國立嘉義大學生命科學院

學生學術研究成果優良海報評選獲獎名單

時間:107 年 5 月 30 日

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GSK-3β pathway is involved in amphiregulin-induced resistance to vincristine therapy in oral squamous cell carcinoma

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Abstract

Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer. Despite advances in surgery, radiotherapy, and chemotherapy, the overall 5-year survival rate of patients with OSCC have not significantly improved. In addition, the prognosis of OSCC patients with advanced-stage is still poor. Therefore, it is necessary to develop novel therapeutic modalities. Vincristine (VCR), a naturally occurring vinca alkaloid, is a classical microtubule-destabilizing agent and is widely used in the treatment of many cancers. Despite the treatment of VCR proven benefits for antitumor activity, one of the major reasons for the failure of treatment is drug resistance. Drug resistance is a complex process involving reciprocal interplay among different types of cells within tumor microenvironment. Secreted proteins may be responsible for the cross talking among cells to induce drug resistance.

The aim of the present study was to identify which secreted proteins are involved in VCR resistance. Our results show that amphiregulin (AREG) is highly expressed and secreted in VCR resistant cells. Pretreatment of exogenous recombinant AREG markedly increased drug resistance against VCR in OSCC cells. Furthermore, we also investigated whether knockdown or overexpression of AREG in cells would mediate VCR sensitivity. The results indicate AREG indeed contributes to VCR resistance in OSCC cells. Meanwhile, we also demonstrate that GSK-3β pathway might be involved in AREG-induced VCR resistance. These findings may provide rationale to combine VCR with blockade of AREG-related pathways for the effective treatment of OSCC.

Results

1. Cytotoxic effects of VCR on parental (SAS) and VCR-resistant (SAS-VCR) cells.

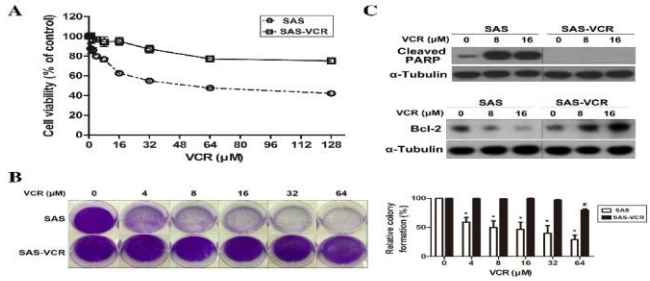


Fig 1. (A) Cells were treated with indicated concentrations of VCR for 48 h and cell viability was measured by MTT assay. (B) The long-term effects of VCR were assessed by colony formation assay. A representative image shows that SAS cells formed fewer colonies compared to SAS-VCR cells. Lower panel is the result of densitometric data of the clonogenic growth of SAS and SAS-VCR cells. (C) Cells were treated with indicated concentration of VCR (0-16 μM) for 24 h and the expression levels of apoptosis-related proteins (cleaved PARP, and Bcl-2) were examined by Western blot. α-tubulin was used as a loading control. The data show the mean ± SEM of at least 3 independent experiments. * P <0.05, compared with the control (0 μM).

2. Growth factors secreted by resistant cells (SAS-VCR) could promote cell resistance to VCR.

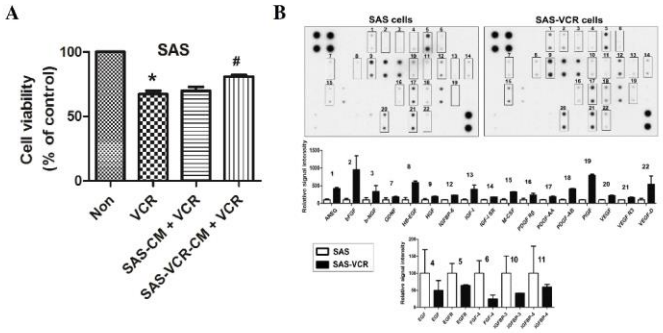


Fig 2. (A) Cells were cultured in complete medium (supplemented with 10% fetal bovine serum) until cells were approximately 80% confluent; at that time the medium was replaced with serum-free medium. After 2 days, the conditioned medium (CM) was collected. SAS cells were activated by CM from SAS and SAS-VCR cells, and then treated with 8μM VCR for 24 h. Cell viability was determined by MTT assay. Graphs show mean ± SEM of three independent experiments. * P < 0.05, compared with untreated group (Non); # P < 0.05, compared with VCR group. (B) Comparison of growth factors in CM from SAS and SAS-VCR cells were performed by a human growth factor antibody array. The marked molecules are significantly changed as indicated by numbers. Lower panel is the result of densitometric data of the pair of duplicate spots representing each marked protein.

3. AREG is highly expressed and secreted in SAS-VCR (resistant cell) line of VCR and involved in the progression of cancer in oral cancer.

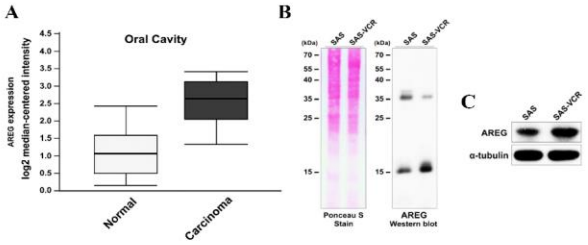


Fig 3. (A) Box plots derived from gene expression data from the Oncomine cancer database (http://www.oncomine.com/) comparing expression of AREG gene in normal (left plot) and carcinoma (right plot) tissue. The fold change is 2.668 and p-value is 6.15E-9. (B) An equal volume of conditioned media (CM) from SAS and SAS-VCR cells was analyzed for secreted AREG by Western blot. Loading quantities were shown on left side by Ponceau S staining. (C) Analysis of AREG levels in total cell lysate of SAS and SAS-VCR were conducted by Western blot. α-Tubulin was used as a loading control.

4. AREG is also involved in VCR resistance in other oral cancer cells (SCC9 cell line).

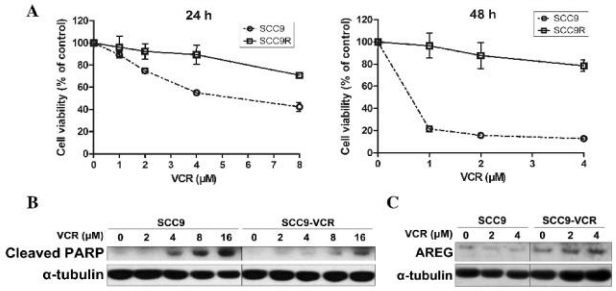


Fig 4. (A) Cells were treated with the indicated concentrations of VCR for 24 and 48 hours and cell viability was measured by MTT assay. (B) Cells were treated with indicated concentration of VCR (0-16 μM) for 24 h and the expression levels of cleaved PARP were examined by Western blot. α-tubulin was used as a loading control. The data show the mean ± SEM of at least 3 independent experiments. * P <0.05, compared with the control (0μM). (C) Cells were treated with indicated concentration of VCR (0-4 μM) for 24 h and the expression levels of AREG were examined by Western blot. α-tubulin was used as a loading control. The data show the mean ± SEM of at least 3 independent experiments. * P <0.05, compared with the control (0μM).

5. AREG is able to promote VCR resistance in oral cancer.

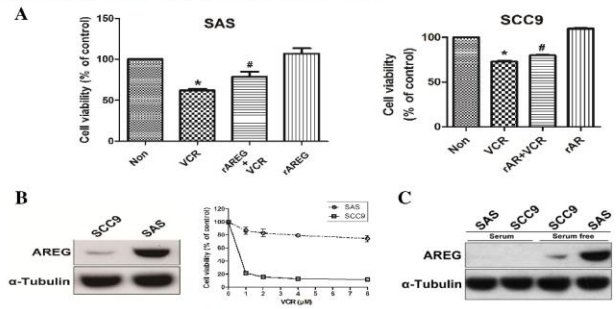


Fig 5. (A) Pretreatment of exogenous recombinant AREG (rAREG) confers VCR resistance in OSCC cells. SAS cells were pretreated with rAREG (50 ng/ml) for 4 h followed stimulation with 16 μM VCR for 48 h. SCC9 cells were pretreated with rAREG (100 ng/ml) for 4 h followed stimulation with 4 μM VCR for 24 h. Cell viability was examined by MTT assay. Results are expressed as mean ± SEM. *P < 0.05 compared with untreated group (Non); #P < 0.05 compared with VCR-treated group. (B) AREG levels of cell lysate from SCC9 and SAS cells were analyzed by Western blot (left panel). Serum-starved SCC9 and SAS cells were exposed to indicated doses of VCR for 24 h and assayed for survival by MTT (right panel). (C) Effects of serum starvation on AREG expression in OSCC cells. Cells were cultured in serum withdrawal medium for 24h.

6. Effects of knockdown or overexpression of AREG on VCR sensitivity in OSCC cells.

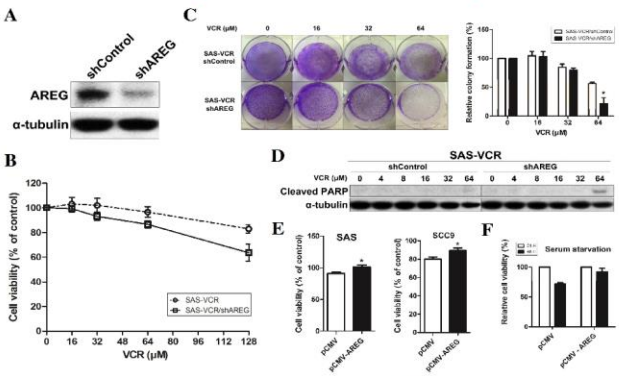


Fig 6. (A) The efficiency of AREG knockdown was verified by Western blot. (B) Cells were exposed to increasing concentrations of VCR for 48 h and subsequently evaluated by MTT assay. *P < 0.05 compared with SAS-VCR/shControl. (C) SAS-VCR silencing AREG and control cells were subjected to colony formation assays. (D) SAS-VCR/shControl and SAS-VCR/shAREG cells were treated with increasing concentrations of VCR for 24 h, and the cleaved PARP was analyzed by Western blot. (E) Overexpression of AREG induces VCR resistance in SAS and SCC9 cells. (F) Effects of overexpression of AREG on serum starvation-induced death were analyzed by MTT assay. *P < 0.05 compared with pCMV control plasmid.

7. GSK-3β signaling pathways might be involved in the effect of AREG on VCR sensitivity.

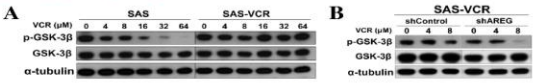
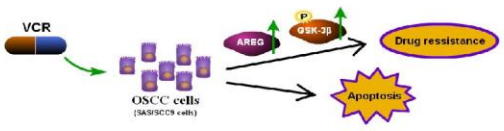


Fig 7. Cells were treated with different concentrations of VCR (0-32μM) for 24 h. The p-GSK3-β was analyzed by Western blot with specific antibodies. The total protein of GSK3-beta is also shown. (A) SAS and SAS-VCR cells (B) SAS-VCR/shControl and SAS-VCR/shAREG cells.

Summary



Selection of acid-fast yeast *Zygosaccharomyces sp.* EVY01 for inoculation in papaya ferment to promote anti-inflammatory and antioxidant capacities

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Introduction:

Fruit and vegetable are consumed as a dietary supplement for the source of nutrition; however, such functional activity might lose during storage, preparation or processing. Inoculation of probiotics is amongst the ways to maintain and increase their nutrition and bioactivity. In order to develop the fruit and vegetable ferments with higher bioactivity for promoting manufacturer competition, this study was firstly aimed to screen the acid-fast yeasts from the aging broth, which were collected from various fruit and vegetable ferments and aged at least for 1 year in factory. Five isolates were obtained by the screening procedure as showed in Fig. 1 and their growth performance in papaya ferments (P) containing yuan zhi powders (Z) were identified (Fig. 2). One of these isolates (YH3) was further subjected to the acid tolerance test in YM broth at pH 5.4, 4.8 or 4.2 (Fig. 3). Furthermore, YH3 was inoculated in 16 different fruit and vegetable fermants at 150 rpm, 30°C for 7 days. Only papaya referment showed yeast counts more than 10^6 CFU/ml (Table. 1) and anti-inflammatory activity during refermentation (data not shown). Finally, the refermentation performance, antioxidant and anti-inflammatory capacity of referments inoculated YH3 or *Rhodosporidium toruloides* (R1) in papaya ferments with or without Z at 30°C for 7 days were identified (Fig. 4).

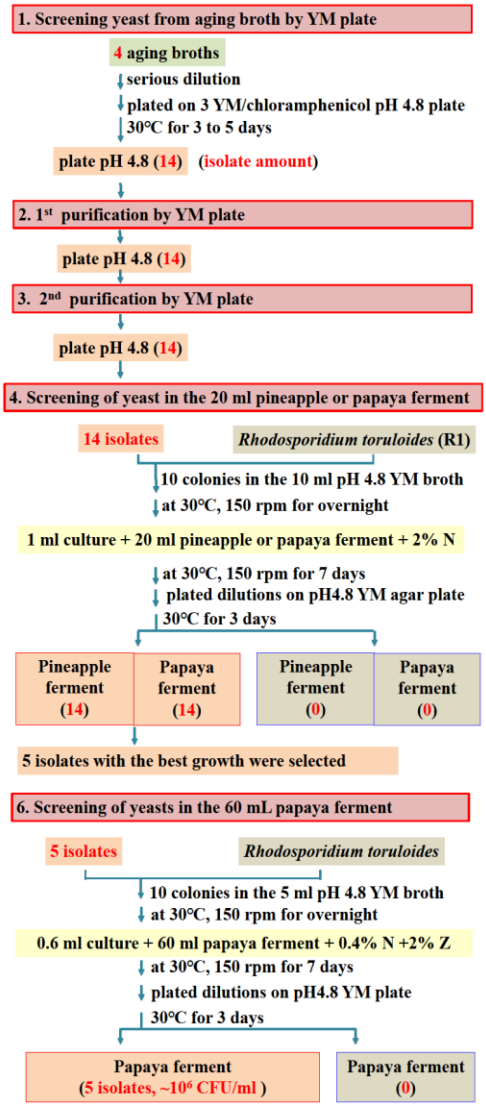

Fig. 1. Screening procedure of acid-fast yeasts from aging broth. N: peptone and yeast extract ; Z: yuan zhi powder .

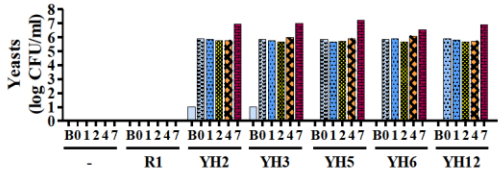

Fig. 2. Yeast counts of referments inoculated with isolates YH2, YH3, YH5, YH6, YH12 and R1 in papaya ferments (60 ml; pH 3.1) supplemented with N (0.2%) and Z (2%) at 30 °C and 150 rpm for 7 days.

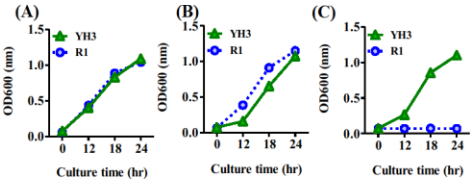

Fig. 3. Growth curves of inoculations of YH3 or R1 in YM medium at pH 5.4 (A), 4.8 (B) or 4.2 (C), 30°C and 150 rpm for 24 hr.

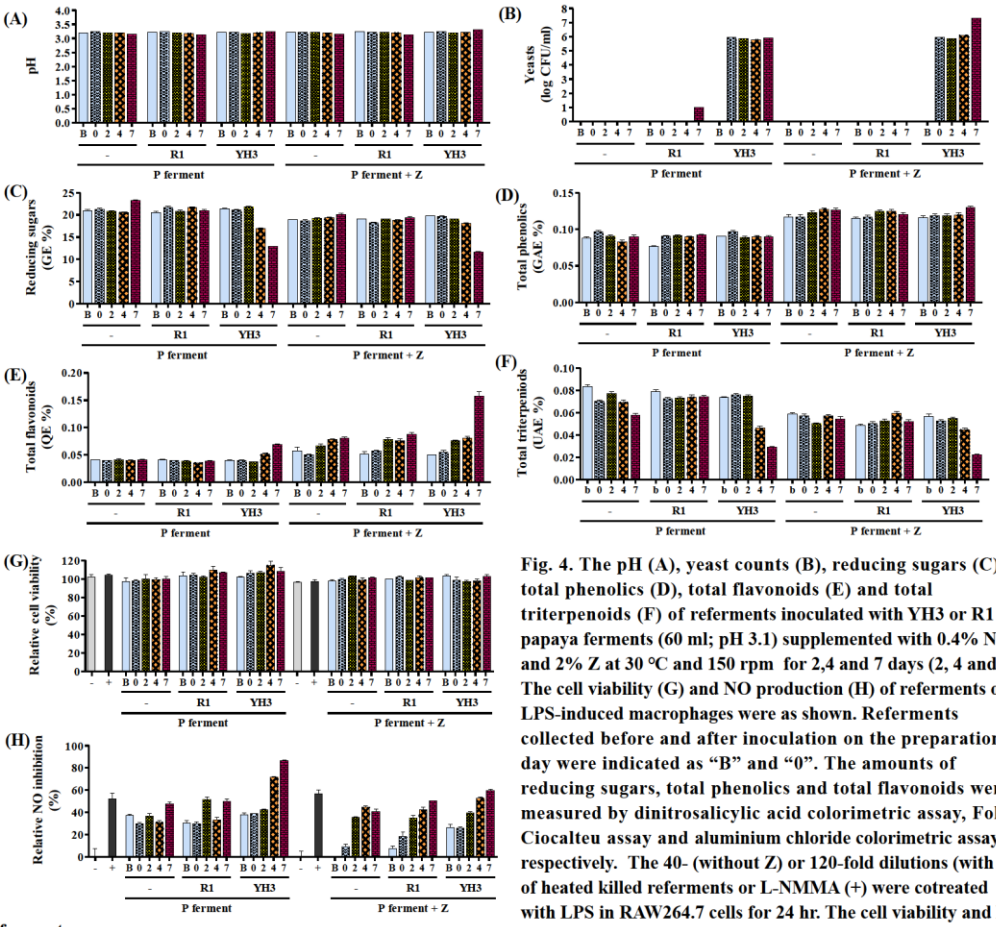

Fig. 4. The pH (A), yeast counts (B), reducing sugars (C), total phenolics (D), total flavonoids (E) and total triterpenoids (F) of referments inoculated with YH3 or R1 in papaya ferments (60 ml; pH 3.1) supplemented with 0.4% N and 2% Z at 30 °C and 150 rpm for 2,4 and 7 days (2, 4 and 7). The cell viability (G) and NO production (H) of referments on LPS-induced macrophages were as shown. Referments collected before and after inoculation on the preparation day were indicated as "B" and "0". The amounts of reducing sugars, total phenolics and total flavonoids were measured by dinitrosalicylic acid colorimetric assay, Folin–Ciocalteu assay and aluminium chloride colorimetric assay, respectively. The 40- (without Z) or 120-fold dilutions (with Z) of heated killed referments or L-NMMA (+) were cotreated with LPS in RAW264.7 cells for 24 hr. The cell viability and NO production were determined by MTT and Griess colorimetric assay, respectively. The values are the mean ± SEM (n=3) with relative cell viability or NO inhibition of cells without any treatment or treated with LPS only (-) being set as 100% or 0%, respectively. GE: glucose equivalent; GAE: gallic acid equivalent ; QE: quercetin equivalents; UAE: ursolic acid equivalents.

Table. 1 Inoculation of YH3 in different fruit and vegetable fermants.

Ferment		P	PT	PE	N	Cm	A	M	B	J	FQS	TO	V	W	T	f15	f17
log CFU/ml	day 0	5.80	5.70	5.71	5.49	5.40	5.71	4.92	4.15	5.78	5.50	5.92	5.83	5.85	5.50	5.56	4.81
	day 7	> 6.30	2.48	5.14	N.D.	5.03	1.00	N.D.	3.85	1.00	N.D.	6.30	5.04	4.97	N.D.	N.D.	N.D.
pH	day 0	3.17	3.45	3.20	3.40	3.37	3.32	3.20	3.25	3.72	3.36	3.26	3.37	3.25	3.35	3.29	3.31
	day 7	3.29	3.47	3.28	3.43	3.36	3.34	3.23	3.61	3.72	3.40	3.31	3.43	3.29	3.42	3.32	3.30

Results:

1. Five isolates were obtained from the aging broth and showed better growth in papaya ferments containing Z, whereas no yeast were found in R1-inoculated referment (Fig. 2).
2. Isolate YH3 (EVY01) were identified as *Zygosaccharomyces sp.* using PCR amplification and sequencing the 18S rRNA genes .
3. EVY01 showed the acid tolerance in YM medium at pH 5.4, 4.8 and 4.2 after 24 h incubation at 30 ˚C, while R1 cannot survive at pH 4.2 (Fig. 3).
4. As compared with R1-inoculated referments, EVY01-inoculated referments with or without Z both showed the obvious decreased reducing sugars and increased total flavonoids during 7-days refermentation (Fig 4C and 4E). However, the obvious increased anti-inflammatory activity only found in EVY01-inoculated referment without Z during refermentation (Fig 4H).

Selection of acid-fast *Lactobacillus acetotolerans* CWP217 for inoculation in fruit and vegetable ferments to promote anti-inflammatory

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Introduction:

Fruit and vegetable are consumed as a dietary supplement for the source of nutrition; however, such functional activity might lose during storage, preparation or processing. Inoculation of probiotics is amongst the ways to maintain and increase their nutrition and bioactivity. Our previous studies had shown that lactic acid bacteria (LAB) cannot survive or grow well in acidic (~pH 4) fruit and vegetable ferments. In order to develop the fruit and vegetable ferments with higher bioactivity for promoting manufacturer competition, this study was firstly aimed to screen the acid-fast LAB from the aging broth, which were collected from various fruit and vegetable ferments and aged at least for 1 year in our factory. The E2 and E3 isolates were obtained by the screening procedure as showed in Fig. 1 and had the best growth performance in pineapple or papaya ferments (A or P) containing yuan zhi powders (Z; Fig. 2). The acid tolerance of E2 and E3 isolates were evaluated in MRS medium at pH 5.4, 4.8 or 4.2 (Fig. 3). Finally, the E3 isolate was further inoculated in 5 ferments supplemented with Z, the refermentation performance and anti-inflammatory capacity of these referents were showed in Fig. 4.

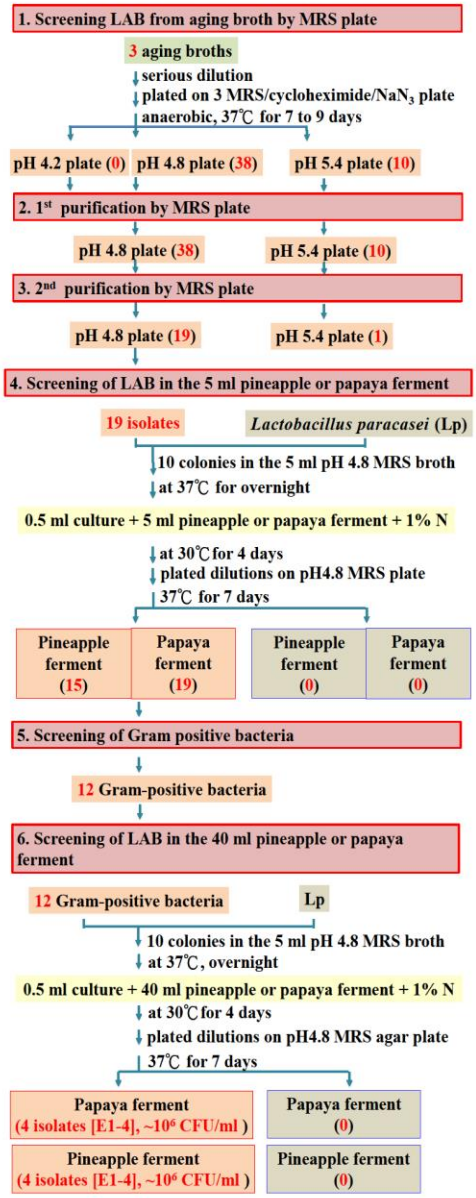

Fig. 1. Screening procedure of acid-fast LAB from aging broth. N: peptone and yeast extract ; Z: dry powder of yuan zhi.

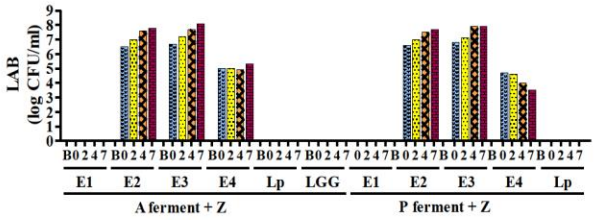

Fig. 2. LAB counts of referments inoculated with isolates E1–E4, Lp or *Lactobacillus rhamnosus* LGG (LGG) in pineapple (A) or papaya (P) ferments (40 ml; pH<3.3) supplemented with peptone and yeast extract (1%) and Z (2%) at 30 ℃ for 7 days.

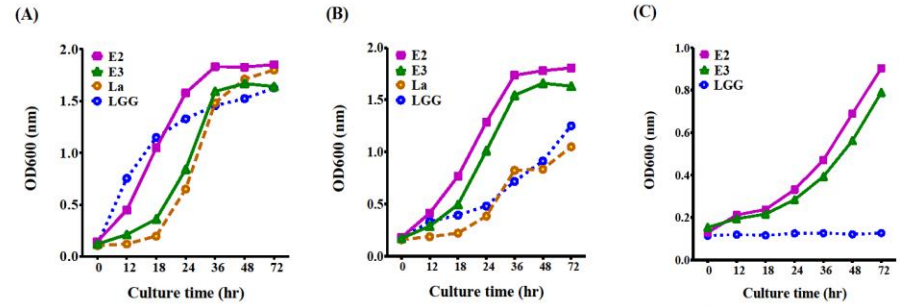

Fig. 3. Growth curves of inoculation of E2, E3, La and LGG in MRS medium at pH 5.4 (Panel A), 4.8 (Panel B) or 4.2 (Panel C) and 37℃ for 3 days. La: *Lactobacillus acetotolerans* BCRC#17709.

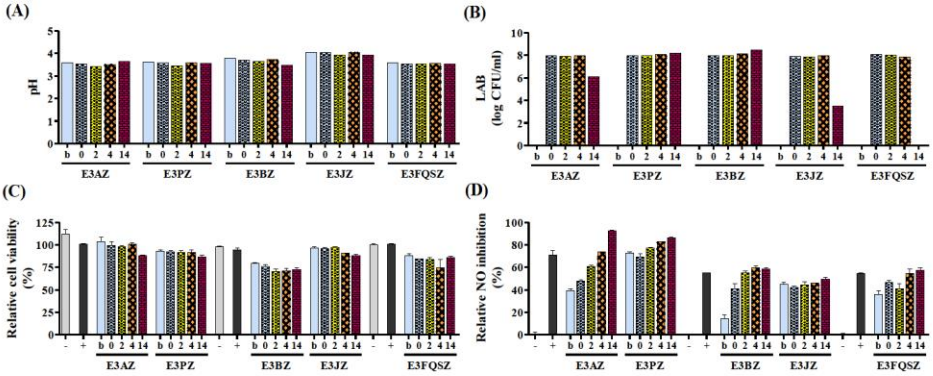

Fig. 4. The pH (Panel A)and LAB counts (Panel B) of referments inoculated with E3 in 40 ml A, P, B (bean sprout), J (Jaboticaba) and FQS (grapefruit, pumpkin and sweet orange) ferments supplemented with 1% peptone and yeast extract and 2% Z at 30 ℃ for 2, 4 and 14 days (2, 4 and 14). The cell viability (Panel C) and NO production (Panel D) of referments on LPS-induced macrophages were as shown. The 200- (ferment J) or 75-fold dilutions (the others) of heated killed referments or L-NMMA (+) were cotreated with LPS in RAW264.7 cells for 24 hr. Referments collected before and after inoculation on the preparation day were indicated as "b" and "0". The cell viability was determined by MTT colorimetric assay. NO production was determined by Griess colorimetric assay and calculated from a linear regression equation using sodium nitrite as standards. The values are the mean ± SEM (n=3) with relative cell viability or NO inhibition of cells without any treatment or treated with LPS only (-) being set as 100% or 0%, respectively.

Results:

1. Two Gram positive bacteria were obtained from the aging broth and showed better growth both in the pineapple and papaya ferments (pH < 3.3) containing yuan zhi powders, while neither LAB strains of *Lactobacillus paracasei* (Lp) nor *Lactobacillus rhamnosus* LGG (LGG) can survive (Fig. 2).
2. Isolate E3 (CWP217) were identified as *Lactobacillus acetotolerans* using PCR amplification and sequencing the 16S rRNA genes .
3. *L. acetotolerans* CWP217 showed the acid tolerance in MRS medium at pH 5.4, 4.8 and 4.2 after 72 h incubation at 37 ˚C (Fig. 3). However, neither La nor LGG can grow as well as CWP217 at pH 4.8, and LGG can not survive at pH 4.2 were found (Fig. 3).
4. After 14 days incubation at 30˚C in the supplementation of Z, CWP217 showed the best growth in P and B ferments; however, A and B referments showed obvious increased effects on anti-inflammatory activity during refermentation.