# Food & Function

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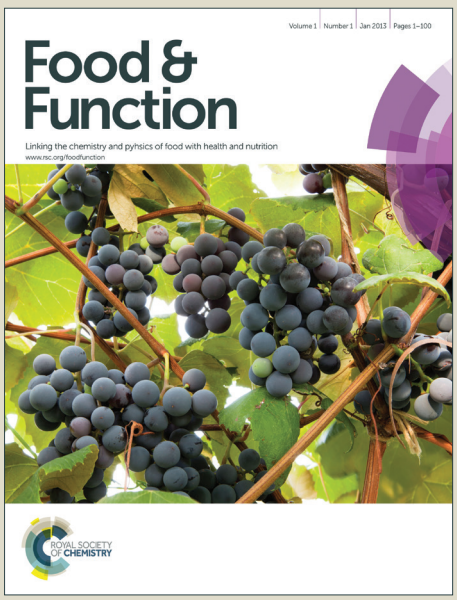


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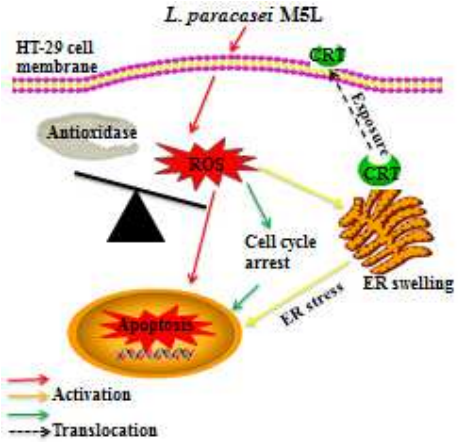


***L. paracasei* M5L induces cell cycle arrest and calreticulin translocation via the generation of ROS in HT-29 cell apoptosis**

Food & Function

ARTICLE

# *Lactobacillus paracasei* supsp. *paracasei* M5L induces cell cycle arrest and calreticulin translocation via the generation of reactive oxygen species in HT-29 cell apoptosis

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*Lactobacillus paracasei* supsp. *paracasei* M5L (*L. paracasei* M5L) was isolated and co-cultured with HT-29 colon cancer cells to study its anti-colorectal cancer effects and mechanism. Using the MTT assay we found that *L. paracasei* M5L significantly inhibits HT-29 cell proliferation. Morphological and biochemical characteristics of apoptosis were observed and confirmed by hematoxylin and eosin (HE) staining and transmission electron microscopy (TEM). Lactobacillus could change the cell cycle distribution and induce calreticulin (CRT) translocation from the endoplasmic reticulum to the surface of the cytomembrane. We also determine that vast reactive oxygen species (ROS) were generated, while the activities of the superoxide dismutase (SOD) and catalase (CAT) were noticeably diminished following *L. paracasei* M5L treatment. This study reveals that *L. paracasei* M5L induces apoptosis in HT-29 cells through ROS generation followed by CRT accompanied endoplasmic reticulum (ER) stress and S phase arrest. These results provide new insights into the possible molecular mechanism of *L. paracasei* M5L as a novel probiotic with the potential for further application.

## 1. Introduction

Colorectal cancer is one of the most common human malignant gastrointestinal cancers, and it continues to be a major healthcare concern worldwide, with its incidence increasing annually.[1-3] Many researchers have reported that probiotics can reduce or inhibit the growth of colon cancer cells by several ways, including lowering the risk of cancer,[4,5] stimulating the host's immune system,[6,7] and preventing infection by pathogenic microorganisms.[8] Lactic acid bacteria (LAB) are a group of bacteria that are widely applied to dairy and meat products. They are commonly used in foods and coupled with their long historical applications worldwide, are considered to be safe bacteria with a 'generally regarded as safe' (GRAS) status for human consumption.[9] The organic acids, including lactic acid, produced by these bacteria may act as natural preservatives and flavor enhancers. Moreover, in recent years, many studies have been carried out on LAB strains with inherent functional properties such as antitumor functions.[10] The protective properties of these bacteria were attributed to different mechanisms including binding of genotoxic carcinogens,[11] their impact on the proliferation and differentiation of cancer cells,[12] interactions with the immune system,[13] reduction of the formation of secondary bile acids[14] and protection against DNA damage[15]. Another important mechanism could be the detoxification of reactive oxygen species (ROS),[16,17] which appears to be the most important aspect considering their different physiological features. Lactobacilli could protect the human body from aging,[18] inflammation,[18] and even reducing cancer risk.[19] Several hypotheses concerning the impact of diet on the incidence of human cancer have been developed, and it has been postulated that ROS formation plays an important role in tumorigenesis.[20] In *vitro* studies have shown that LAB strains possess antioxidant properties and inactivate ROS via enzymatic and non-enzymatic mechanisms.[21, 22] Human and animal studies have demonstrated that strains that inactivate ROS are resistant to radicals and decrease the biochemical parameters of oxidative stress.[23,24] The therapeutic success observed in patients with inflammatory bowel diseases (which are characterized by increased endogenous ROS formation) treated with these strains can be taken as an indication for the antioxidant effects in humans.[25] Studies have also confirmed that during apoptosis, apoptotic tumor cells are highly immunogenic and capable of activating dendritic cells (DCs) in an inflammatory or stressful microenvironment,[26] thus eliciting tumor-specific immunity.[27] It has also found the upregulation and surface translocation of calreticulin (CRT) are likely involved in the enhanced immunogenicity of stressed apoptotic tumor cells.[28] More research into the immunogenic sequences of apoptotic tumor cells is needed to provide new strategies for cancer therapy.

Traditional Chinese fermented foods are made by the local population in many regions throughout northwestern China. The traditional style of fermentation has been maintained because of the specific plateau climate. Due to their unique fermentation styles, these products could be a valuable source of native lactobacilli.[29] There are several reports regarding immunomodulating activities of LAB.[30] Our research group has confirmed that *Lactobacillus paracasei* supsp. *paracasei* M5L (*L. paracasei* M5L) displays strong antibacterial activities towards pathogenic[31] and live bacteria specifically exhibiting immunomodulating activities at the cell wall and genomic DNA.[32] However, to the best of our knowledge, little

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work has examined these properties in active and inactive LAB strains. Additionally, no studies have been completed to identify the antitumor mechanisms of these organisms. Thus, in this study, we aimed to identify the *in vitro* antitumor activities of *L. paracasei* M5L, already knowing their application in fermented dairy products.

## 2. Materials and Methods

### 2.1 Bacterial strains and culture condition

The strain *L. paracasei* M5L was isolated from kumiss made by local households in Sinkiang. It was identified as *L. paracasei subp. paracasei* according to its conservative and polymorphous 16S rDNA.[32] PCR products were sent to the Sangon Biotech Co., Ltd (Shanghai, China) for sequencing of the 16S regions. The strain was cultured in de Man, Rogosa, and Sharpe (MRS) broth at 37 ℃ and stored at 4 ℃. The strain was subcultured twice at 37 ℃ for 18 h before use. The colony forming units (cfu/mL) of the strains were determined by plating a 10-fold serial dilution on MRS-agar.

### 2.2 Cell line and materials

Human colon cancer HT-29 cells were obtained from the Cancer Institute of the Chinese Academy of Medical Science. The cells were cultured in RPMI-1640 medium (Thermo Scientific Hyclone, Shanghai, China) supplemented with 10% fetal bovine serum (Thermo Scientific Hyclone, Shanghai, China), penicillin (100 IU/mL), and streptomycin (100 lg/mL) (Gibco BRL, Life Technologies, Grand Island, NY) in a humidified atmosphere with a 5% carbon dioxide incubator (HEPA class 100, Thermo Scientific HyClone, USA) at 37 ℃.

### 2.3 Preparation of live bacteria and inactive lactobacillus

After an 18h incubation, the *L. paracasei* M5L cells from the lactobacilli cultures were harvested by centrifugation (10,000 g, 15 min, 4 ℃), washed twice with PBS (pH 7.2) and resuspended in RPMI 1640 medium. The lactobacilli were obtained after adjusting concentration to $10^9$ cfu/mL. To acquire the inactive lactobacilli, the cells were killed by heat treatment at 100 ℃ for 15 min.

### 2.4 Cell proliferation assay

MTT assay was used to assess the viability of HT-29 cells. Briefly, cells were seeded in 96-well culture plates at a density of $1\times10^5$ cells/mL in RPMI 1640 culture medium that contained 10% FBS and then incubated at 37 ℃ under 5% $CO_2$. After 24h incubation, the cells were exposed to various infection ratios (1:1, 5:1, 10:1, 50:1 and 100:1) of active or inactive lactobacilli for 24, 48 or 72 h, respectively. MTT solution (0.5 mg/mL in media) was added and the plates were then incubated for 4 h at 37 ℃. After washing, the formazan dye precipitates, which are proportional to the umber of live cells, were dissolved in 150 uL DMSO. The absorbance at 490 nm was then read using an enzyme-linked immunosorbent assay plate reader (Bio-Rad-500, Bio-Rad Laboratories In, USA).

### 2.5 HE staining of HT-29 cells treated with *L. paracasei* M5L

Active or inactive lactobacilli were added to HT-29 cells at an infection factor of 100:1 24, 48 and 72 h, respectively, and then $4\times10^5$ cells were washed with PBS (pH 7.3) and fixed. The fixed cells were immersed in hematoxylin for 10 min and then washed with distilled water. The cells were immersed in eosin for 3 min and washed with varying grades of ethanol. Cell morphology was determined by microscopy.

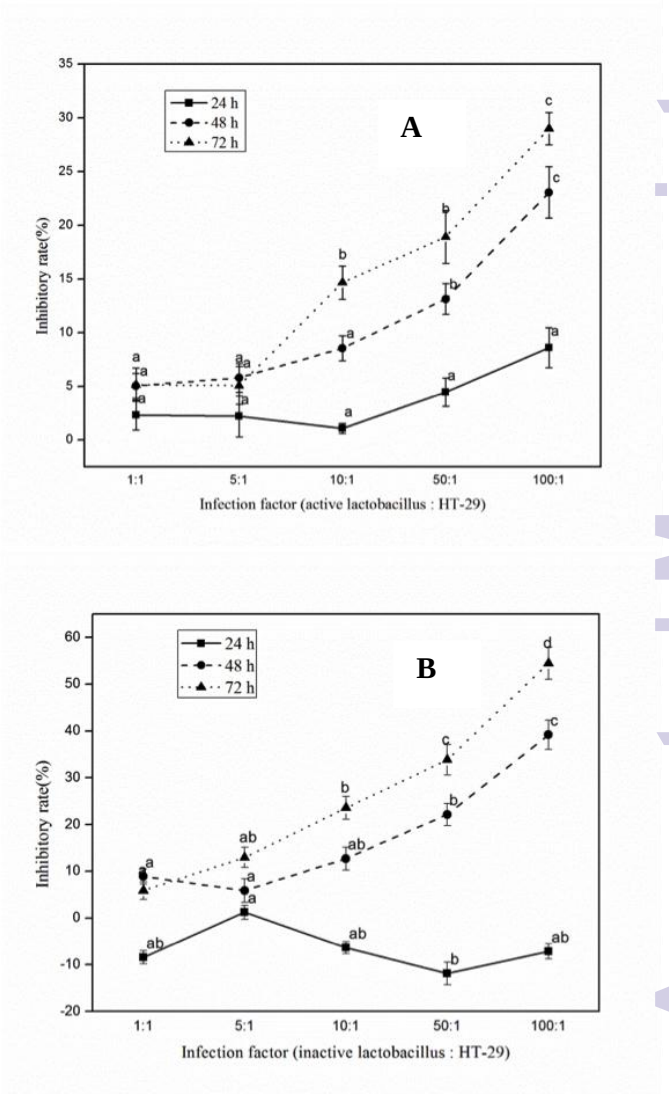



**Fig.1** Growth inhibition of cultures of cells treated with active lactobacilli (A) or inactive lactobacilli (B). Under the various infection factors (1:1, 5:1, 10:1, 50:1 and 100:1) of lactobacilli, HT-29 cells were incubated for 24 h, 48 h, 72 h, respectively. Cultured cells without treatment of bacteria for each time intervals were used as control. Results represented the mean ± SD of triplicate cultures and were expressed as percentage of control. Error bars represented the standard deviation.

### 2.6 Transmission electron microscopy for cell morphology

After 72 h of treatment at an infection factor of 100:1, HT-29 cells ($1\times10^6$ cells) were harvested by trypsinization, washed twice with PBS, fixed in 2.5% glutaraldehyde for 90 min at room temperature, and post-fixed in 1% osmium tetroxide for 30 min. After washing with PBS, the cells were progressively dehydrated in ascending grades of ethanol solutions (50, 70, 95 and 100%), and embedded in epon 812 resins. The blocks were cut into ultra-thin sections with a microtome, and were then stained with saturated uranyl acetate and lead citrate. The ultrastructure of the cells was then examined with a transmission electron microscope (JEM-I230, Jeol, Japan).

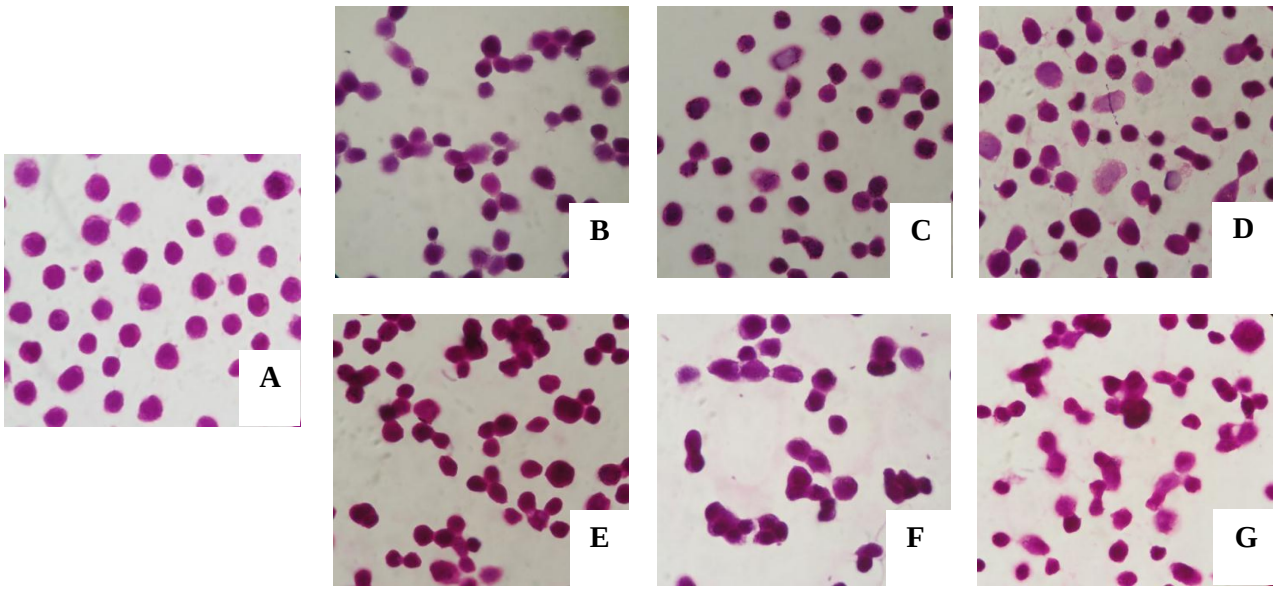



**Fig.2** Morphology changes of HT-29 cells effected by active or inactive lactobacilli evaluated by HE staining. HT-29 cells were cultured with 100:1 infection factor of active or inactive lactobacilli for 24 h (B, C), 48 h (D, E) or 72 h (F, G), respectively. Untreated cells were used as control (A). After treatment, cells were then stained with hematoxylin and eosin. Cell morphology was determined by microscopy (magnification, ×400).

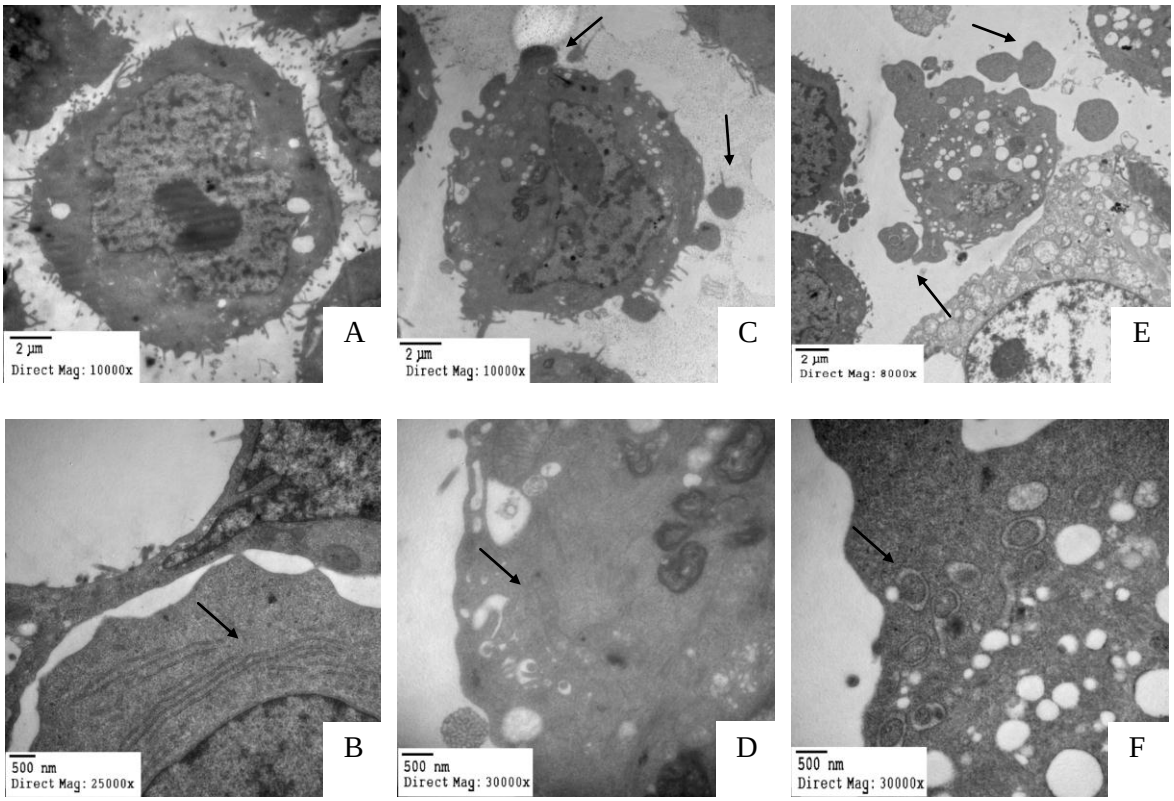



**Fig.3** Effect of active or inactive lactobacilli on morphology of HT-29 cells and ER stress using transmission electron microscope. HT-29 cells were treated with 100:1 infection factor of active lactobacilli (C, D) or inactive lactobacilli (E, F) for 72 h, untreated cells were used as control (A, B). Typical apoptotic morphology and endoplasmic reticulum damage as indicated by the arrow were observed.

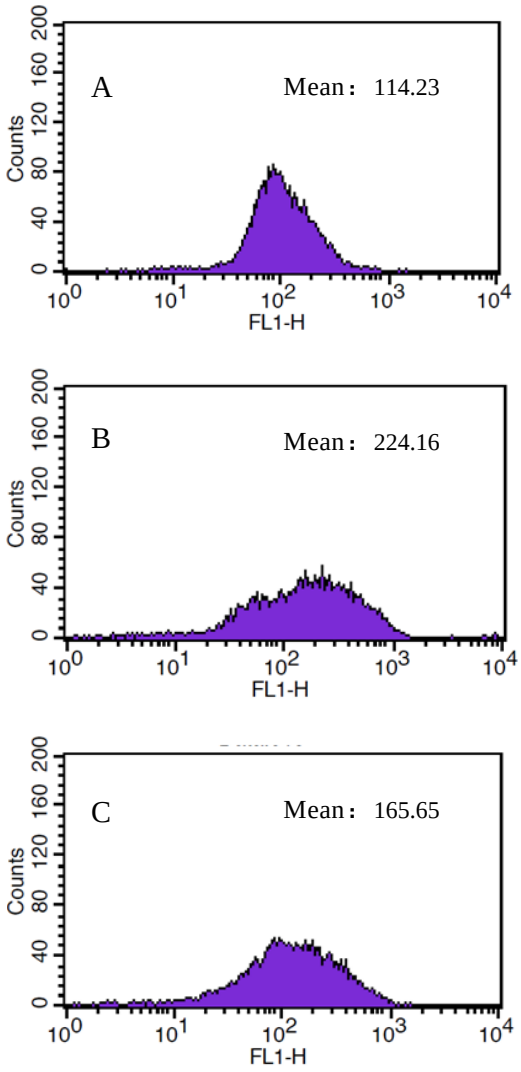



**Fig.4** Effect of lactobacilli on the fluorescence intensity of CRT in HT-29. HT-29 cells were treated with 100:1 infection factor of active lactobacillus (B) or inactive lactobacillus (C) for 72 h; Untreated cells were used as control (A). After staining with anti-CRT antibody, the fluorescence intensity of surface CRT was determined by flow cytometry.

### 2.7 Fluorescence detection of cell surface CRT

*L. paracasei* M5L-treated HT-29 cells (active or inactive lactobacilli : HT-29 ratio of 100:1, 72h) were harvested after centrifugation(1000 rpm, 5 min), and then washed three times with cold PBS before fixing and closing with 75% ethanol and 1% BSA, respectively. The cells were then incubated with a 1:200 dilution of rabbit anti-mouse CRT antibody (Shanghai Biyuntian Biological Co.,Ltd., Shanghai, China) at 25 ℃ for 2 h. The cells were washed another three times with PBS. A 1:50 dilution of FITC conjugated goat anti-rabbit lgG secondary antibody was used and incubated in dark place for 30min. Finally, the HT-29 cells were washed three times and analyzed for surface CRT with a FACS calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

### 2.8 Flow cytometry analysis

Cell cycle phase was analyzed via flow cytometry. HT-29 cells were seeded at a density of $5\times10^5$ cells/mL in 6-well plates, and cultured for 24 h in RPMI-1640. After culturing, the cells were treated with active or inactive lactobacilli (100:1) for 72h. The cells were collected and centrifuged at 10,000 rpm for 5 min, and 300 μL of cold PBS was added to the pellets. The cells were fixed in ice-cold 70% ethanol and stored at 4℃ overnight. After centrifugation at 13,000 rpm for 5 min, 1 ml of cold PBS was added to the pellets and they were centrifuged at 10,000 rpm for 5 min. Before analysis, the cells were stained with a propidium iodide (PI) solution consisting of 100 μg/mL propidium iodide, 0.2% Triton X-100, and 100 μg/mL RNaseA, followed by incubation at 37℃ for 30 min. After staining, the cells were sorted via flow cytometry and analyzed for cell cycle phase.

### 2.9 Determination of intracellular ROS in HT-29 cells

Intracellular production of ROS was measured using the Reactive Oxygen Species Assay Kit via oxidation of 2', 7'-dichloro fluorescin diacetate (DCFH-DA) to fluorescent 2', 7'-dichlorofluorescin (DCF) according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). HT-29 cells were seeded at a density of $5\times10^5$ cells/mL in a 6-well plate. After treatment with active or inactive lactobacilli (100:1) for 72 h, the HT-29 cells were washed with serum-free 1640 medium three times, incubated with 10μM of DCFH-DA in the dark for 20 min at 37 ℃, and then washed twice with cold PBS. The qualitative analysis of ROS generation was completed using a fluorescent microscope.

### 2.10 Determination of enzyme activities

Cells were incubated with active or inactive lactobacilli for 24, 48 and 72 h, respectively, and lysed in cell lysis buffer. The supernatant was obtained after centrifugation at 12,000 g and collected for the determination of the enzyme activities. The antioxidant activities of superoxide dismutase (SOD) and catalase (CAT) were measured by spectrophotometrical methods. SOD activity was assayed using the xanthine-oxidase method. CAT activity was assayed using the ammonium molybdate method. The commercial kits used in these assays were supplied by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

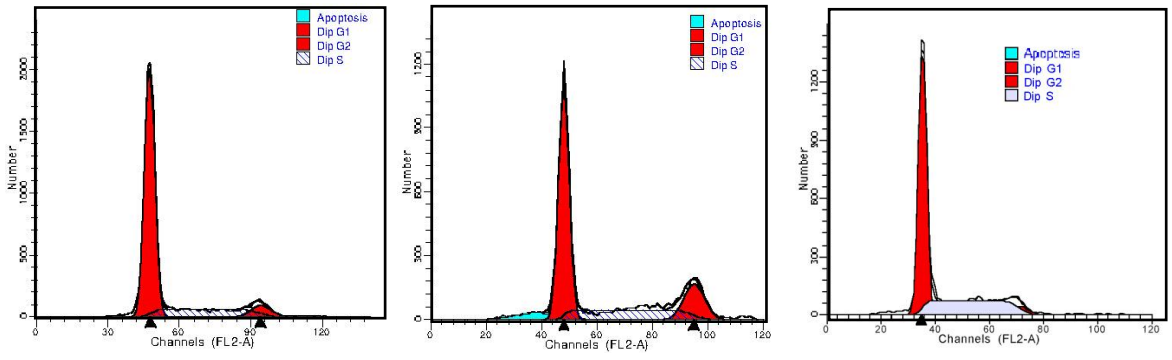
### 2.11 Statistical analysis

All of the experiments were performed in triplicate and all of the results were expressed as the mean ± standard deviation (SD). The observed differences were assessed by one-way analysis of variance (ANOVA), and in all of the comparisons, the differences were considered to be statistically significant at a value of $p < 0.05$.

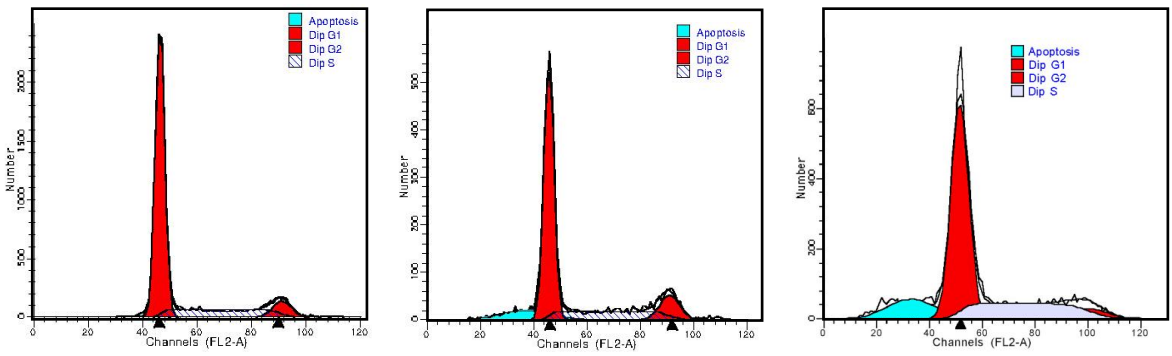
## 3. Results

### 3.1 *L. paracasei* M5L inhibited the proliferation of HT-29 cells in a dose- and time-dependent manner

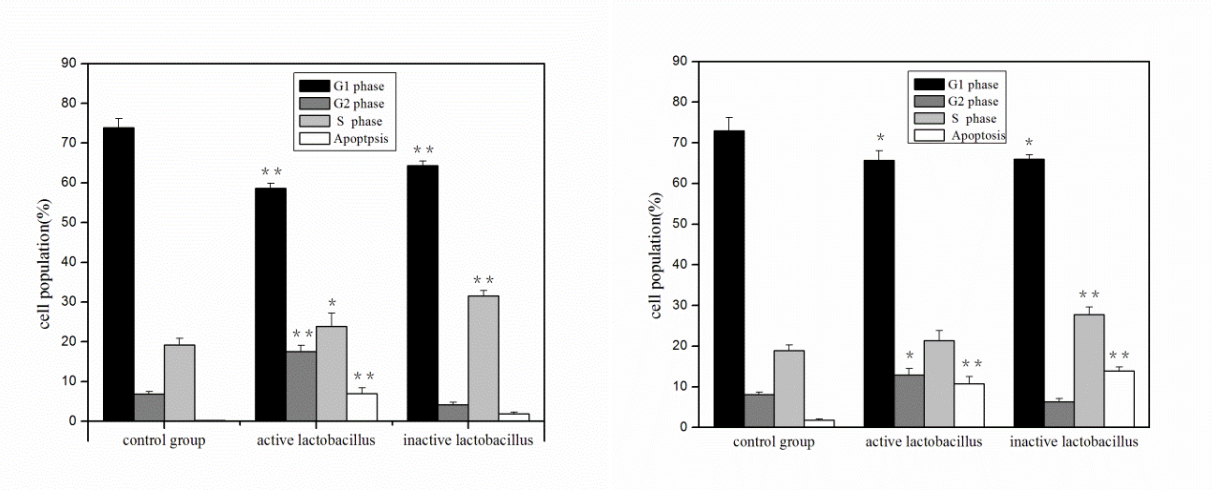
Active or inactive lactobacilli were added to HT-29 cells with an infection ratios of 1:1, 5:1, 10:1, 50:1 or 100:1, respectively. The antiproliferative effect of LAB on HT-29 cells was detected by the MTT assay described in 2.4. As shown in Figs.1A-B, after a 24h incubation, active and inactive lactobacilli displayed almost no inhibitory effects on the HT-29 cells. After 48h, the inhibition rate was higher than after 24h, and as the length of infection increased, the inhibitory rate enhanced. When the infection ratios were 50:1 and 100:1, the cells showed dramatically higher ($p<0.05$) anti-proliferative activities. After 72h of treatment, the results were more




A.48 h control group C. 48h treatment of active lactobacilli E. 48h treatment of inactive lactobacilli




B. 72 h control group D. 72h treatment of active lactobacilli F. 72h treatment of inactive lactobacilli



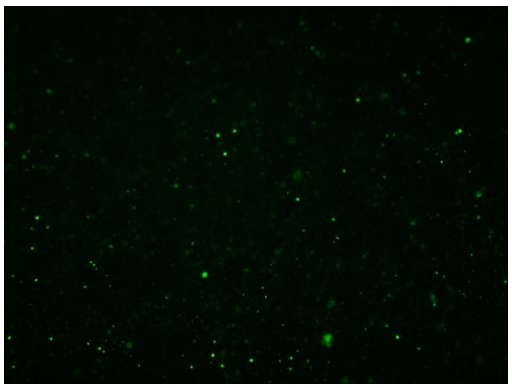

G. 48h-treatment of cell cycle H.72h-treatment of cell cycle

**Fig.5** Effect of active and inactive lactobacillus on cell cycle distribution in HT-29 cells. Results are expressed as mean ± SD (n=3). $^{*}P<0.05$, $^{**}P<0.01$ compared with the control group.

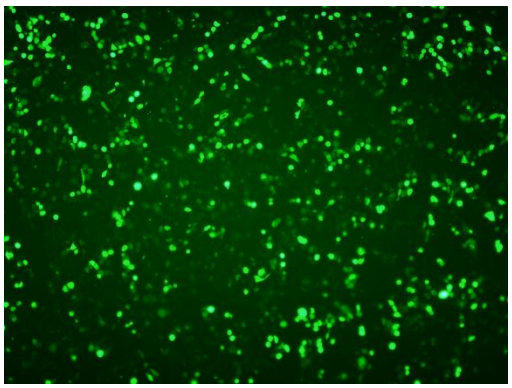
obvious, thus an infection factor of 100:1 was used for the subsequent experiments. In general, compared with the active bacteria, the inactivate bacteria showed a higher inhibition rate, indicating that the inactive bacteria could significantly inhibit the growth of HT-29 cells. In conclusion, the active and inactive *L. paracasei* M5L inhibited the proliferation of HT-29 cells in a dose- and time-dependent manner.

### 3.2 Morphological changes of HT-29 cells

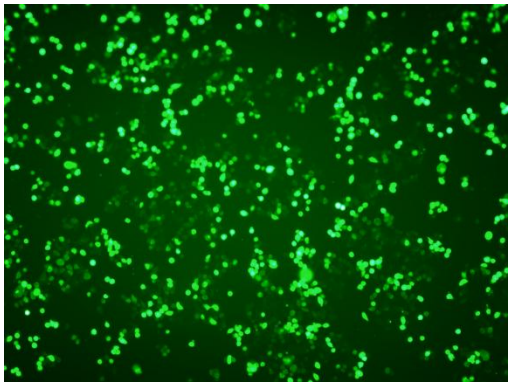
HT-29 cells were treated with active or inactive lactobacilli (100:1 infection ratios) for 24, 48 or 72 h, respectively, as shown in Fig.2 A, HT-29 cells without lactobacilli treatment displayed uniform size, regular and globular shape, and had intact nuclear envelopes. In contrast, after treatment with active or inactive LAB for 24 h (Figs.2 B-C), cell morphology was changed to an elliptical ball or irregular shape. After 48 h of treatment (Fig.2 D-E), more cells showed irregular shape, with some cells getting wider, a few with shrunken or vanished nuclei and several cells adhered to one other. After 72 h of incubation (Fig.2 F-G), more irregularly shaped HT-29 cells



A. Control group



B. Active lactobacillus group



C. Inactive lactobacillus group

**Fig.6** Intracellular ROS generation in HT-29 cells exposed to *L. paracasei* M5L. Fluorescence microscope (100×) of cells treated with 100:1 infection factor of active (B) or inactive (C) *L. paracasei* M5L for 72h.

appeared, the nucleus membrane was less clear in the cells, and some cells became larger in size (approximately two to three times the size of normal cells). HE staining demonstrated that apoptosis was probable following *L. paracasei* M5L treatment of HT-29 cells.

### 3.3 Apoptosis in HT-29 cells detected by transmission electron microscopy

To clarify whether apoptosis was occurring in the HT-29 cells, transmission electron microscopy (TEM) was performed to detect the cells treated with active or inactive *L. paracasei* M5L (100:1) after 72h. As shown in Figs. 3A and B, the HT-29 cells without lactobacilli treatment exhibited normal cytoplasm, organelle and nucleus morphologies. Following treatment with LAB (Fig. 3C), typical apoptotic morphology, including nuclei deformation, chromatin shrinkage, apoptotic bodies, and chromatin leaks were observed. In Fig. 3D, we can see that the endoplasmic reticulum is swelling, its cellular structure was badly damaged, and that the cell surface villus has also decreased. As shown in Figs. 3 E-F, the most prominent morphological changes in the inactive lactobacilli-treated cells were the formation of abundant autophagic vacuoles sequestrating the cytoplasm and organelles. In addition, giant autophagosomes filled with degraded organelles and autolysosomes were frequently observed. In conclusion, TEM was used to detect apoptosis, demonstrating that both active and inactive *L. paracasei* M5L could successfully induce apoptosis in the HT-29 cells and leading to the disappearance of cell surface villus, mitochondria degeneration, endoplasmic reticulum (ER) swelling, functional decline, and damage to organelles.

### 3.4 *L. paracasei* M5L induces CRT exposure on the surface of HT-29 cells

As a $Ca^{2+}$-binding protein, CRT has a high capacity to buffer $Ca^{2+}$ located in the lumen of the ER, also involved in $Ca^{2+}$ homeostasis and the modulation of $Ca^{2+}$ signaling. CRT is a crucial determinant in the phagocytosis in dying tumor cells by macrophages and DCs when bound to the plasma membrane of cells upon apoptosis initiation. In our experiments, CRT in lactobacilli-treated HT-29 cells was determined by flow cytometry. As shown in Fig. 4 A-C, when the cell surface was stained with fluorochrome-conjugated antibodies against CRT, HT-29 cells treated with active or inactive lactobacilli exhibited significant increases in CRT fluorescence compared to the control group, indicating the CRT shift from the ER to the cell surface. Moreover, we can see the active lactobacilli more powerfully enhanced the fluorescence compared with the inactive lactobacilli, with changes in intensity from 114.23 to 224.16 and 165.65, respectively. In conclusion, the CRT membrane surface translocation in lactobacilli-treated apoptotic HT-29 cells illustrate that either active or inactive lactobacilli have the capacity to induce CRT translocation from the ER to the surface of the cytomembrane in HT-29 cells.

### 3.5 Cell cycle analysis in HT-29 cells treated with *L. paracasei* M5L

Suppression of growth in the HT-29 cells by lactobacilli can be explained by cell cycle progression. In this experiment, flow cytometry was used to analyze cell-cycle distribution to determine the possible mechanism responsible for lactobacilli-mediated inhibition of culture growth. Based on Figs. 5 C D G and H, we can see that following treatment with active lactobacilli, the portion of cells in $G_1$ phase significantly decreased, while the portion of cells in $G_2$ phase and S phase increased. Moreover, active lactobacilli could induce apoptosis in the HT-29 cells, and as amount of time increased the effect is more remarkable. We concluded that active lactobacilli inhibit the growth of HT-29 cells by preventing the cells from moving into $G_2$ and S phase.As shown in Figs. 5 E F G and H, after treatment with inactive lactobacilli (100:1) for 48h and 72h, S phase populations increased from 19.23±1.65% and 18.93± 1.32% to 31.53± 1.37% and 27.73± 1.87%($p<0.05$), respectively. During this time, a marked decrease in the amount of cells in the $G_1$ and $G_2$ phase was observed. Moreover,

after 72h of incubation, inactive lactobacilli significantly induced apoptosis in the HT-29 cells, with apoptotic rate of 13.87± 0.95% compared with 1.75± 0.13% in the 0 h cells ($p<0.05$). We can conclude that the increased number of cells in S phase may be associated with inactive lactobacilli-mediated HT-29 cell growth

inhibition. Both the active and inactive lactobacilli could influence cell cycle distribution with inhibition of $G_2$ and S phase and S phase, respectively.

### 3.6 ROS is involved in lactobacillus-induced apoptosis in HT-29 cells

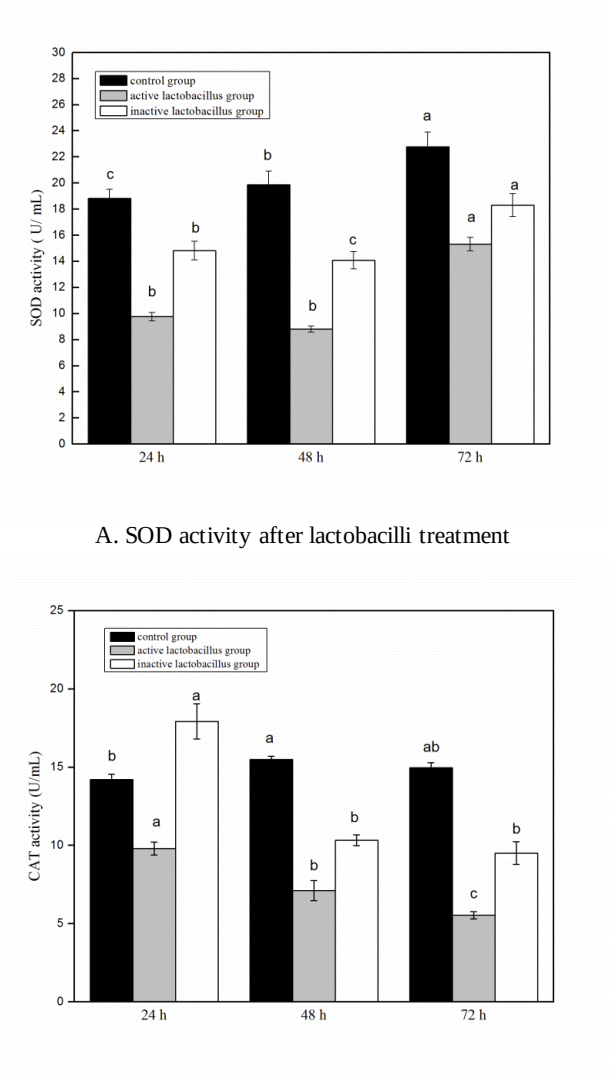
In our study, we used the fluorescent dye DCF-DA to determine intracellular ROS levels, as it is generally considered to play a vital role in apoptosis in various cells types. As shown in Fig. 6, active or inactive *L. paracasei* M5L were added to HT-29 cells with an infection factor of 100:1 for 72 h. We can see that *L. paracasei* M5L significantly induced ROS production in the HT-29 cells compared with the control group. The ROS level of HT-29 cells treated with the inactive *L. paracasei* M5L was higher than those treated with the active *L. paracasei* M5L.

### 3.7 Effect of lactobacillus on the oxidative metabolism balance

The activities of antioxidant enzymes in HT-29 cells following treatment with active or inactive lactobacilli are presented in Fig. 7. As shown in Fig. 7 A, we can see that SOD activities in the lactobacilli-treated groups were lower than in the control groups at each of the time intervals. SOD activity for the control group increased significantly from 24 to 72 h. However SOD activities in the active or inactive lactobacilli-treated group increased significantly only after 72 h of incubation. CAT activity in the control group did not change over time (Fig. 7 B), whereas the CAT activity of the active lactobacilli-treated group was lower than the control group throughout the experiment, and decreased in a time-dependent manner. However, the CAT activity of the cultures after 24 h of incubation with the inactive lactobacilli showed higher values compared with the control group and was lower after 48 and 72 h of incubation. According to these data, both the active and inactive *L. paracasei* M5L can significantly reduce the activity of antioxidant enzymes.

## 4. Discussion

In this study, we investigated the effects of active and inactive lactobacillus from *L. paracasei* M5L on human colon cancer HT-29 cells. The results demonstrated that both the active and inactive bacteria had remarkable inhibitory effects in a time- and dose-dependent manner on the cancer cells (Fig. 1). Typical apoptosis morphological and biochemical changes including irregular and wider cell shape, chromatin shrinkage, nuclei deformation, apoptotic bodies, and especially chromatin leaks were observed by HE staining and transmission electron microscopy (Figs. 2-3). We also identified damage to the endoplasmic reticulum (ER), such as ER swelling, formation of abundant autophagic vacuoles and giant autophagosomes filled with degraded organelles and autolysosomes indicating the occurrence of ER stress (Fig. 3). ER stress (ERS), which is known to trigger cellular adaptive responses via the accumulation of unfolded or misfolded proteins in response to oxidative stress mediated by ROS, is one pathway of launching cell apoptosis.[33] The ER is the major intracellular $Ca^{2+}$ store, and thus cellular calcium homeostasis and relevant calcium signalling pathways are closely related to the status of the ER.[34,35] In our study, CRT, one of the major binding proteins in the ER lumen, shifted from the ER to the cell surface following treatment with *L. paracasei* M5L indicating that CRT is possibly involved in inducing HT-29 apoptosis through ER stress (Fig. 4). We also examined the contribution of the redox system to *L. paracasei* M5L induced HT-29 cell apoptosis. We found that ROS were largely generated during *L. paracasei* M5L induced apoptosis in human HT-29 cells especially when treated with inactive *L. paracasei* M5L (Fig. 6). In




A. SOD activity after lactobacilli treatment

B. CAT activity after lactobacilli treatment

**Fig.7** Effect of lactobacilli on activity of enzymes of SOD and CAT of HT-29 cells. Cells were incubated with active or inactive lactobacilli for 24 h, 48 h and 72 h and lysed by cell lysis buffer. The supernatant was obtained after centrifugation, and SOD and CAT concentration was measured according to the instruction of the kit. (Mean ± SD, n = 3). Bars with no letters in common are significantly different ($p < 0.05$).

contrast, the activities of the antioxidases SOD and CAT noticeably decreased after 48 and 72 h treatment with *L. paracasei* M5L (Fig. 7) compared with the control group, which might be due to the generation of ROS. Active lactic acid bacteria could produce hydrogen peroxide, which would consume the antioxidasea as exogenous free radicals and lead to a decrease in SOD and CAT activities. In this study, we demonstrated lower intracellular ROS levels and lower SOD and CAT enzymatic activities in the active lactobacillus group. Endogenous ROS including superoxide ($O^{-}_2$), hydrogen peroxide ($H_2O_2$), hydroxyl radicals (OH) and singlet oxygen ($^1O_2$)[36] not only play a role in redox regulation during normal physiological functions but are widely recognized as intracellular messengers that regulate the intensity and duration of ER stress and could be possibly be upstream signals in ERS

mediated cell apoptosis.[37-39] Furthermore intracellular ROS may play an important role in the regulation of cell cycle progression by either activating growth factor receptors or influencing phosphorylation and ubiquitination pathways.[40, 41] In this study, an increase of the percentage of HT-29 cells in S phase (Figs. 5 G and H) showed that cell cycle checkpoints are activated, indicating that the cell cycle is arrested at S phase, which blocks DNA synthesis and S to $G_2$ progression. ROS levels are essential for this progression, thus, when intracellular potential is abnormally high, cycling are dephosphorylated and the cell cycle is arrested in S phase leading to apoptosis.[42]

## 5. Conclusion

In summary, this study reveals that *L. paracasei* M5L can induce apoptosis in HT-29 cells through ROS generation followed by CRT accompanied ER stress and S phase arrest. Once generated in cells, endocellular ROS-eliminated antioxidases lead to an imbalance in the redox system. CRT is exposed due to ROS-induced ER stress and the cell cycle is paused causing apoptosis. These results provide new insights into the possible molecular mechanism of *L. paracasei* M5L as a novel probiotic with the potential for further usage.

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## References

1. X. Qu, R. Xie, L. Chen, C. Feng, Y. Zhou, W. Li, H. Huang, X. Jia, J. Lv, Y. He, Y. Du, W. Li, Y. Shi and W. He, *Genomics*, 2013, **104**, 242-248.
2. Y. M. Khayyat and E. M. Ibrahim, *Qatar medical journal*, 2014, **1**, 17-24.
3. J. Chai and M. M. Jamal, *World journal of gastroenterology: WJG*, 2012, **18**, 6521.
4. R. F. Orlando A, *Journal of gastrointestinal cancer*, 2013, **44**, 491.
5. K. Shida and K. Nomoto, *The Indian journal of medical research*, 2013, **138**, 808-814.
6. F. Russo, M. Linsalata and A. Orlando, *World journal of gastroenterology : WJG*, 2014, **20**, 13258-13272.
7. A. T. Borchers, C. Selmi, F. J. Meyers, C. L. Keen and M. E. Gershwin, *Journal of gastroenterology*, 2009, **44**, 26-46.
8. Seema Patel , A. Majumder and A. Goyal, *Indian Journal of Microbiology*, 2012, **52**, 3-12.
9. Sabrina da Silva Sabo, Michele Vitolo, José Manuel Domínguez González and a. R. P. d. S. Oliveira, *Food Research International*, 2014, **64**, 527-536.
10. B. G. Spyropoulos, E. P. Misiakos, C. Fotiadis and C. N. Stoidis, *Digestive diseases and sciences*, 2011, **56**, 285-294.
11. S. Lebeer, J. Vanderleyden and S. C. De Keersmaecker, *Microbiology and Molecular Biology Reviews*, 2008, **72**, 728-764.
12. Y. Tuo, L. Zhang, H. Yi, Y. Zhang, W. Zhang, X. Han, M. Du, Y. Jiao and S. Wang, *Journal of dairy science*, 2010, **93**, 2362-2366.
13. J. Villena, M. L. S. Oliveira, P. C. Ferreira, S. Salva and S. Alvarez, *International immunopharmacology*, 2011, **11**, 1633-1645.
14. H. Jensen, S. Grimmer, K. Naterstad and L. Axelsson, *International journal of food microbiology*, 2012, **153**, 216-222.
15. A. J. Burns and I. R. Rowland, *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 2004, **551**, 233-243.
16. W. Liao, Z. Ning, L. Chen, Q. Wei, E. Yuan, J. Yang and J. Ren, *Journal of agricultural and food chemistry*, 2014, **62**, 8648-8654.
17. W. Liao, Z. Ning, L. Ma, X. Yin, Q. Wei, E. Yuan, J. Yang and J. Ren, *Rejuvenation research*, 2014, **17**, 422-429.
18. H.-S. Lee, S.-Y. Han, E.-A. Bae, C.-S. Huh, Y.-T. Ahn, J.-H. Lee and D.-H. Kim, *International immunopharmacology*, 2008, **8**, 574-580.
19. M. Thirabunyanon, P. Boonprasom and P. Niamsup, *Biotechnology letters*, 2009, **31**, 571-576.
20. F. Weinberg, R. Hamanaka, W. W. Wheaton, S. Weinberg, J. Joseph, M. Lopez, B. Kalyanaraman, G. M. Mutlu, G. S. Budinger and N. S. Chandel, *Proceedings of the National Academy of Sciences*, 2010, **107**, 8788-8793.
21. Alberto Amaretti, Mattia di Nunzio, Anna Pompei, Stefano Raimondi, Maddalena Rossi and a. A. Bordoni, *Applied microbiology and biotechnology*, 2013, **97**, 809-817.
22. Dominici L., Moretti M., Vlilarini M., Vannin S., Cenci G., Zampino C. and a. T. G., *International Journal of Probiotics and Prebiotics*, 2011, **6**, 179-186.
23. M. Bermudez-Brito, J. Plaza-Diaz, S. Munoz-Quezada, C. Gomez-Llorente and A. Gil, *Annals of Nutrition and Metabolism*, 2012, **61**, 160-174.
24. D. Martarelli, M. C. Verdenelli, S. Scuri, M. Cocchioni, S. Silvi, C. Cecchini and P. Pompei, *Current microbiology*, 2011, **62**, 1689-1696.
25. R. M Jones, J. W Mercante and A. S Neish, *Current medicinal chemistry*, 2012, **19**, 1519-1529.
26. H. Feng, Y. Zeng, M. W. Graner and E. Katsanis, *Blood*, 2002, **100**, 4108-4115.
27. H. Feng, Y. Zeng, L. Whitesell and E. Katsanis, *Blood*, 2001, **97**, 3505-3512.
28. H. Feng, Y. Zeng, M. W. Graner, A. Likhacheva and E. Katsanis, *Blood*, 2003, **101**, 245-252.
29. S.-N. Liu, Y. Han and Z.-J. Zhou, *Food Research International*, 2011, **44**, 643-651.
30. S. Salva, J. Villena and S. Alvarez, *International journal of food microbiology*, 2010, **141**, 82-89.
31. Y.-C. Zhang, L.-W. Zhang, M. Du, H.-X. Yi, C.-F. Guo, Y.-F. Tuo, X. Han, J.-Y. Li, L.-L. Zhang and L. Yang, *Microbiological research*, 2011, **167**, 27-31.
32. Y. F. Tuo, L. W. Zhang, X. Han, M. Du, Y. C. Zhang, H. X. Yi, W. Q. Zhang and Y. H. Jiao, *World J Microbiol Biotechnol*, 2011, 505-511.
33. G. S. Hotamisligil, *Cell*, 2010, **140**, 900-917.
34. A. Görlach, P. Klappa and T. Kietzmann, *Antioxidants & redox signaling*, 2006, **8**, 1391-1418.
35. N. Dejeans, N. Tajeddine, R. Beck, J. Verrax, H. Taper, P. Gailly and P. B. Calderon, *Biochemical pharmacology*, 2010, **79**, 1221-1230.
36. D. Gough and T. Cotter, *Cell death & disease*, 2011, **2**, e213.
37. R. Rao, H. Ellerby and D. Bredesen, *Cell Death & Differentiation*, 2004, **11**, 372-380.

38. X. Duan, Y. Zhou, X. Teng, C. Tang and Y. Qi, *Biochemical and biophysical research communications*, 2009, **387**, 694-699.
39. F. Martinon and L. H. Glimcher, *Current opinion in immunology*, 2011, **23**, 35-40.
40. J. Boonstra and J. A. Post, *Gene*, 2004, **337**, 1-13.
41. W.-S. Wu, *Cancer and Metastasis Reviews*, 2006, **25**, 695-705.
42. R. C. Rancourt, P. C. Keng, C. E. Helt and M. A. O'Reilly, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 2001, **280**, L617-L626.