis. Pipette 0.00, 0.20, 0.40, 0.60, 0.80, and 1.00 mL of rutin standard solution (200 μg/mL) into 10 mL volumetric flasks. After adding 60% ethanol to 3 mL in each vial, add 1 mL of 5% sodium nitrite (mixed and left to stand for 6 minutes), 1 mL of 10% aluminum nitrate (mixed and left to stand for 6 minutes), 4 mL of 4% sodium hydroxide solution, and

then adjust the volume to 10 mL with 60% ethanol, and leave it to stand for 15 minutes. Measure the absorbance at 510 nm and plot the standard curve.

Accurately pipette 1.0 mL of *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu to be tested in a 10 mL volumetric flask. Follow the above steps to determine the absorbance. The total flavonoid content in the sample was calculated using the standard curve.

2.2.7. Determination of Antitumor Activity

The *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu was centrifuged at 1118 ×g for 15 min, and the supernatant was collected and filtered through a 0.22 μm sterile membrane [33]. The filtrate was aliquoted into sterilized centrifuge tubes and diluted with sterile water to create a concentration gradient (0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} mg/mL), then stored at 4°C for further use.

A-549 cells were digested with trypsin, washed with PBS to remove enzyme residues, and counted using a hemocytometer. The cell suspension was adjusted to a density of 1×10^4 cells/mL and seeded into a PEF3590 96-well plates at 100 μL per well. The cells were incubated in a CLM-1708-8-NF cell culture incubator at 37°C with 5% CO_2 for 12 h to allow adherence. After adherence, 20 μL of the diluted filtrate (with the same final concentration of each well as above) was added to each well, and the cells were further cultured for 44 h. Blank control wells (containing medium and the compound but no cells) were also prepared. Subsequently, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h. The supernatant was then carefully removed, and 150 μL of dimethyl sulfoxide (DMSO) was added to each well. The plates were shaken for 10 min to ensure complete dissolution of the formazan crystals. The absorbance was measured at 490 nm using an iMark microplate reader, with the blank control wells used for zero adjustment. The inhibition rate was calculated according to Formula 1.

$$IR = \frac{A_c - A_d}{A_c} \times 100\%\# \quad (1)$$

IR represents the inhibition rate. A_c represents the average absorbance value of the control group, and A_d represents the average absorbance value of the sample group.

2.2.8. Data Analysis

All data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was adopted. When the p-value was less than 0.05, it was statistically significant. All statistical analyses were carried out using SPSS 26.0 software.

3. Results and Discussion

3.1. Results of the Single-factor Experiments

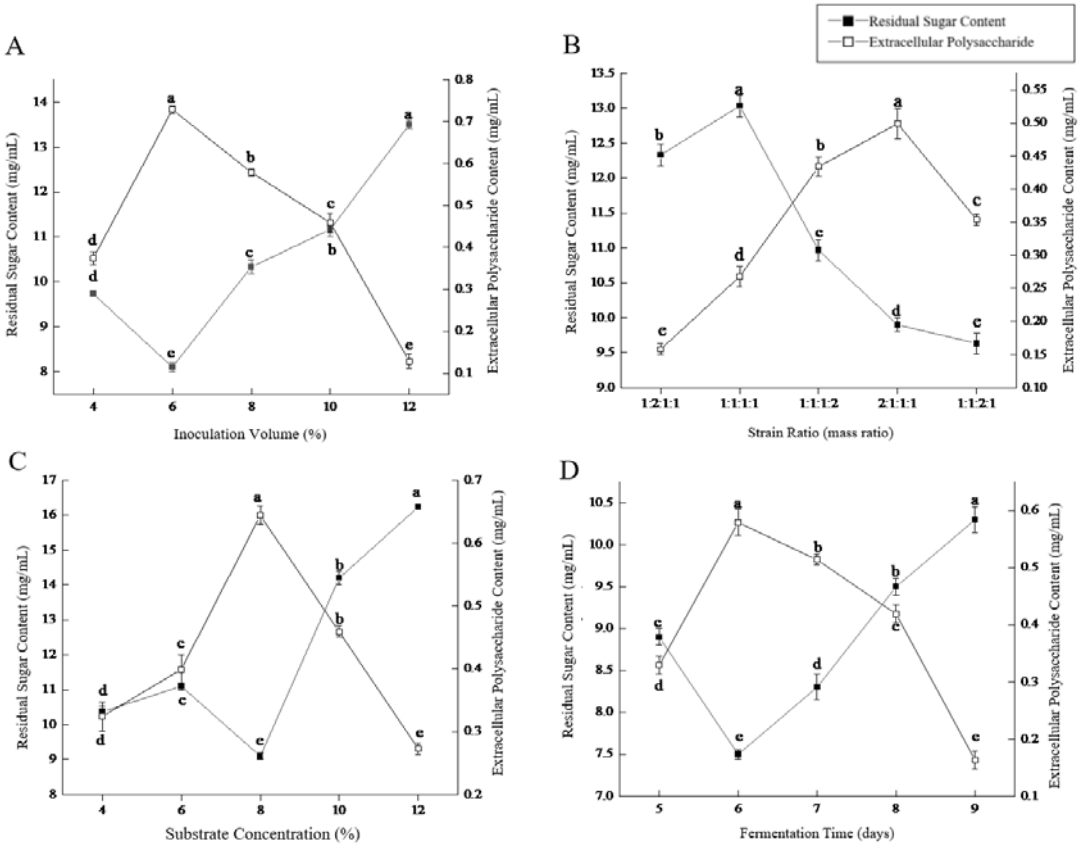

Figure 1. Effects of (A) inoculum amount (%), (B) substrate concentration (%), (C) strain ratio (mass ratio), and (D) fermentation time (days) on residual sugar content and extracellular polysaccharide content

In the formulation optimization of *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu, the selection of extracellular polysaccharide content and residual sugar amount as indicators is of great significance. Extracellular polysaccharide is a functional component produced by microbial metabolism, with antioxidant [34], immune regulation [35] and other biological activities, which directly affect the efficacy and quality of Jiaosu. The content of residual sugar reflects the efficiency of substrate utilization, and low residual sugar indicates that the raw material is fully utilized, which can avoid the contamination of stray bacteria and waste of resources caused by residual sugar, and the combination of the two can comprehensively assess the fermentation effect and product quality.

In the fermentation process, the impact of the inoculum amount on microbial growth and product formation is more complex. Although an increased inoculum amount is conducive to the output of the product, but too much will lead to rapid bacterial growth, nutrient loss, premature death, increased viscosity in the fermenter, and a decline in fermentation ability, affecting the output rate of lactic acid production and the active ingredient [36].

As shown in Figure 1A, the extracellular polysaccharide content of the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu exhibited an initial increase followed by a decline with rising inoculum levels, whereas residual sugar displayed an inverse trend. Higher the extracellular polysaccharides content correlates with enhanced antioxidant and immunomodulatory activities, while lower residual sugar indicates improved substrate utilization efficiency in the fermentation system. Experimental results showed that when the inoculum amount was 4%, the extracellular polysaccharide content was 0.35 ± 0.01 mg/mL, and the residual sugar amount was 10.00 ± 0.01 mg/mL. When the inoculum reached 6%, the extracellular polysaccharide production peaked at 0.75 mg/mL, showing significant differences from other groups, while residual sugar decreased to 8.20 mg/mL. Considering the commonly used inoculum levels for microbial fermentation ranging from 5% to 10% [29], these three inoculum volumes (6%, 7%, and 8%) were selected as the experimental factors for orthogonal optimization of the fermentation process.

As shown in Figure 1B, the substrate concentration had a significant effect on the fermentation process of the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu. With the increase of substrate concentration from 4% to 12%, the extracellular polysaccharide production first increased and then decreased, reaching the maximum value at 8% substrate concentration, while the amount of residual sugar reached its minimum value at 8% substrate concentration. Under moderate substrate concentration, microorganisms were able to exhibit optimal substrate conversion efficiency via balanced catabolic-anabolic pathways for growth and metabolite synthesis. When the substrate concentration increased to 12%, an excess of substrate may have occurred, leading to increased osmotic pressure, inhibiting the growth and metabolic activity of microorganisms, and thus resulting in a decrease in polysaccharide production. In addition, too high a substrate concentration may lead to a substrate inhibition effect, causing the residual sugar amount to increase. Based on the above experimental results and considering factors such as substrate utilization efficiency and product yield, three levels (7%, 8%, and 9%) were selected for the subsequent orthogonal experimental design in this study.

The strain ratio plays a key regulatory role in the fermentation system. Among the combinations of *S. thermophilus*, *L. bulgaricus*, *B. adolescentis*, and *L. acidophilus* involved in the present study, when the ratio was 1:1:1:2, the fermentation effect was relatively optimal, which was manifested by the high content of extracellular polysaccharides and the low amount of residual sugar (Figure 1C). This optimized effect may be related to the synergistic effect among different strains. The increase in the ratio of *L. acidophilus* may promote the growth and metabolic activity of other strains, thereby increasing the efficiency of substrate utilization and product yield [37]. Based on this, three levels (1:1:1:2, 2:1:1:1, and 1:1:2:1) were selected for the orthogonal test.

The experiment on fermentation time is shown in Figure 1D. The results showed that the residual sugar amount reached its lowest value (7.50 mg/mL) on the 6th day and then gradually increased as fermentation time extended. The extracellular polysaccharide production peaked (0.60 mg/mL) on the 6th day and then slightly decreased on the 7th day, indicating that the fermentation was most effective at this time. The residual sugar amount was highest on the 9th day, and the extracellular polysaccharide content was highest on the 6th day. During the fermentation of blueberry Jiaosu [38], the effect of fermentation time on the total phenol content was significant, with the total phenol content reaching a peak at 60 h and then gradually decreasing. This was similar to the trend of the effect of fermentation time on residual sugar and extracellular polysaccharide in this experiment. This trend may reflect the dynamic balance of microbial growth and metabolism during fermentation: in the early stage (0-6 days), the microorganisms were in the logarithmic growth phase, and extracellular polysaccharide synthesis was active. In the late stage (>6 days), due to nutrient depletion and metabolite accumulation, the microorganisms gradually entered the decline phase, resulting in a decrease in product synthesis rate [39]. Therefore, three levels (6 days, 7 days, and 8 days) were selected for orthogonal tests.

3.2. The Results of the Orthogonal Test

According to Table 2, using the extracellular polysaccharide content of the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu as an indicator, the influence of each factor, in descending order, is B>C>A>D. Based on this, the optimal program was determined to be $A_3B_3C_2D_1$: 8% inoculum, a strain ratio (*S. thermophilus*: *L. bulgaricus*: *B. adolescentis*: *L. acidophilus* = 1:1:1:2), 9% substrate concentration, and a 6-day fermentation time. Under these conditions, the extracellular polysaccharide content reached 0.67 ± 0.01 mg/mL. This contrasts with the parameters used by Ge R. et al. [29], where cinnamon Jiaosu was produced with a 9% substrate concentration, 9% inoculum, a strain ratio (*S. thermophilus*: *L. bulgaricus*: *B. adolescentis*: *L. acidophilus* = 1:1:2:1), and 8-day fermentation, yielding 1.11 mg/mL extracellular polysaccharides.

The reason for this is that the differences in the raw material composition of *L. ruthenicum*, *C. lacryma-jobi*, and cinnamon make the types and contents of available carbon and nitrogen sources different in the fermentation process, together with the differences in the metabolic pathways and synergistic effects of the strains, which jointly affect the synthesis efficiency of extracellular polysaccharides.

In summary, the process protocol identified in this study demonstrated good extracellular polysaccharide output in the production of composite Jiaosu, although it was different from the parameters and results of cinnamon Jiaosu, which reflects the uniqueness and complexity of different raw materials and process conditions in Jiaosu production. In the future, we can further expand the scope of research, such as adjusting the nutrient supplementation during fermentation to further enhance the extracellular polysaccharide production and promote the development and application of related Jiaosu products.

From the ANOVA results in Table 3, factors A, B, C, and D were significant at the α=0.05 level, indicating that substrate concentration and strain ratio were the key factors affecting extracellular polysaccharide production, while inoculum amount and fermentation time played an important role in regulating the growth and metabolic processes of microorganisms.

3.3. Determination of the Flavonoid Content of the *L. ruthenicum* and *C. lacryma-jobi* Composite Jiaosu

The standard curve for rutin was established by UV-Vis spectrophotometry. The standard curve was plotted with the mass concentration of rutin standard (mg/mL) as the horizontal coordinate (x) and the absorbance value as the vertical coordinate (y). The linear regression equation obtained was $y = 0.87x + 0.02$ ($R^2 = 0.99$). This indicated a good linear relationship within the determined concentration range.

After a 10-fold dilution of the sample solution, the absorbance value was measured at 510 nm and substituted into the standard curve regression equation for calculation. The result showed that the flavonoid content was 0.10 ± 0.00 mg/mL. Compared with the results of Wei Xueqin et al. [40] on *L. ruthenicum* and Jujube composite Jiaosu (flavonoid content of 1.40 mg/mL), the flavonoid content of the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu in this study was relatively low. This difference may be mainly attributed to the following factors: first, in terms of raw material composition, they used a mixture of *L. ruthenicum*, brown sugar, and water (mass ratio 1:3:10), whereas *L. ruthenicum* and *C. lacryma-jobi* (mass ratio 1:1) were used as the fermentation substrate in this study. Studies have shown that saccharides can be used as a high-quality carbon source for microbial fermentation and promote the biotransformation of flavonoids [41]. Secondly, in terms of fermentation process parameters, they used a fermentation cycle of up to 12 months, whereas only 6 days of fermentation was performed in this study. The longer fermentation time favored the accumulation of microbial metabolites and the full release of flavonoids [42]. The synergistic effect of these factors may be the main reason for the difference in flavonoid content between the two studies. However, in this study, a fermentation time of 6 days was chosen to optimize the fermentation conditions in a shorter timeframe to achieve relatively high polysaccharide yields, which is more practical for industrial applications.

Table 2. Orthogonal test $L_9(3^4)$

Test number	Factor				Extracellular polysaccharide content/(mg/mL)
	A Inoculation amount/%	B Fermentation substrate concentration/%	C Strain ratio	D Fermentation time/days	
1	1	1	1	1	0.40 ± 0.23^{d}
2	1	2	2	2	0.29 ± 0.23^{e}
3	1	3	3	3	0.47 ± 0.01^{c}
4	2	1	2	3	0.39 ± 0.02^{d}
5	2	2	3	1	0.31 ± 0.02^{e}
6	2	3	1	2	0.25 ± 0.02^{f}
7	3	1	3	2	0.51 ± 0.02^{b}
8	3	2	1	3	0.20 ± 0.01^{g}
9	3	3	2	1	0.67 ± 0.01^{a}
k_1	0.39	0.43	0.29	0.46	
k_2	0.32	0.27	0.45	0.35	
k_3	0.46	0.46	0.43	0.36	
R	0.14	0.20	0.17	0.11	
Optimal level	A_3	B_3	C_2	D_1	

Table 3. Analysis of variance table for the orthogonal test (α=0.05)

Factor	Sum of squares of deviations	Degrees of freedom	Mean square	F	Significance
A	0.09	2	0.04	176.70	*
B	0.20	2	0.10	396.90	*
C	0.15	2	0.07	291.90	*
D	0.07	2	0.04	138.90	*
Error	0.01	18	0.00		

a. ($R^2 = 0.99$)

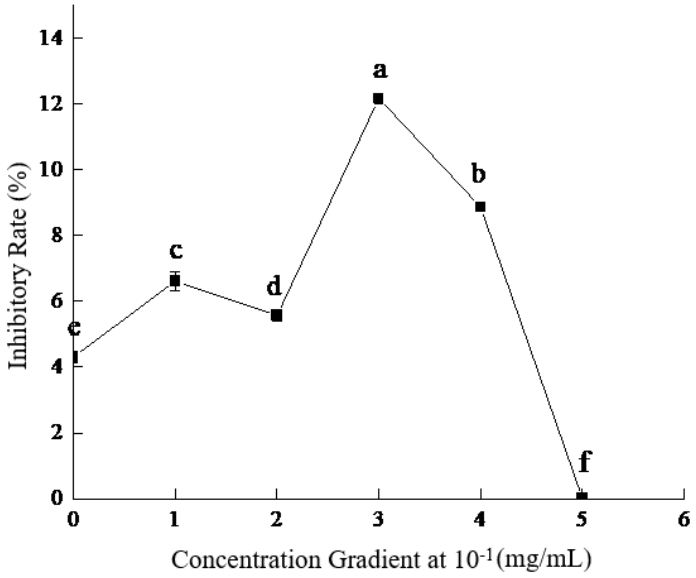

Figure 2. Antitumor ability of the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu

3.4. Determination of the Antitumor Ability Against Lung Cancer Cells

As shown in Figure 2, the inhibitory ability of the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu against A-549 cells at different concentrations exhibited an overall trend of first increasing and then decreasing. At the concentration of 10^{-3} mg/mL, the inhibition rate of A-549 cells reached a peak of 12.17%, showing a significant difference compared to the other test groups ($p<0.05$). This indicates that the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu exhibited the strongest anti-tumor activity at this concentration. This phenomenon may result from the synergistic effect of the active ingredients in *L. ruthenicum* and *C. lacryma-jobi.*

First of all, the polysaccharide LBP3 of LBP exerts antitumor effects by inhibiting the IRE1α -XBP1 pathway of ER stress and enhancing the function of tumor-associated dendritic cells [43]. Meanwhile, *C. lacryma-jobi* extract combined with sorafenib enhanced apoptosis in HCT116 and HepG2 cells [44]. Existing pharmacological studies further support that *C. lacryma-jobi* has many pharmacological effects and is a natural antitumor agent [45].

In addition to the above identified active components, other functional components in *L. ruthenicum* and *C. lacryma-jobi* may also be involved in the antitumour process. For example, anthocyanins inhibit the progression of lung adenocarcinoma by down-regulating TP53I3 and inhibiting the PI3K/AKT/mTOR pathway [46]. *C. lacryma-jobi* oil induces apoptosis in PANC-1 PC cells by regulating mitochondrial dysfunction and related apoptotic molecules via PTEN [47]. Moreover, the microbial fermentation process may further enhance the bioavailability and synergistic effects of the components by degrading the cell wall, releasing the bound state active ingredients, or generating new substances (e.g., short-chain fatty acids) [48]. At a concentration of 10^{-5} mg/mL, the inhibitory ability of Jiaosu on A-549 cells was close to zero, a phenomenon that may be related to the fact that the active ingredients were not sufficient to trigger the signaling pathway cascade response at low concentrations or were limited by the permeability efficiency of the cell membrane.

In conclusion, the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu exhibited the most significant inhibitory effect on A-549 cells at specific concentrations, particularly at 10^{-3} mg/mL. The results provide a scientific basis for the potential antitumor applications of the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu. This anti-tumor effect may depend on the joint action of multiple material components of Jiaosu products, and the specific mechanism of action remains to be studied in depth.

4. Conclusion

In this study, *L. ruthenicum* and *C. lacryma-jobi* were used as composite raw materials, which were pre-treated and fermented with selected probiotic strains. The process parameters were optimized through one-way and orthogonal tests, the optimal fermentation conditions were determined, and the anti-tumor activity and total flavonoid content of the composite Jiaosu were evaluated. The results showed that the optimal fermentation process for the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu included a strain ratio (*S. thermophilus*: *L. bulgaricus*: *B. adolescentis*: *L. acidophilus* = 1:1:1:2, mass ratio), an inoculum amount of 8%, a fermentation time of 6 days, and a substrate concentration of 9%. Under these conditions, the extracellular polysaccharide content of the composite Jiaosu was 0.67 mg/mL, and the flavonoid content was 0.10 mg/mL. The MTT colorimetric assay analysis of A-549 cell survival rates showed that the composite Jiaosu exhibited optimal antitumor activity at a concentration of 10^{-3} mg/mL, with an inhibition rate of 12.17%.

This study not only provides a scientific basis for the development of the *L. ruthenicum* and *C. lacryma-jobi* composite Jiaosu but also lays a theoretical foundation for the further investigation of its anti-tumor action mechanism. Additionally, it serves as an important reference for the development of related functional products.

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Statement of Competing Interests

The authors declare that there are no known financial interests, personal relationships, business or financial relationships that could influence the results of the study and be considered a potential conflict of interest.

List of Abbreviations

ANOVA, One-way analysis of variance; DMSO, dimethyl sulfoxide; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide; A-549 cells, human A-549 non-small cell lung cancer cells.

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