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Lactobacilli metabolites restore E-cadherin and suppress MMP9 in cervical cancer cells

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ABSTRACT

Cervical cancer is leading cause of cancer death in females worldwide. Vaginal lactobacilli colonizing cervical area are known to play an important role in maintaining cervical physiological conditions to ward away vaginal infections including bacterial vaginosis (BV) and cancer prevention. There are limited studies to study effect of Lactobacilli isolated from different sources on cervical cancer. The objective of the study was to investigate the potential of cell-free culture supernatants (CFCs) or metabolites of twelve well-characterized *Lactobacillus* species from different microenvironments for their anti-proliferative properties on HPV16 and HPV18 cervical cancer cells and to investigate the mechanisms of anti-proliferative and anti-metastatic activities. *Lactobacillus* metabolites exerted a dose, strain and cell line-dependent effect on cervical cells as demonstrated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. The metabolites from *vaginalis* and *L. salivarius* exhibited the lowest half-maximal inhibitory concentration (IC50) on HeLa (131 and 167 ng/ml) respectively and SiHa (149 and 205 ng/ml) respectively. Lactobacilli demonstrating greater inhibitory effect produced majorly L-lactic acid and hydrogen peroxide (H_2O_2). Treatment with lactobacilli CFCs significantly upregulated E- cadherin levels in HeLa ($p = 0.0451$) and SiHa ($p = 0.0051$) cells and downregulated matrix metalloproteinase 9 (MMP9) levels in Hela cells ($p = 0.0465$) as measured by ELISA. *Lactobacillus*-derived metabolites could be explored as biotherapeutics for the control of HPV infections and cervical cancer.

Introduction

Cervical cancer is the fourth most common cancer in women worldwide with an estimated 604,000 new cases of cervical cancer reported globally in 2020, and approximately 342,000 deaths (Sung et al., 2021). Infection by high oncogenic risk- Human Papillomavirus (HPV) is the main attributable factor for cervical cancer development (Walboomers et al., 1999). Two HPV types, high-risk strains (16 and 18) cause 70 % of cervical cancers and pre-cancerous cervical lesions. The majority of the HPV infections and induced neoplastic lesions are transient and resolve spontaneously (Burd, 2013) indicating other environmental and host factors like cervicovaginal microflora may be involved in the progress of low grade squamous intraepithelial lesion (LSIL) to invasive cancer (Xie et al., 2020; Lin et al., 2022). Currently, the treatment used for cervical cancer includes chemotherapeutic interventions and radiation therapy which can result in cytotoxicity side effects to the host, recurrence and resistance to infection (Kuku et al., 2013). Hence, there is a need of enhancing the quality of life in cervical cancer patients using anticancer therapies with minimal side effects (Pourmollaei et al., 2020; Jahanshahi et al., 2020; Damani et al., 2021).

The healthy vaginal microenvironment is inhabited by lactobacilli which protect the vagina from reproductive and sexually transmitted infections. These beneficial microbes modify the cervicovaginal ecosystem by the production of antimicrobials, competition with pathogens and improving the epithelial barrier function (Graver and Wade, 2011; Spurbeck and Arvidson 2011; Pramanick and Aranha, 2020). Depletion of these commensals leads to increased infectiousness by various pathogens like *Gardnerella vaginalis*, *Prevotella*, *Trichomonas vaginalis, Neisseria gonorrhea* and *Chlamydia trachomatis* (Spurbeck and Arvidson, 2011; Pramanick et al 2022). The persistence of these infections could lead to the production of deleterious metabolites which increase the risk of oncogenic HPV and promote cervical cancer (Ho et al., 1995; Schiffman et al., 2016; Kwasniewski et al., 2018). Hence there is an indirect relationship between vaginal *Lactobacillus* and cervical cancer. This cycle of infection could be reversed with the restoration of the normal vaginal flora. However, the effect of lactobacilli has

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been demonstrated to be strain specific both in vitro (Pramanick and Aranha, 2020) and in vivo (Guo et al., 2012).

Studies have shown certain lactobacilli and their metabolites can inhibit the proliferation of cervical cancer cells and thus could play an important role in cancer prevention and treatment (Brotman et al., 2014, Yang et al., 2018, Maghsood et al 2020). Nami et al. (2014a,b) reported that *L. plantarum* and *L. acidophilus* exhibited desirable probiotic properties and remarkable anticancer activity against human cancer cell lines, HeLa, MCF7, HT29 with no significant cytotoxic effects on normal cell line (HUVEC). Also, vaginal lactobacilli, including *L. crispatus, L. rhamnosus and L. gasseri,* have been shown to exert cytotoxic effects on cervical tumor cells (Nouri et al., 2016; Anton et al., 2018) but not on normal cells. Kim et al. (2015) stated that metabolites of *L. casei* had no significant effect on the growth of Ca Ski and HeLa cells. However, *L. casei* and *L. paracasei* strains isolated from human breast milk are shown to be effective against HeLa (Riaz Rajoka et al., 2018). Hence, the anticancer effects of different lactobacilli species from different environments on cervical cancer cell lines are still debated.

Earlier studies suggest that epithelial-mesenchymal transition (EMT) is involved in carcinogenesis, cancer progression and metastasis (Thiery, 2002). E-cadherin is the crucial factor involved in Epithelial-Mesenchymal Transition (EMT) and helps in cell connection. E-cadherin downregulation could be an important factor for cancer cell migration and metastasis (Ishiyama et al., 2010; Wang et al., 2017) and re-expression of this molecule is known to block invasiveness (Birchmeier and Behrens, 1994). In addition, clinical studies report Matrix metalloproteinase (MMP9) expression with progression of gynecological cancers. MMPs degrade various components of the ECM, including collagen, laminin, fibronectin, vitronectin, elastin and proteoglycans (Quintero-Fabián et al., 2019). Since MMPs play a critical role in cancer invasion, migration, metastasis and tumorigenesis, blocking tumor cell expression of MMPs can significantly reduce tumor invasion and metastasis in cervical cancers (Roomi et al., 2009). Studies have also shown the relationship between MMP and E-cadherin (Hsu et al., 2016; Gao et al 2017).

The present study attempted to investigate the ability of CFCs from twelve different species of lactobacilli from different microenvironments to inhibit HPV 16 and HPV 18 infected human cervical cancer using HeLa and SiHa cell lines respectively and explore the mechanisms by which lactobacilli exerting their anti-proliferative and anti-metastatic activities. We also studied the secretion of total lactic acid, their isomers and hydrogen peroxide produced by *Lactobacillus* species in exerting the antiviral activity. We also explored the potential anti-metastatic effects of lactobacilli metabolites by measuring levels of the E-cadherin and MMP9 in treated cervical cells.

Materials and methods

Bacterial strains

Twelve standard *Lactobacillus* species obtained from the American Type Culture Collection (ATCC) described in Table 1 were used for the study.

Culture conditions and isolation

Bacterial species stored at −80 °C in De Man, Rogosa, and Sharpe (MRS) broth (Hi-Media) containing 20 % glycerol were streaked on lactobacilli MRS agar and incubated at 37 °C for 48 h anaerobically. Each isolated colony was observed for morphological characterization. Further, the cultures of all these isolates were gram stained and checked for gram reaction and cell morphology. These cultures were propagated in the MRS broth anaerobically at 37 °C for 48 h and growth was determined by measuring OD_{600} nm spectrophotometrically and pH checked using HiIndicator™ pH papers (Himedia).

Table 1
Lactobacillus strains used in this study.

Species- Present nomenclature	Source
L. salivarius subsp. *salivarius* (*ATCC® 11741™*)	saliva
L. reuteri (*ATCC® 23272™*)	Feces, human
Limosilactobacillus fermentum (*ATCC® 9338™*)	feces.
Lactiplantibacillus plantarum (*ATCC® 8014™*)	Not stated
L. vaginalis (*ATCC® 49540™*)	Vagina of patient with trichomoniasis
L. johnsonii (*ATCC® 33200™*)	Human blood
L. delbreckii (*ATCC® 7830™*)	Emmenthal Cheese
L. acidophilus (*ATCC® 314™*)	Infant feces
Lactobacillus gasseri (*ATCC® 19992™*)	Feces
L. jensenii (*ATCC® 25258™*)	Human vaginal discharge
L. crispatus(*ATCC® 53545™*)	Human stool
L. rhamnosus (*ATCC® 9595™*)	Not stated

Preparation of the CFCs from Lactobacillus cultures

Lactobacillus isolates were inoculated in MRS broth and incubated at 37 °C for 48 h under anaerobic conditions. The MRS broth cultures at the end of the exponential growth phase were centrifuged at 8000 rpm for 10 mins. Bacterial cells pellets were washed twice with sterile PBS and concentration was adjusted to 1 at OD_{600} nm. These cells were further conditioned with Dulbecco's Modified Eagle medium (DMEM) (Gibco). These cultures were incubated in a shaker for 4 h followed by static overnight incubation at 37 °C. Further, the DMEM CFCs were obtained by centrifugation at 8000 rpm for 10 min at 4 °C followed by filter sterilization of cultures using 0.22 mm syringe filters (Axiva, India). The CFCs were stored at −80 °C till tested. Sterility checking was confirmed by inoculating aliquots of CFCs on MRS agar and incubating at 37 °C for 24 h.

Quantification of protein by Bradford Assay

Total protein quantitation was carried out by Bradford's Protein Assay. Typically, BSA (1 mg/mL) was used as a standard and a calibration curve based on the concentration of BSA (ranging from, 0.3–4 μg/ml) was used to determine the unknown protein concentration of supernatants. In a 96 well microtitre plate 50 μl of the standard/sample was mixed with 50 μl of Bradford's reagent. The plates were further incubated for 10-15mins and read at 595 nm using a microplate reader (Synergy H1, Biotek, USA). The experiment was performed in triplicates.

Cell culture and maintenance

Human cervical cancer cell lines HeLa (HPV 18+) (ATCC-CCL-2™) and SiHa (HPV 16+) (ATCC-HTB-35™) obtained from American Type Culture Collection (ATCC) were maintained as monolayer cultures at 37 °C under 5 % CO_2in DMEM medium (Gibco) supplemented with 10 % heat inactivated fetal bovine serum (FBS) (Gibco) and 1 % penicillin/streptomycin mixture (Invitrogen).

Cell cytotoxicity 1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay

Cytotoxicity effects of *Lactobacillus* metabolites on cervical cancer cell lines were demonstrated by tetrazolium (MTT) assay (Mosmann, 1983). Here, 2×10^4cells in 96 well microplates were treated with the filtered CFCs of *Lactobacillus* species at different concentrations ranging from 10 % to 100 % (v/v). Untreated cells served as control to determine the background levels of cell death under the experimental conditions used. Plates were incubated at 37 °C under 5 % CO_2 for 24 h. 10 μl of MTT solution (5 mg/mL in PBS) was added and incubated further for 4 h at 37 °C. 100 μl of DMSO was added and incubated for 30 mins. The absorbance was measured at 570 nm using a microplate reader. Cell viability and cytotoxicity were determined by formula:

Cell viability % = (Absorbance Sample − Absorbance Blank)/ (Absorbance Control − Absorbance Blank) × 100.

And, Cell cytotoxicity% = 100 − cell viability %.

Detection of lactic acid

Qualitative measurement

Lactic acid producers were screened by streaking *Lactobacillus* isolates on the sterile MRS agar plate having Bromo-cresol purple (BCP) (Himedia, India). On incubation at 37 °C for 48 h anaerobically, lactic acid producing bacteria showed the change in the colour of the media from purple to yellow.

Quantitative measurement

Lactic acid produced by lactobacilli in CFCs was estimated by using D-/L-Lactic Acid (D-/L-Lactate) (Rapid) test kit (Megazyme). Manufacturer's instructions were followed to carry out the assay. The experiments were performed in duplicates.

Detection of hydrogen peroxide

Qualitative measurement of H_2O_2

Semi quantitative assay for isolates producing Hydrogen peroxide (H2O2) was assessed using method given by Pendharkar et al (2013) with slight modifications. Briefly Lactobacillus isolates were streaked onto MRS agar plate containing 3,3′,5,5′-tetramethylbenzidine (TMB) and horseradish peroxidase (HRP) (Sigma-Aldrich). Plates were incubated in anaerobic condition for 48 h and were exposed to air for variable time period. Isolates were scored as weak (>60 min), intermediate (15–60 min) and strong producing strains (<15 min); on the basis of the time required for the blue coloration to appear.

Quantitative estimation of, H_2O_2

The concentration of hydrogen peroxide was determined by measuring absorbance induced with the chromophore- o-dianisidine (Sigma, USA) described by Martín and Suárez (2010).

Enzyme linked immunosorbent assay for E-cadherin and MMP9

Human E-Cadherin ELISA Kit (cat. no. ab233611, Abcam), and Human MMP9 ELISA kit (cat. no. ab100610, Abcam) were used to detect E-cadherin and MMP-9 in the culture supernatants respectively. Samples were processed according to the manufacturer's instructions. Untreated cells served as a control to determine the background levels of e-Cadherin and MMP under the experimental conditions used.

Statistical analysis

Data analysis was performed by Graph Pad Prism 8.4.2 software. Data are represented as means ± standard deviation (SD). Differences across groups were estimated by multiple comparisons of the data as calculated by One-way analysis of variance (ANOVA) with Kruskal-Wallis testfollowed by Dunn's multiple comparison test for comparison between species. P value of <0.05 considered statistically significant. Correlation was carried out by using Spearman's rank correlation.

Results

Colony characteristics of the standard Lactobacillus species

Cultural characteristics of twelve well characterized reference *Lactobacillus* species were studied by observing the well isolated colonies that appeared on the MRS agar plate. Further, gram staining was carried out for these *Lactobacillus* species. Colony characteristics of lactobacilli showed variation in size, shape, margin, elevation, opacity and consistency. Gram stained *Lactobacillus* colonies showed Gram-positive nature along with variable morphology characteristics like short, medium, long rods, to their appearance in chains and clusters.

Determination of growth

The mean growth of *Lactobacillus* cultures in MRS measured in 24 h was 1.2 ± 0.1247 (range 0.98–1.34) and in DMEM media was 1.04 ± 0.1532 (range 0.81–1.26) (Fig. 1a and b). Significant differences in optical density was observed between MRS and DMEM ($p = 0.0317$). Also significant differences were observed across species of the lactobacilli in MRS ($p = 0.0420$) and DMEM ($p = 0.0284$). *L. crispatus* showed maximum growth and *L. plantarum* showed minimum growth in DMEM (Fig. 1b).

Determination of pH

Overall, all the *Lactobacillus* species reduced the pH of the MRS medium The mean pH of lactobacilli in MRS media was 3.67 ± 0.2 and in DMEM media was 4.37 ± 0.6 with significant difference ($p = 0.0004$). In MRS medium, all *Lactobacillus* isolates lowered pH to 3.5 except species of *L. reuteri, L. fermentum, L. johnsonii* and *L. acidophilus*, which lowered pH to 4. While in DMEM media, *L. salivarius, L. crispatus, L. reuteri* and *L. fermentum* isolates acidified media weakly. MRS and DMEM CFCs showed significant differences in pH among the species of the lactobacilli ($p = 0.0177$) (Supplementary Fig. 1).

Quantification of protein

Concentration of protein in DMEM supernatants determined by Bradford Assay varies in different species of *Lactobacillus* (Fig. 1c). The protein concentration was observed in the range of 2.47–25.47 μg/mL. *L. fermentum* (25.48 ± 1.14) produced maximum amount of protein followed by *L. plantarum* (22.72 ± 1.06), *L. reuteri* (21.72 ± 1.32), and *L. delbrueckii* (21.72 ± 2.01) *as* compared to other *Lactobacillus* species. *L. vaginalis* produced less amount of protein (2.47 ± 0.25). Protein production by *Lactobacillus* species differed significantly among the isolates ($p = 0.0005$) while, significant difference was observed between the protein produced by *L. fermentum* and *L. vaginalis* ($p = 0.0131$). Significant negative correlation was observed between growth and protein concentration in DMEM ($r = -0.68$, $p = 0.01$) (Supplementary Fig. 2).

Antiproliferative effects of lactobacilli supernatants on cancer cells

Dose dependent cytotoxicity of the CFCs was observed for both the cell lines. CFCs of the majority of the species were effective at <50 to 60 % on HeLa and SiHa respectively. *L. rhamnosus, L. reuteri* and *L. fermentum* required a higher volume of CFC on HeLa and SiHa to show the desired effect compared to other *Lactobacillus* species. *L. plantarum* and *L. salivarius* were potent against both cervical cancer cell lines at the concentration of 20 % (v/v) (Fig. 2). However, the metabolites from *L. vaginalis* and *L. salivarius* exhibited the lowest IC50 on HeLa (131 and 167 ng/ml) respectively and SiHa (149 and 205 ng/ml) respectively (Table 2). CFCs of lactobacilli were more active against HPV18 as compared to HPV16; however, no significant differences were observed.

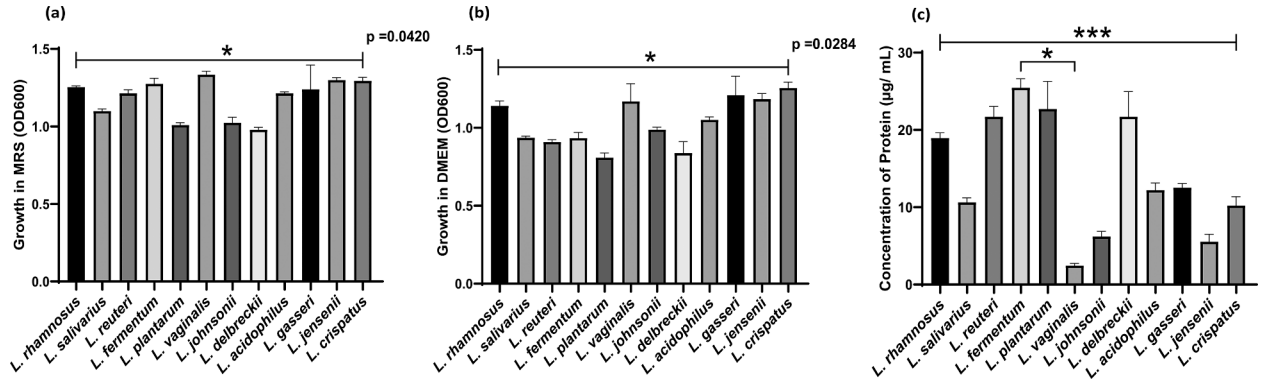

Fig. 1. The growth of different *Lactobacillus* species was measured at 600 nm in (a) MRS and (b) DMEM media (c) Protein concentration in DMEM CFCs determined by Bradford assay. Data are represented as mean ± SD, *p < 0.05, ***p < 0.001 indicating statistical significances after performing the Kruskal-Wallis test. The upper line indicates the Kruskal-Wallis test and downward line indicates Dunn's multiple comparison test.

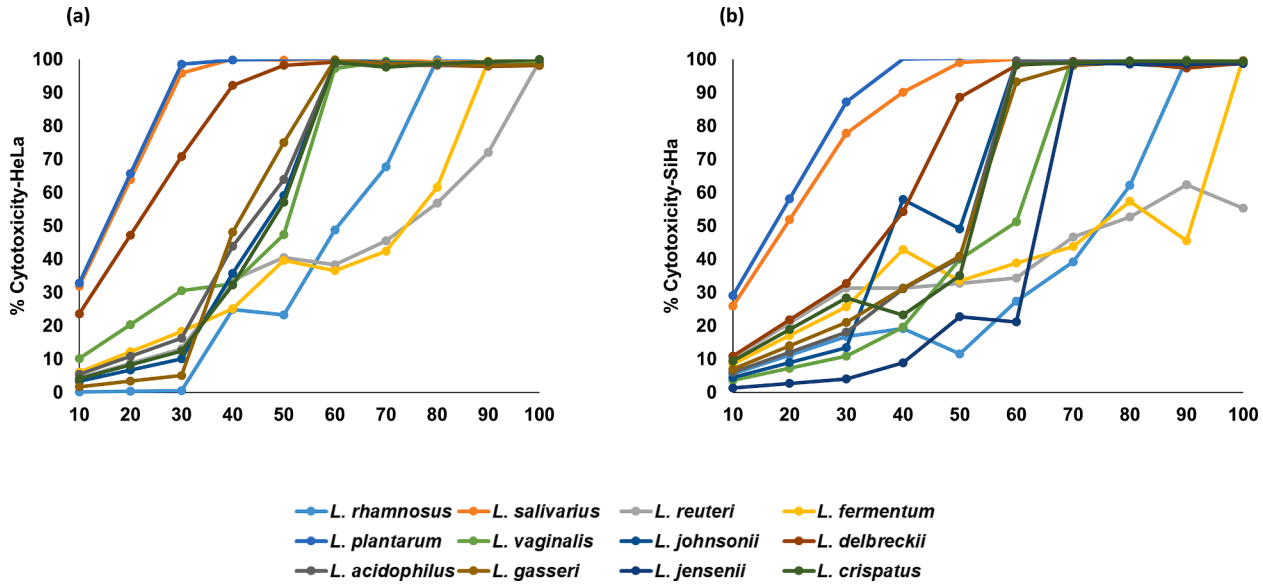

Fig. 2. Effect of cell free DMEM supernatants of *Lactobacillus* species (v/v) on (a) HPV18-HeLa and (b) HPV 16-SiHa cell lines in various concentrations ranging from 10 to 100 %. Cell proliferation was determined using the MTT colorimetric assay, measured at an optical density of 570 nm.

Table 2
Protein concentrations of CFCs of different *Lactobacillus* species and the half maximal inhibitory concentrations (IC50) of lactobacilli CFCs on both HeLa and SiHa cells. Statistical significances were determined by using Kruskal-Wallis test.

Lactobacillus species	Total Protein in DMEM CFCs (µg/mL) Mean ± SD	IC50 concentration (V/V%)		IC 50 Protein concentration (µg/mL)		Significance P value
		HeLa	SiHa	HeLa	SiHa	
L. rhamnosus	18.975 ± 0.66	61.488	75.00	1.167 ± 0.04	1.423 ± 0.05	0.100
L. salivarius	10.642 ± 0.56	15.651	19.27	0.167 ± 0.01	0.205 ± 0.01	0.100
L. reuteri	21.725 ± 1.32	76.889	80.00	1.670 ± 0.10	1.738 ± 0.11	0.400
L. fermentum	25.475 ± 1.14	75.000	80.00	1.911 ± 0.09	2.038 ± 0.09	0.200
L. plantarum	22.725 ± 1.06	15.227	17.20	0.346 ± 0.05	0.391 ± 0.06	0.400
L. vaginalis	2.475 ± 0.25	52.743	60.00	0.131 ± 0.01	0.149 ± 0.02	0.200
L. johnsonii	6.225 ± 0.66	42.301	50.00	0.263 ± 0.03	0.311 ± 0.03	0.200
L. delbreckii	21.725 ± 2.01	21.177	40.00	0.460 ± 0.07	0.869 ± 0.13	0.100
L. acidophilus	12.225 ± 0.90	45.568	52.00	0.557 ± 0.04	0.636 ± 0.05	0.200
L. gasseri	12.558 ± 0.52	41.554	51.50	0.522 ± 0.02	0.647 ± 0.03	0.100
L. jensenii	5.558 ± 0.95	53.000	60.00	0.295 ± 0.05	0.334 ± 0.06	0.400
L. crispatus	10.225 ± 1.14	50.000	55.00	0.511 ± 0.06	0.562 ± 0.06	0.400
P value	0.0005			0.0004	0.0003	

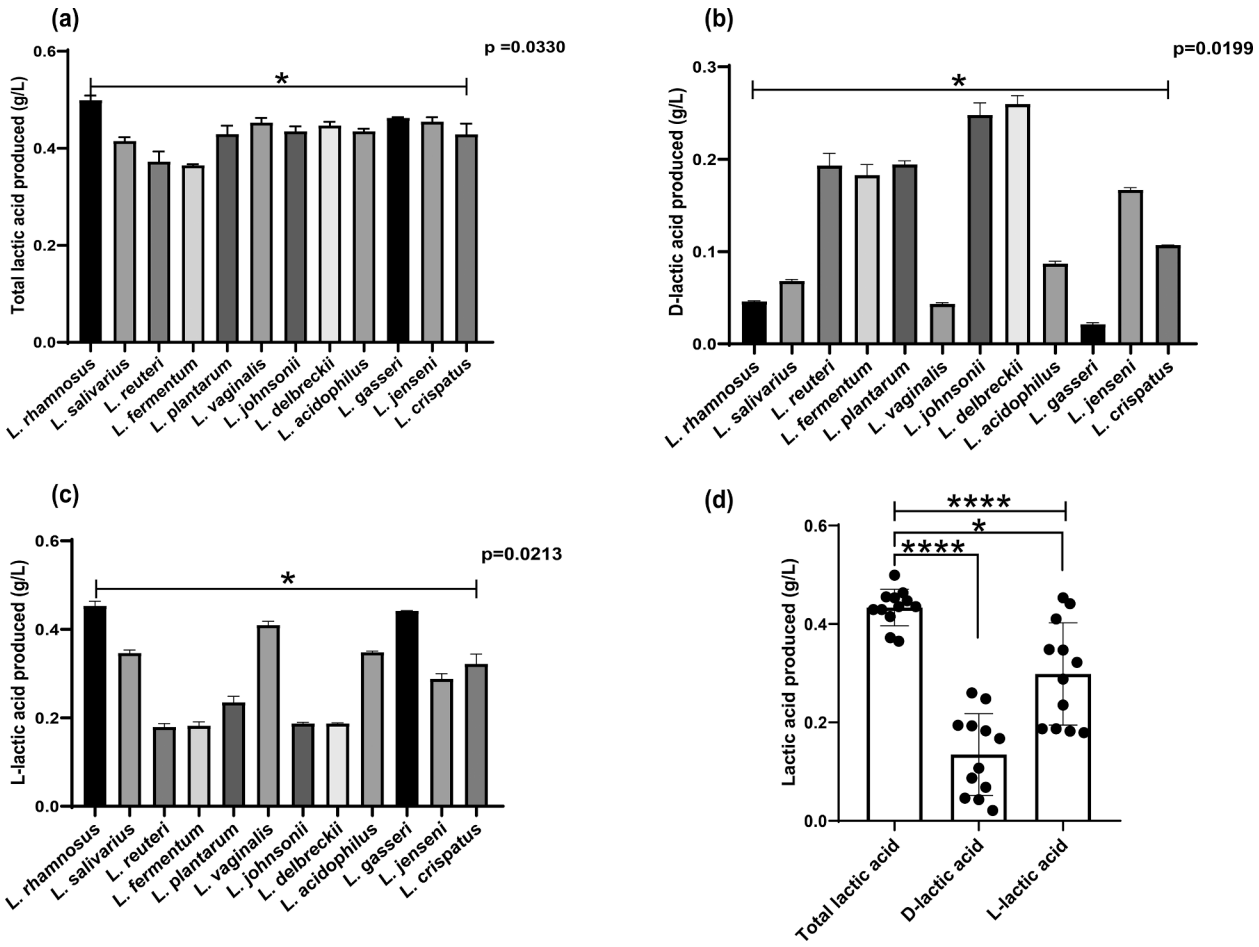

Fig. 3. Quantitative determination of (a) Total lactic acid, (b) D-lactic acid (c) L-lactic acid by different lactobacilli in DMEM CFCs after 24 h incubation at 37 °C were measured in duplicate using Lactate colorimetric kits. (d) Distribution of total lactic acid, D- and/L- isomers of lactic acid by lactobacilli in DMEM CFCs. Data are represented as mean ± SD, *P < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 showing statistical significances after using the Kruskal Wallis test. The upper line indicates the Kruskal Wallis test and downward line indicates Dunn's multiple comparison test.

Lactic acid production

Lactic acid produced by *Lactobacillus* isolates was determined qualitatively on MRS-BCP agar. All lactobacilli evaluated were able to change the colour of bromocresol purple in MRS agar to yellow. The average total lactic acid in culture supernatants of all the isolates was 0.4 g/L. *L. rhamnosus* (0.499 g/L) produced maximum total lactic acid while *L. fermentum* (0.364 g/L) produced minimum total lactic acid (Fig. 3a). Notably, *L. reuteri, L. fermentum, L. plantarum, L. johnsonii,* and *L. delbrueckii* produced more D-isomer of lactic acid (Fig. 3b) whereas *L. rhamnosus, L. gasseri, L. vaginalis, L. crispatus and L. salivarius* produced mainly L-lactic acid (Fig. 3c). Significant differences were observed

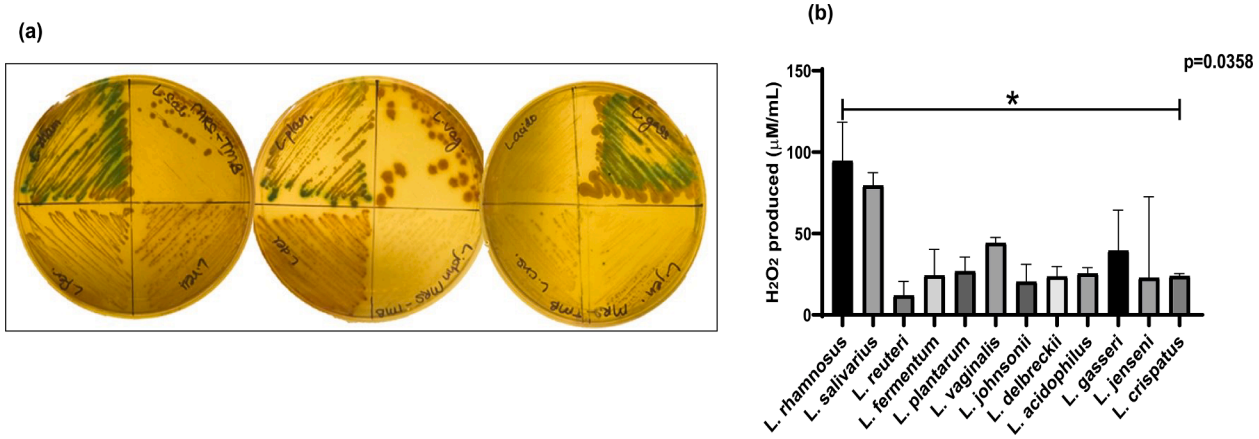

Fig. 4. (a) Semi-quantitative H_2O_2 determination of *Lactobacillus* species on MRS-TMB and HRP agar media (b) Quantitative estimation of Hydrogen peroxide by *Lactobacillus* species in DMEM media using o-dianisidine colorimetric assay. Data represented as mean ± SD. Kruskal Wallis test was used for comparisons among species of lactobacilli *P < 0.05.

across the species of the lactobacilli, for total lactic acid ($p = 0.0330$), D-lactic acid ($p = 0.0199$), and L-lactic acid ($p = 0.0213$) and (Fig. 3a,b,c). Variations were observed in the production of total, D- and L- lactic acid ($p = <0.0001$) Quantification of the lactic acid demonstrated that, though both isomers were produced, L-lactic acid produced was more compared to D- lactic acid in the culture medium (Fig. 3 d). Thus, acidity in the medium was primarily contributed by total lactic acid and L-lactic acid was the major determinant.

Hydrogen peroxide production

H_2O_2 producing lactobacilli were checked on MRS-TMB HRP agar qualitatively. *L. rhamnosus*, *L. gasseri, L. plantarum, L. vaginalis, L. salivarius*, were strong H_2O_2 producers.*L. delbrueckii* and *L. fermentum* were medium producers H_2O_2 (Fig. 4a). Significant differences were observed for H_2O_2 levels in the DMEM media by *Lactobacillus* species. Detectable concentration of H_2O_2 observed in the range of 12–94.67 μM/mL respectively. Of the 12 species of lactobacilli, *L. rhamnosus* (94.67 μM) and *L. salivarius* (80 μM) produced maximum H_2O_2 in DMEM. Significant differences were observed across the species of the lactobacilli ($p = 0.0358$) (Fig. 4b).

ELISA for measurement of E-Cadherin and MMP9

We explored the potential anticancer effects of lactobacilli by measuring levels of the E-cadherin and MMP9 using ELISA assays. As shown in Fig. 5(a) and (b), after 24 h, treatment with lactobacilli upregulated E- cadherin levels in HeLa ($p = 0.0451$) and SiHa ($p = 0.0051$) cells significantly when compared with their respective controls (untreated cells). Furthermore, *Lactobacillus* supernatants significantly downregulated MMP9 levels in Hela cells ($p = 0.0465$) (Fig. 6-a). No significant differences were observed in downregulation of expression of MMP9 in SiHa cells treated with *Lactobacillus* supernatants (Fig. 6-b).

Discussion

Cervical cancer, caused by HPV is the most common gynecologic cancer among all women. Vaginal dysbiosis has been associated with risk of HPV infection and cervical cancer (Brusselaers et al., 2019). *Lactobacillus* as probiotics have been explored as a prophylactic and therapeutic for urogenital infections (Ballini et al., 2015; Chee et al., 2020). Antitumor activity by lactobacilli have been demonstrated in breast cancer (Motevaseli et al., 2018), human myeloid leukemia (Tuo et al., 2015) and colon cancer (Yue et al., 2020). Recent studies by Palma et al. (2018) have shown the effectiveness of long-term usage of probiotics in preventing HPV infection; whereas Ou et al. (2019) reported the absence of positive effect on HPV. However, protection of women by lactobacilli against HPV infection and cervical cancer may be species specific (Nouri et al., 2016; Brotman et al., 2014).

Several investigators have demonstrated the antiproliferative activity of metabolites of one or two *Lactobacillus* species on cancer cell growth (Motevaseli et al., 2013; Nouri et al., 2016; Wang et al., 2017; Nami et al., 2014a,b; Kim et al., 2015). The effect of lactobacilli may be mediated by cells, or their secreted products (Maghsood et al., 2020). To our knowledge, this is the first study investigating the effect of twelve different *Lactobacillus* species, commonly described in the vaginal environment, and isolated from different sources for their antiproliferative activity on HPV 16 and HPV18 positive cervical cancer cells. HPV 16 and 18 are well-established precursors for cervical cancers, anogenital cancers, oropharyngeal and non-oropharyngeal squamous cell carcinomas (Kobayashi et al., 2018).

Distinctly, these CFCs exerted cytotoxic effects in concentration dependent manner, a similar concentration dependent effect observed by Tiptiri-Kourpeti et al. (2016) on colon carcinoma cells by *L. casei.* Though all species exerted antiproliferative effect on cervical cells, selective species were more efficient in their cytotoxicity. Our studies including that of others (Chuah et al., 2019; Happel et al., 2020; Pramanick and Aranha, 2020) have demonstrated different strains of the same species also vary in their functional properties, hence investigation of several species need to be undertaken to identify the promising probiotic for particular function. Studies have shown that probiotics isolated from different environments or from the same environmental niche have varying functional properties ((Bazireh et al., 2020; Kahraman et al., 2022). Our study further showed HeLa cells were more sensitive than SiHa cells; likewise, sensitivity of HeLa to chemotherapeutic agents has been observed by Xu et al. (2012). This differential sensitivity of cells to anticancer drugs could be correlated to p21Waf1/Cip1 levels (Funaoka et al., 1996) or due to differences in bacterial genome versatility and diversity among the strains (Truong et al., 2017; Sela et al., 2018). Whole genome sequencing of these isolates would further give a better insight of the differences in genomic diversity and validate the functional properties of different species.

Among the lactobacilli used in our study, *L. plantarum* and *L. salivarius* showed the maximum antiproliferative activity when crude supernatant was used for evaluating antiproliferative activity. The bioactivity is attributed due to the cumulative effects of lactic acid, hydrogen peroxide, bacteriocins, biosurfactants and exopolysaccharides in crude supernatant. CFCs from different *Lactobacillus* species showed variations in the amounts of protein in different species. When protein content was evaluated for antiproliferative activity it was observed that *L. salivarius* and *L. vaginalis* were effective at lower protein concentrations. Notably *L. vaginalis* is commonly seen in vagina whereas *L. salivarius* is seen in the oral, intestine and female reproductive tract. Interestingly, HPV is implicated in cervical, anal and oropharyngeal

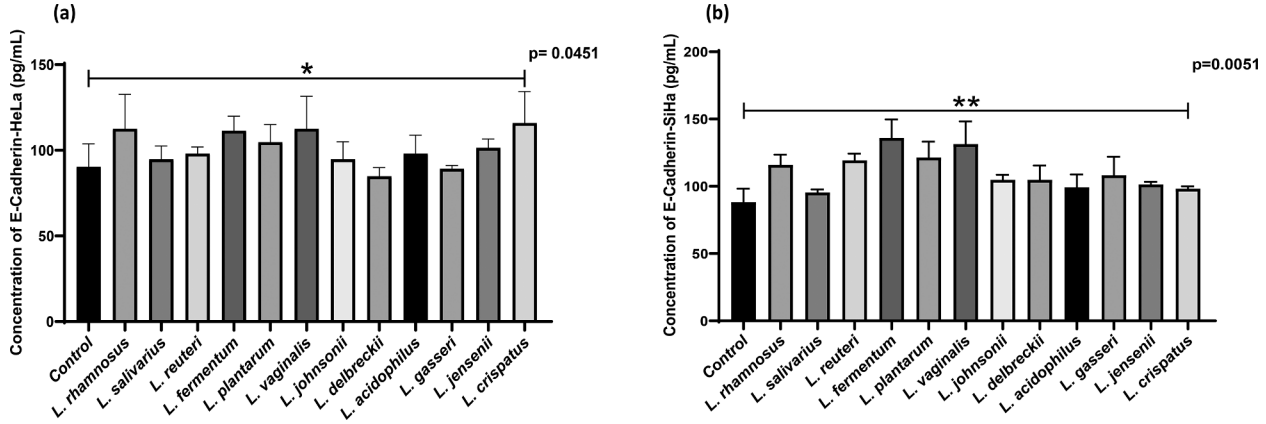

Fig. 5. Effect of metabolites of *Lactobacillus* species on E-Cadherin levels in cervical cancer cell lines (a) HeLa and (b) SiHa. Data are represented as mean ± SD, *P < 0.05, ** $p < 0.01$ indicating statistical significances after performing the Kruskal-Wallis test.

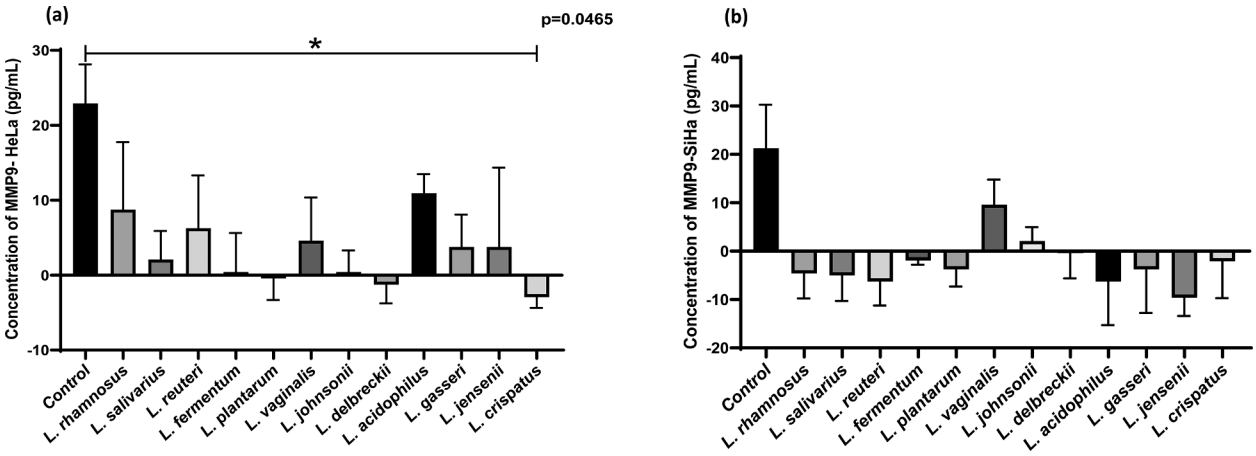

Fig. 6. Effect of CFCs of *Lactobacillus* species on MMP9 levels in cervical cancer cell lines (a) HeLa and (b) SiHa. Data are represented as mean ± SD, *P < 0.05, indicating statistical significances after performing the Kruskal-Wallis test.

cancers (Schiffman et al., 2016). *L. salivarius* and *L. vaginalis* are the microbiota commonly identified in the vagina of healthy women and known to play a protective role against urogenital infections (Madhivanan et al., 2014; Pino et al., 2019; Pramanick and Aranha, 2020). So it would be interesting to evaluate the indigenous microbiota of healthy individuals for their protective activity against HPV cancers of oral and cervico-vaginal origin.

The antiproliferative activity of lactobacilli studied was independent of pH and protein. Study by Motevaseli et al. (2013) reported anticancer activity was found to be independent of pH. Witkin and Linhares (2016) stated that lactobacilli produce both isomers of lactic acid. Our results showed that, though both isomers were produced, levels of L-isomer of lactic acid was more than D-isomer of lactic acid. L-lactic acid has been found to be 17-fold more potent than D-lactic acid in inactivating HIVBa-L in vitro (Aldunate et al., 2013). Likewise, the L.isomer of Lactic acid appears to be more bioactive than D-lactic acid as most of the isolates exerting greater bioactivity were L-lactic acid producers. It would be useful to investigate the two isomers in pure form for their anti-proliferative activity on cervical cancer cells.

Hydrogen peroxide producing lactobacilli are critical in maintaining a healthy vaginal ecosystem. Earlier experimental studies showed that women with H_2O_2 producing lactobacilli were at lower risk of dysbiosis (Mitchell et al., 2015). Production of H_2O_2 differed within species of *Lactobacillus; L. plantarum, L. vaginalis*, and *L. salivarius* were characteristic in producing more H_2O_2 in MRS media and exerting anticancer activity.

E-cadherin is 12 kDa calcium dependent membrane glycoprotein, has notable physiological activities regarding the cell–cell adhesion, structural integrity and epithelial tissue polarity (Li et al., 2017). Previous research has demonstrated that invasion and metastasis are important for tumor progression which results in the reduction of E-cadherin in cancers (Kourtidis et al., 2017). Increasing evidences has shown that E-cadherin levels are usually downregulated in several human cancers, including oral squamous carcinoma, lung and skin cancer, as well as cervical cancer. Hence, depletion of E-cadherin levels has been considered as a cause of the poor prognosis (Dohadwala et al., 2006). Our study showed that *Lactobacillus* supernatants notably upregulated E-cadherin levels in cervical cancer cells, indicated that this effect may be a factor responsible for anticancer effects in cervical cancer cells.

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent proteolytic enzymes. These enzymes are normally involved in the breakdown of the extracellular matrix within the context of physiological tissue remodelling and angiogenesis which have been linked to the aggressiveness of gynaecological cancers such as cervical cancer (Liu et al., 2018) MMPs-mediated degradation of extracellular matrix is strongly implicated in the invasion and metastasis of malignant cells. The expression of MMPs is elevated in some carcinomas and accelerates tumor progression (Quintero-Fabián et al., 2019). We found that treatment of cervical cancer cells with lactobacilli supernatants downregulated MMP9 concentration when compared with control cells. Therefore, the results suggested that lactobacilli metabolites may have the potential to inhibit cell migration and invasion in cervical cancer cells via regulation of EMT-associated factors. However, this needs to be confirmed using suitable cell migrations assays. Besides, E-cadherin and MMP9 detection in samples of patient treated with lactobacilli would validate and strengthen our findings.

Thus, our study shows that *Lactobacillus* supernatants could be explored as anti-proliferative and anti-metastatic agents. Earlier studies have also confirmed that *Lactobacillus* secreted metabolites and cell components also have probiotic properties (Maghsood et al., 2020; Teame et al., 2020). Though there is considerable evidence supporting the potential role of probiotic live LAB cells in improving health being of individuals, it is a challenge to effectively deliver the probiotic in the formulation without compromising the viability of probiotics (Terpou et al., 2019). Hence targeting against infections and cancers through use of probiotic metabolites could be an option that could be further focused and explored.

Further work is required to study the other mechanisms through which lactobacilli exhibits anti-cancer effects on expression of E6 and E7 oncogenes, and cancer related-genes and pathways. Also, the different specific components of CFCs like exopolysaccharides, biosurfactants could be isolated and evaluated for their antitumor activity. Nonetheless, our study showed the potential of anti-cervical cancer activity exhibited by different *Lactobacillus* species on HPV 16 and 18 cervical cancer cells. Studies of the immunologic responses, in vivo effects in cancer models can be further explored.

Conclusion

Anticancer activity of the CFCs from different *Lactobacillus* species evaluated was species specific and cell line specific. These metabolites could be used in isolation or integrated as prebiotics with beneficial probiotic bacteria as an effective adjunct strategy for cervical cancer. Further the metabolites could be explored as prophylaxis to control vaginal dysbiosis and thus modulate the vaginal microbiota to prevent transition from neoplasia to cervical cancer.

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CRediT authorship contribution statement

Krupali Pawar: Investigation, Formal analysis, Writing – original draft. **Clara Aranha:** Conceptualization, Methodology, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2022.100088.

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