



Apple cider vinegar: Effective adjuvant treatment for aerobic vaginitis

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Abstract



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Aerobic vaginitis (AV) is a vaginal infection caused mainly by overgrowth of *Escherichia coli* and a reduction of Lactobacilli in the vagina. The infection is linked with adverse pregnancy outcomes, such as preterm birth. The current medical therapy for AV with antibiotics is associated with adverse effects and necessitates the use of alternative treatments. Apple cider vinegar (ACV) is a natural fermented product produced from apples and is reported to exhibit potent antibacterial activity. ACV also contains lactic acid bacteria, which can act as probiotics. Symptoms of AV can be improved by restoring the disturbed microbial imbalance rather than exposing the vagina to synthetic drugs. Hence, an attempt was made to investigate whether ACV could support growth of the beneficial bacteria and inhibit growth of the pathogenic bacteria such as *E. coli* in the vagina. *In vitro* evaluation of the anti-bacterial potential of ACV against *E. coli* showed a potent antibacterial activity, recording a zone of inhibition diameter of 32.9 ± 0.5 mm; however, no zone of inhibition was observed against *Lactobacillus acidophilus*. Turbidimetric analysis was used to ensure growth of the bacteria in a simulated vaginal fluid (SVF) by using a nephelometer as a Nephelometric Turbidity Unit (NTU). Both *E. coli* and *L. acidophilus* were grown individually in SVF containing ACV. The ACV expressed antibacterial efficacy against *E. coli* and favoured growth of *L. acidophilus* in the SVF. Conclusively, ACV can help in growth of the beneficial bacteria and restore the natural microbiota of the vagina, thus proving to be beneficial in the management of AV.

Keywords: *E. coli*, *L. acidophilus*, Apple cider vinegar, Simulated vaginal fluid, Aerobic vaginitis, Turbidimetry

1. Introduction

Aerobic vaginitis (AV) is a vaginal infection caused due to disturbance in the common vaginal microbiota, specifically due to the decrease in the Lactobacilli as a dominant microflora ([Donders *et al.*, 2002](#); [Donders, 2007](#)). AV is characterized by an increase in the vaginal discharge that is sticky, yellowish or yellowish green in color, with foul smell and inflamed vaginal mucosa ([Donders *et al.*, 2017](#)). In AV the vaginal pH is raised to ≥ 5 . The occurrence of AV is associated with adverse pregnancy outcomes such as chorioamnionitis; preterm birth and miscarriage, as it is linked to the increase in the level of interleukins IL1 β , IL-6 and IL-8 ([Donders *et al.*, 2011](#)). Unlike the bacterial vaginosis, AV is characterized by inflammation of the vaginal mucosa ([Geng *et al.*, 2015](#)), and hence it is more associated with the risk of sexually transmitted diseases and HIV. The infection is caused due to the overgrowth of certain bacterial pathogens such as *E. coli* and *Staphylococcus aureus*, which results in reduction of the number of Lactobacilli in the vagina ([Wang *et al.*, 2016](#); [Rumyantseva *et al.*, 2016](#))

Lactobacilli contribute to nonspecific antibacterial host defence in the normal vagina ([Eschanbach *et al.*, 1989](#)). They inhibit adherence of the pathogenic microbes to the vaginal region and suppress their growth, by competing for binding onto the receptors located on the host vaginal epithelial cells ([Marcone *et al.*, 2010](#)). Lactobacilli produce several antimicrobial substances such as lactocin, bacitracin, lactic acid and hydrogen peroxide ([Mendling, 2016](#)). Bacitracin and lactocin are the proteins that have bactericidal activity against *E. coli* ([Kale *et al.*, 2005](#); [Turovskiy *et al.*, 2009](#); [Razzak *et al.*, 2011](#)). Lactobacilli can interact with the immune cells and stimulate the host immune response ([Lebeer *et al.*, 2008](#)). Moreover, they are reported to inhibit the growth and biofilms formation by *E. coli*. According to [Tempera *et al.*, \(2006\)](#), in patients suffering from AV; increased level of *E. coli* and decreased level of Lactobacilli have been reported.

Growth of *E. coli* and the other aerobic microbes are reported to increase 3-5 folds in case of AV, and are associated with inflammation of the vaginal mucosa. Any changes in the microbiota of the vagina cause reduction in the *Lactobacillus* spp., thus favoring the growth of other pathogens such as *E. coli* that causes vaginal infection.

Treatment of AV requires the administration of local anti-bacterials and anti-inflammatory agents ([Bertuccini *et al.*, 2017](#)). However, the chances of development of resistance to antibiotics and occurrence of re-infection limit the chemical antibacterial therapy. Furthermore, the risk of side effects development caused by the use of antibiotics along with eradication of the beneficial bacteria are the main reasons for the unsuccessful treatments of AV with the use of synthetic anti-bacterials and antibiotics ([Eade *et al.*, 2012](#); [Deshkar *et al.*, 2022](#)). Hence, recently the health care system is more inclined towards the use of herbal and natural remedies for the management of AV.

Apple cider vinegar (ACV) is a natural apple product produced by the fermentation process, and consists of sugar, apple and yeasts ([Del Campo *et al.*, 2008](#); [Medina and Pieper, 2016](#)). The antibacterial potency of ACV on bacterial species such as *E. coli* is well established, and is also found effective against the Gram positive bacteria. This activity is attributed to the presence of a wide range of components such as organic acids; flavonoids, polyphenols, vitamins and minerals ([Xia *et al.*, 2020](#)). There are two types of Lactic acid bacteria (LAB) most commonly identified in apple juice by-products mainly; *Lactobacillus* sp. and *Oenococcus* sp. ([Cousin *et al.*, 2017](#)). Presence of these LABs in ACV will help to improve the dysbiosis condition in AV, which is caused by the increase in numbers of *E. coli* and the decrease in numbers of Lactobacilli. Moreover, acetic acid that is present in ACV is reported to have potent antibacterial efficacy

([Halstead et al., 2015](#)). Therefore, the objectives of the present work were to investigate the efficacy of Apple cider vinegar to promote growth of Lactobacilli and simultaneously inhibit *E. coli* in the vagina, in order to treat AV naturally.

2. Materials and methods

2.1. Materials

Bragg's Apple cider vinegar was purchased from Organic India store, Pune, India. On the other hand, *L. acidophilus* and *E. coli* strains were provided by the National Collection of Industrial Microorganisms (NCIM), Pune, India.

2.2. Growth of *L. acidophilus* and *E. coli*

A single colony of *E. coli* was taken carefully and emulsified with 2 ml sterile saline in a test tube and its turbidity was adjusted equivalent to 0.5 McFarland. This bacterial suspension was further used to carry out the antibacterial activity using a cup plate assay ([Balouiri et al., 2016](#)). This bacterial suspension of *E. coli* (1 ml) was inoculated aseptically into 10 ml of sterilized nutrient broth (NB) tubes, and incubated at 37 °C for 24 h, for carrying out the turbidimetric analysis ([Balouiri et al., 2016](#)).

Lactobacillus acidophilus is a normal Gram-positive anaerobic micro-organism, which grows at pH 4.5 at 37 °C ([Mousavi et al., 2013](#)). The bacterial suspension of *L. acidophilus* was prepared in the same manner as described earlier for *E. coli*. About 1 ml of this bacterial suspension was inoculated into 10 ml of sterile nutrient broth (NB) tubes, and grown anaerobically under aseptic condition in a desiccator at 37 °C for 24 h, to carry out the turbidimetric analysis. Anaerobic condition in the desiccator was maintained by keeping a burning candle inside it. Combustion of the candle consumed the oxygen present in the desiccator and thus anaerobiosis was generated ([Patole and Pandit, 2018](#)).

2.3. Antibacterial activity

The antibacterial potential of ACV was determined using the cup-plate assay. Using the previously prepared bacterial suspensions of *E. coli* and *L. acidophilus* of 1.5×10^8 cfu/ ml each, approximately (0.1 ml) of each bacterial suspension was individually inoculated into the surface of nutrient agar (NA) petri plates, and spread uniformly with the aid of a sterile glass spreader. Individual cups each of 6 mm diameter were punched in the NA plates using a sterile cork borer, and then 1 ml of ACV (5 % acidity) was filled aseptically into these cups. Petri-plates were kept in refrigerator for 15 min. to diffuse the solution, and then incubated at 37 °C for 24 h. The assay was carried out in triplicates. After incubation, the zone of inhibition was measured using a calibrated ruler ([Balouiri et al., 2016](#)).

2.4. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of ACV

MIC is defined as the lowest concentration of an antibacterial agent that inhibits the growth of a bacterial strain. On the other hand, MBC is the lowest concentration of this agent required to kill or reduce the viability of an initial bacterial inoculum by ≥ 99.9 % over a fixed period, and under a specific set of conditions. MBC is complementary to the MIC.

The same standard concentration of 1×10^7 cfu/ml of both *E. coli* and *L. acidophilus* was used to assess their susceptibilities to the different concentrations of ACV, according to [Edris et al., \(2017\)](#). To define the MIC of ACV against both of the tested bacterial strains, different sets of NB treatments were prepared individually. The sets included negative control tubes (NB without *E. coli* or *L. acidophilus* and without of ACV); positive control tubes (NB tubes inoculated individually only with *E. coli* or *L. acidophilus* without ACV); and the treatment tubes (NB tubes inoculated individually with *E. coli* or *L. acidophilus*, and containing different concentrations of ACV, mainly; 0.5, 1, 1.5 and 2 ml of ACV). The MBC was defined by sub-culturing 1 ml of the tube that represented MIC of each bacterium individually into

the surface of NA petri plates, and then spread uniformly using a sterile glass spreader ([Edris et al., 2017](#)). Plates were incubated at 37°C for 24-48 h. After incubation, the number of bacteria (cfu\ ml) or (cfu\ g of sample) was evaluated as follows:

cfu\ ml = (Number of colonies)/ (Dilution × volume of inoculum)

MBC was recorded if no growth was observed on the NA plates, which confirmed that the effect of AVC on the tested bacterial strains was bactericidal and not bacteriostatic ([Edris et al., 2017](#)).

2.5. Measurement of turbidity using Nephelometer

The turbidimetric assay is a simple and rapid quantitative determination method of the bacterial population ([Metzger et al., 2018](#)). In Nephelometry, the particle density is a function of the scattered light directed to the detector. The amount of scattered light is directly proportional to the amount of insoluble particles in a solution. The higher the amount of particles scattering the light beam, the more light reaches the detector, the more Nephelometric Turbidity Units (NTU) is recorded ([Dalgaard et al., 1994](#)). The turbidimetric analysis was carried out to assess the individual growth of *E. coli* and *L. acidophilus* in the simulated vaginal fluid (SVF) medium, as when the bacterium grows in the SVF medium, more amount of bacterial cells were detected in this medium. When these treated SVF were subjected to turbidimetric analysis, more amount of light was scattered reflecting the increase in the bacterial population, and hence indicated the growth of *E. coli* and *L. acidophilus*. Aerobic vaginitis occurs due to the imbalance between the levels of *E. coli* and *L. acidophilus* in the vagina. Therefore, growth of these bacterial strains was studied in SVF. Simulation of the vaginal environment was created using SVF medium for growth of the anaerobic bacterium ([Borges et al., 2012](#)). The SVF was prepared as follows: 3.51 g/ l sodium chloride, 1.40 g/ l potassium hydroxide, 0.222 g/ l calcium hydroxide, 0.018 g/ l bovine serum albumin (BSA), 2 g/ l lactic acid, 1 g/ l acetic acid,

0.16 g/ l glycerol, 0.4 g/ l urea and 5 g/ l glucose, in reference to [Owen and Katz, \(1999\)](#).

The effect of ACV on the growth of *E. coli* and *L. acidophilus* in SVF was studied using turbidimetric analysis. In this study, *E. coli* (10⁷ cfu\ ml) and *L. acidophilus* (10⁷ cfu\ ml) were grown individually in 10 ml of SVF containing 1 ml of ACV. Control treatments were also used in absence of ACV. Different dilution groups were made namely Groups I to IV. The composition of Group I: 10 ml SVF+ 1 ml of NB culture of *E. coli* (10⁷ cfu\ ml); Group II: 10 ml SVF + 1 ml of NB culture of *L. acidophilus* (10⁷ cfu\ ml); Group III: 10 ml SVF + 1 ml of NB culture of *E. coli* (10⁷ cfu\ ml) + 1 ml ACV; Group IV: 10 ml SVF + 1 ml of NB culture of *L. acidophilus* (10⁷ cfu\ ml) + 1 ml ACV. All the test tubes were incubated at 37°C for 24 h. The developed turbidity due to bacterial growth was measured at 600 nm using nephelometer (331, Electronics, India), and was recorded as a Nephelometric Turbidity Unit (NTU). This assay was carried out in triplicates.

2.7. Statistical analysis

The antibacterial effect of AVC on *E. coli* and *L. acidophilus* was assessed by measuring the zone of inhibition, where the results were expressed as the mean value of three independent replicates ± the standard deviation (SD) The data derived from the turbidimetric analysis was subjected to statistical analysis using Graph pad prism version 7 (student t-test), to find the statistical significance of the effect of ACV on growth of *E. coli* and *L. acidophilus*.

3. Results and Discussion

3.1. Study of antibacterial potential

The ACV produced a zone of inhibition diameter of 32.9 ± 0.5 mm against *E. coli* (Fig. 1a). Thus, ACV demonstrated good antibacterial efficacy against *E. coli*, the causative agent of AV. The presence of phenolic compounds and organic acids in ACV contribute to its antibacterial properties ([Halstead et al., 2015](#); [Du et al., 2019](#); [Lagana et al., 2019](#); [Ousaaid](#)

[et al., 2021](#)). The antibacterial effect of ACV against *E. coli* is attributed to the presence of several phenolic compounds including; vanillic acid, chlorogenic acid, caffeic acid, gallic acid, catechin, epicatechingallate and phlorizin; however, the most prominent being gallic acid and tannins ([Yagnik et al., 2018](#)). Gallic acid and tannins express antibacterial potential by binding to the bacterial cell membrane ergosterol, which is responsible for maintaining the integrity of the microbial cell wall, and also by inhibiting the activity of the catalase enzyme ([Lagana et al., 2019](#); [Ousaaaid et al., 2021](#)). Moreover, ACV possess organic acids such as acetic acid and lactic acid, which release protons into the bacterial cell wall and decrease its intracellular pH, causing destabilization of the bacterial cell wall and eventually leads to the cell death ([Lourenço et al., 2019](#)). Furthermore, the organic acids decrease the raised vaginal pH > 5 and

restore it back to its normal level ([Lourenço et al., 2019](#)). Lactic acid is reported to exert antibacterial effect specifically against the Gram- negative bacteria ([Zhang et al., 2021](#)). As being a fermented food product, ACV contains both the lactic acid and acetic acid bacteria, which produce lactic acid and acetic acid, respectively ([Aumiller et al., 2021](#)). Lactates and acetates enzymes are known to promote the growth of *Acetobacters* and *Lactobacilli*, respectively ([Aumiller et al., 2021](#)). *Acetobacters* have the characteristics of probiotics. Thus, *Acetobacters* and *Lactobacilli* share a synergistic relationship and mutually promote the growth of one another through cross feeding ([Aumiller et al., 2021](#); [Guine et al., 2021](#)). Thus, ACV did not present antibacterial potential against *L. acidophilus*; however, it promoted *L. acidophilus* growth, where no zone of inhibition was observed on the treated NA plates (Fig. 1.b).

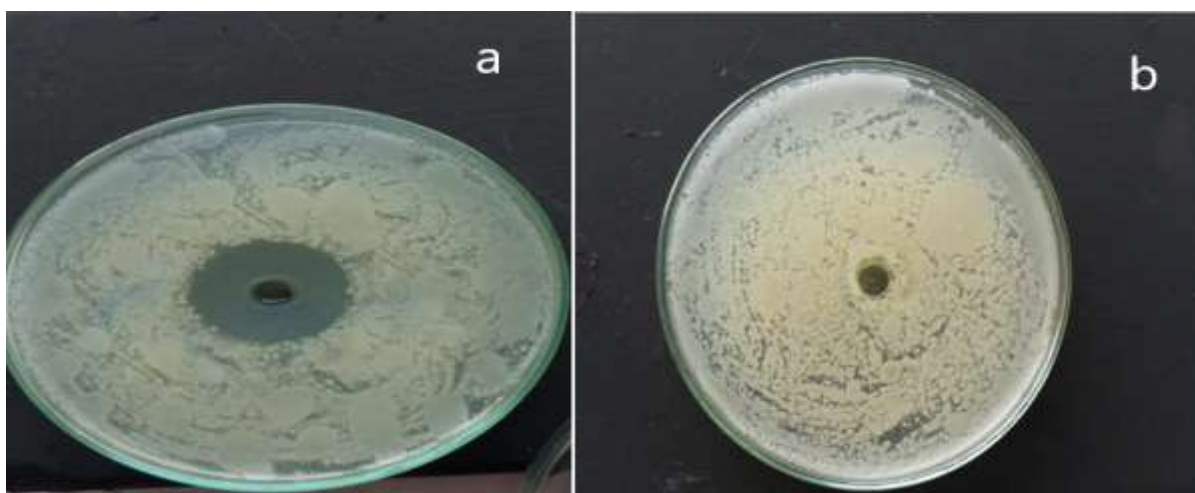


Fig. 1: a)-Antibacterial effect of ACV against *E. coli* (zone of inhibition was observed), b)-Antibacterial potential of ACV against *L. acidophilus* (no zone of inhibition)

3.2. Determination of the MIC and MBC

The absence of bacterial growth in the MIC test and then its growth on the NA plates indicates that although a particular antibacterial agent caused inhibition; however, further plating of the bacteria onto NA still result in bacterial proliferation, which is attributed to the bacteriostatic mode of action of this antibacterial agent (Edris *et al.*, 2017). Results of MIC and MBC are summarized in Table (1). Therefore, the MBC was performed to get the exact antibacterial dose of the ACV. Turbidity was observed in all the tubes containing the tested concentrations of AVC against *L. acidophilus* (Fig. 2). Results of MBC demonstrated that *L. acidophilus* expressed growth on the NA plates when taken from all the tested ACV concentrations

(0.5, 1, 1.5 and 2 ml). However, in the case of *E. coli* (Fig. 3), not all tubes expressed turbidity. Thus, the recorded MIC of ACV tested against *E. coli* was 0.5 ml. Thereafter, the MBC of ACV at a concentration of 0.5 ml was tested against *E. coli*, which did not present any growth. Accordingly, these results revealed the bactericidal action of ACV against *E. coli*; however, no such activity was detected against *L. acidophilus*. These results are in line with the study conducted by Yagnik *et al.*, (2018), reporting the antibacterial potency of ACV against *E. coli*. Meanwhile, the previous study of Cousin *et al.*, (2017) confirmed the presence of lactic acid bacteria in the ACV, which could have supported the growth of *L. acidophilus*. These results were recorded from the current MIC and MBC studies.

Table 1: Determination of MIC for both *E. coli* and *L. acidophilus* induced by ACV

Test Tube	Volume of nutrient broth medium (ml)	Concentration of ACV (ml)	Visual results <i>L. acidophilus</i>
a	5.0	2	Clear
b	5.0	1.5	Clear
c	5.0	1	Clear
d	5.0	0.5	Clear
e	5.0	0.0	Turbid
f	5.0	0.0	Clear
Test Tube	Volume of nutrient broth medium (ml)	Concentration of ACV (ml)	Visual results <i>E. coli</i>
g	5.0	0	Clear
h	5.0	2	Turbid
i	5.0	1.5	Turbid
j	5.0	1	Turbid
k	5.0	0.5	Turbid
l	5.0	0.0	Turbid

Where; the tested concentrations of ACV were represented using the following letters; a: 2 ml; b: 1.5 ml; c: 1 ml; d: 0.5 ml of ACV; e: positive control; f: negative control (tested against *L. acidophilus*). On the other hand, g: negative control; h: 2 ml; i: 1.5 ml; j: 1 ml; k: 0.5 ml of ACV and l: positive control) tested against *E. coli*. *Negative control refers to NB tube without *E. coli* or *L. acidophilus* and without of ACV, while, Positive control refers to NB tube inoculated individually with *E. coli* or *L. acidophilus* and without ACV

3.3. Detection of growth of the tested bacterial strains in SVF using turbidimetric analysis

The turbidity observed for *E. coli* in SVF (Group I) was 1500 NTU, while that for *L. acidophilus* in SVF (Group II) was 1578 NTU. However, the recorded turbidity in Group III was reduced to 900 NTU, which was statistically significant ($p < 0.001$) when compared to Group (I) (Fig. 2). These results demonstrate the antibacterial action of ACV on *E. coli*. Furthermore, the turbidity observed in Group IV was slightly

increased to 1659 NTU, which was statistically insignificant when compared to Group II. Thus, no growth inhibition was recorded by *L. acidophilus* in SVF. However, ACV slightly favored growth of *L. acidophilus*, which was evident from the slight increase in the recorded turbidity of Group IV. The turbidimetric analysis further supported the efficiency of ACV in slightly promoting the growth of *L. acidophilus* and inhibiting the growth of *E. coli* (Table 2).

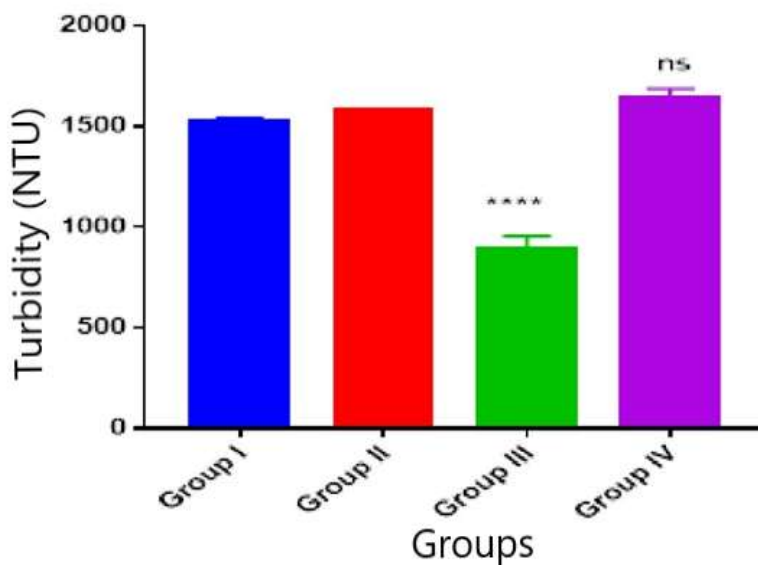


Fig. 2. Effect of ACV on growth of *E. coli* and *L. acidophilus*. Error bars represent the standard error (SE). Data was analyzed by Graph pad prism version 7. (Where; student t test (unpaired) **** ($p < 0.001$) ns: - non –significant). Groups I and III represent the treatment groups for *E-coli*, and Groups II and IV represent the treatment groups for *L. acidophilus*

Table 2: Measurement of *E. coli* and *L. acidophilus* bacterial growth in SVF using turbidity analysis

Group	Dilution fluid + Bacterial culture	Turbidity (NTU)
I	SVF + 1 ml of NB culture of <i>E. coli</i>	1500 ± 0.5
II	SVF + 1 ml of NB culture of <i>L. acidophilus</i>	1578 ± 0.6
III	SVF + 1 ml of NB culture of <i>E. coli</i> + ACV	900 ± 0.2
IV	SVF + 1 ml of NB culture of <i>L. acidophilus</i> + ACV	1659 ± 0.5

Where; (±): indicates standard error. SVF: simulated vaginal fluid, NB: Nutrient broth. Results are means of 3 replicates

The turbidometric results indicate that ACV could help to restore the disturbed vaginal microflora by inhibiting the growth of *E. coli* as observed by reduction in turbidity in Group (III), in accordance with the previous study of [Yagnik et al., \(2018\)](#). Meanwhile, the possible reason for the slight increase in turbidity observed in group (IV) could be attributed to the presence of acetic acid in ACV, which was reported previously to enhance growth of the lactic acid bacteria ([Safari et al., 2017](#)), thereby promoted growth of the *L. acidophilus* in SVF.

Conclusion

AV is a vaginal infection due to imbalance in the number of bacteria that reside in the vagina. Therefore, the line of treatment was to maintain bacterial balance. ACV served dual functions by reducing the growth of *E. coli*, and simultaneously enhancing the growth of *L. acidophilus*. Thus, ACV plays an important role in maintaining the normal flora in the vagina. ACV showed good antibacterial activity against *E. coli*, while favored the growth of *L. acidophilus* in the SVF. Finally, Apple cider vinegar is regarded as a good adjuvant treatment to maintain the microbiota of the vagina, thus could be used effectively to avoid the excessive use of the synthetic anti-bacterials, steroids and antibiotics, which are currently manipulated to treat AV.

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Conflict of interest

The authors declare that they have no conflict of interests.

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Ethical approval

Not applicable.

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