Chapter

Probiotics and Postbiotics from Food to Health: Antimicrobial Experimental Confirmation

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Abstract

The field of probiotics is up-and-coming, especially in management of microbial pathogens. Probiotics confer nutritional benefits, reduce inflammation and infection. Probiotics have also shown to be helpful in the management of microbial pathogens, which include bacteria, fungi, and viruses. To ernes this potential maximumly, there is a need for an elaborate screening system for new isolates. This entails; rigorous screening methods and thorough confirmatory systems. There is need also to come up with standard methods used to evaluate the probiotics mechanism of action both in vivo and in vitro. In summary, there is a need for a standard screening process for probiotic microorganisms that is reproducible. The aim is to ensure that, the candidate microbial cultures are not written off without proper investigations. This will also fasten the screening process and save time and resources wasted in pre-screening experiments.

Keywords: probiotics, screening methods, confirmatory methods, postbiotics, animal model, coculture

1. Introduction

Fermentation is one of the oldest technologies used for food preservation. It involves converting carbohydrates to alcohol, carbon dioxide, and organic acids using microorganisms under anaerobic conditions. The fermentation process improves food by developing diverse flavors, aromas, and textures in food substrates. Also, it enriches food substrates with protein, essential amino acids, essential fatty acids, and vitamins. The primary mechanism of the preservation of foods is the production of acid, which lowers the pH to a level at which most of the spoilage-causing microorganisms cannot grow, hence prolonging the shelf life of such foods [1]. At present, various fermented foods are produced worldwide at household and industrial levels, in both small-scale and large commercial enterprises. Associated with fermentation are beneficial microorganisms known as probiotics. The vast majority of the probiotics are lactic acid microorganisms [2] to produce fermented dairy products.

Among the beneficial effects of probiotics include improved intestinal health, enhancement of the immune response, reduction of serum cholesterol, and cancer prevention [3–5]. There is also substantial evidence to support probiotic use in

treating acute diarrhoeal diseases, prevention of antibiotic-associated diarrhea, and improvement of lactose metabolism [6]. The range of food products containing probiotic strains is vast and still growing. And so is the list of beneficial effects. More so, with an increasing desire for quality life, preference for minimal use of chemicals, and the rising cost of healthcare. Natural products like probiotics is a promising alternative. Related to probiotics are prebiotics. Biogenics involves the use of beneficial bioactive substances produced by probiotic bacteria whose activities are independent of the viability of probiotic bacteria.

This book chapter focuses on the use of probiotics in the management of microbial pathogens, emphasizing the need to have a reproducible standard screening process both *in vivo* and *in vitro*. This will highlight areas in the used technologies that need harmonization, technologies for investigation and confirmation of the antimicrobial activities of probiotics, and finally, the future prospects of probiotics and antimicrobial agents.

1.1 Mechanism of action of probiotics

WHO/FAO defines probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [7, 8]. Prebiotics refer to the substrates that are selectively utilized by host microorganisms that result in conferring a health benefit to the host [8–11]. Furthermore, postbiotics entails the use of beneficial bioactive compounds produced by probiotic bacteria. The activity of postbiotics is independent of probiotic bacteria's viability [11]. The term synbiotics is where both prebiotics and probiotics are utilized simultaneously [11, 12].

The probiotics have myriad of mechanisms in which it protects against infection. These include; (1) they lower pH, (2) pathogen antagonism by producing antimicrobial compounds for example, bacteriocins and or other metabolic products, (3) competitive exclusion with the pathogen for binding sites and receptors sites, (4) competition for substrates that is, nutrients and growth factors, therefore, limiting resources, (5) stimulate immunomodulatory cells, (6) production of enzymes example, enzymes that neutralize toxins produced by pathogens (7) improve the barrier function of the intestinal mucosa, (8) modulate inflammatory responses, (9) aggregate with pathogens, (10) produce hydrogen peroxide (H_2O_2) a strong oxidizing agent that damage nucleic acids and proteins, (11) produce organic acids like lactic acid, acetic acid among others (12) produce CO_2 thus creating anaerobic microenvironment (**Figure 1**) [3, 4, 13–16].

The probiotics are generally regarded as safe [17]. The few results obtained when probiotics are administered together with conventional drugs clinically are promising and include synergy with the drug, half dose of conventional drug needed, and faster healing [18–21]. Further research is needed in this area.

1.2 Probiotics as antimicrobial agents

Besides the health-improving benefits, the antimicrobial activity of probiotics has been well documented, with promising results against microbial pathogens. Probiotics have been deemed as the following most crucial immune defense systems according to WHO [7]. This is due to increasing antibiotic resistance to commonly prescribed antibiotics [22, 23]. There is a need, therefore, to come up with reproducible screening protocols for *in vitro*, *in vivo*, and clinical studies. Therefore, this book chapter will highlight protocols used in screening probiotics and postbiotics, cite their strengths and drawbacks, and point areas that need harmonization.

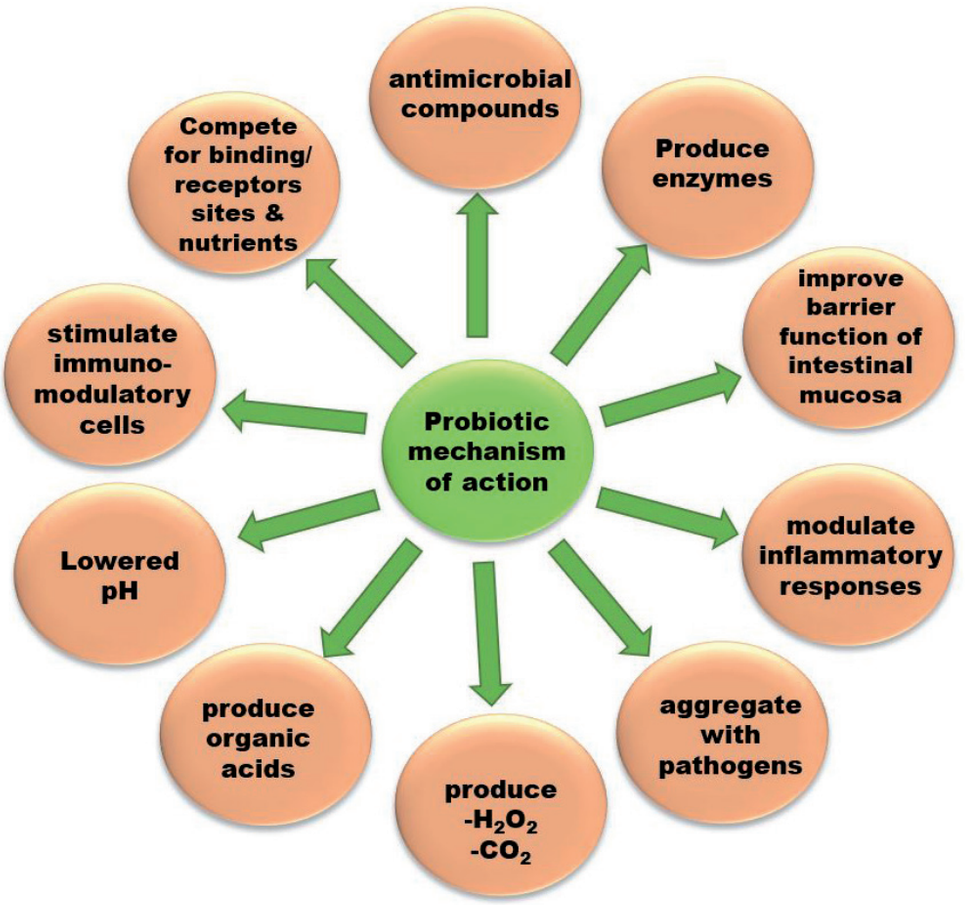

Figure 1.
Mechanisms in which probiotics protect against infection.

2. Areas in the used technology that need harmonization

To obtain reproducible and conclusive results of probiotic antimicrobial activity, standardization of protocols is essential. This section reviews the essential areas that will inform on the choice of indicator pathogen, probiotic microorganism, inoculum size, incubation time and conditions, and technique of production of postbiotics (also referred to as cell-free supernatant (CFS)/ Biogenic/spent media) used in previous research and the need for harmonization.

2.1 The selection of experimental indicator pathogen

The choice has relied on the target disease that the probiotic is thought to treat. Thus, for vulvovaginal candidiasis *C. albicans,* the predominant pathogen has been chosen [24, 25], even though *Candida glabrata* has also been screened [26, 27]. Enterotoxigenic *E. coli* and *Salmonella typhimurium* is the choice for studying gastrointestinal infection [28]. However, while screening new probiotic microorganisms for general antimicrobial activity, major classes of pathogens of medical importance should be representatively tested [29]. For example, studies on fungal pathogens should include at least a dermatophyte, non-dermatophyte, and yeast. Antibacterial should consist of a Gram-positive and a Gram-negative bacterial pathogen. Furthermore, clinical, typed microorganism and drug-resistant strains should be included due to emerging resistance [10].

2.2 The choice of probiotic microorganism and postbiotics

The WHO/FAO has listed the criteria for evaluation of probiotic microorganism, which include; the ability of the microorganism to adhere to epithelial cells, bile salts, resist stomach acid and enzymes, persistence within the system, produce antimicrobial compounds, antibiotic resistance profile inability to confer resistance or genome stability and ability to stabilize the normal microbiota among others [7, 15, 30]. Probiotic antimicrobial activity is strain-specific; therefore, the species level and strain of the selected probiotic should be identified.

2.3 Inoculum size

The actual number of viable indicator pathogens in the inoculum size directly influences the outcome. Too little may lead to false-positive results, while too heavy inoculum may give a false negative result [29, 31]. A foundation for the inoculum size can be suggested by CLSI [32]. Researchers have used different inoculum size, incubation temperature, and time for both probiotic and indicator pathogen in *in vitro*, *in vivo* and clinical studies [9]. We propose that the viability and dose of probiotic microorganisms used (also in the production of postbiotics) be established by dose-dependent experiments. This should be indicated in experimental reports.

2.4 The experimental conditions and incubation time

Lactic acid bacteria and Bifidobacteria are fastidious; subsequently, the media chosen should have a specific nutrient requirement, for example, growth factors. MRS, which is an appropriate media for the growth of probiotic microorganisms, is widely used. MRS is both a selective and an enriched media for the growth and isolation of only lactic acid bacteria and other bacteria. Therefore, if this medium cannot support the indicator pathogen, for example, dermatophytes (J. [33]), probiotic growth factors can be incorporated in any media of choice such as potato dextrose agar (PDA), sabouraud dextrose agar (SDA) and nutrient agar (NA) to favor the growth of both the indicator pathogen and the probiotic microorganism. Proper choice of media and specific modifications is key to a successful experiment [33, 34]. Therefore, media supplemented with growth factors should be screened for the ability to grow both probiotic microorganisms and indicator pathogen. We propose that the specific incubation conditions such as time and oxygen requirements for both probiotic microorganisms and indicator pathogen be optimized before the experiment and confirmed by the growth curve of individual microorganisms (**Figure 2**). Furthermore, fresh media should always be prepared and used for reproductive results, especially in the case of disc diffusion results.

2.5 The technique of production of postbiotics

The postbiotics is also referred to as cell-free supernatant (CFS) or biogenics or spent media. The preparation of postbiotics is varied and attests to the need to harmonize the methods. The process entails the following steps; the probiotic microorganism is inoculated in broth media and incubated in an incushaker [35]. Cells are then removed by centrifuging to obtain the CFS ([36]; J. [33, 35, 37]). The supernatant obtained can then be screened for antimicrobial activity [35], or the supernatant is further filter-sterilized [35, 36]. The CFS is then used to screen for the microbial activity or concentrated to obtain concentrated CFS (cCFS) [36, 38] or freeze-dried [36].

The advantage of using postbiotics is that the properties of the active component can be deduced. To ascertain if the active ingredient is proteinaceous, heat treatment and enzymes are used. If the activity is reduced or is lost compared

to non-treated postbiotic, it infers that the antimicrobial agent is proteinaceous [33, 38]. To ascertain if the antimicrobial activity is pH-related, the postbiotic is neutralized and buffered [30].

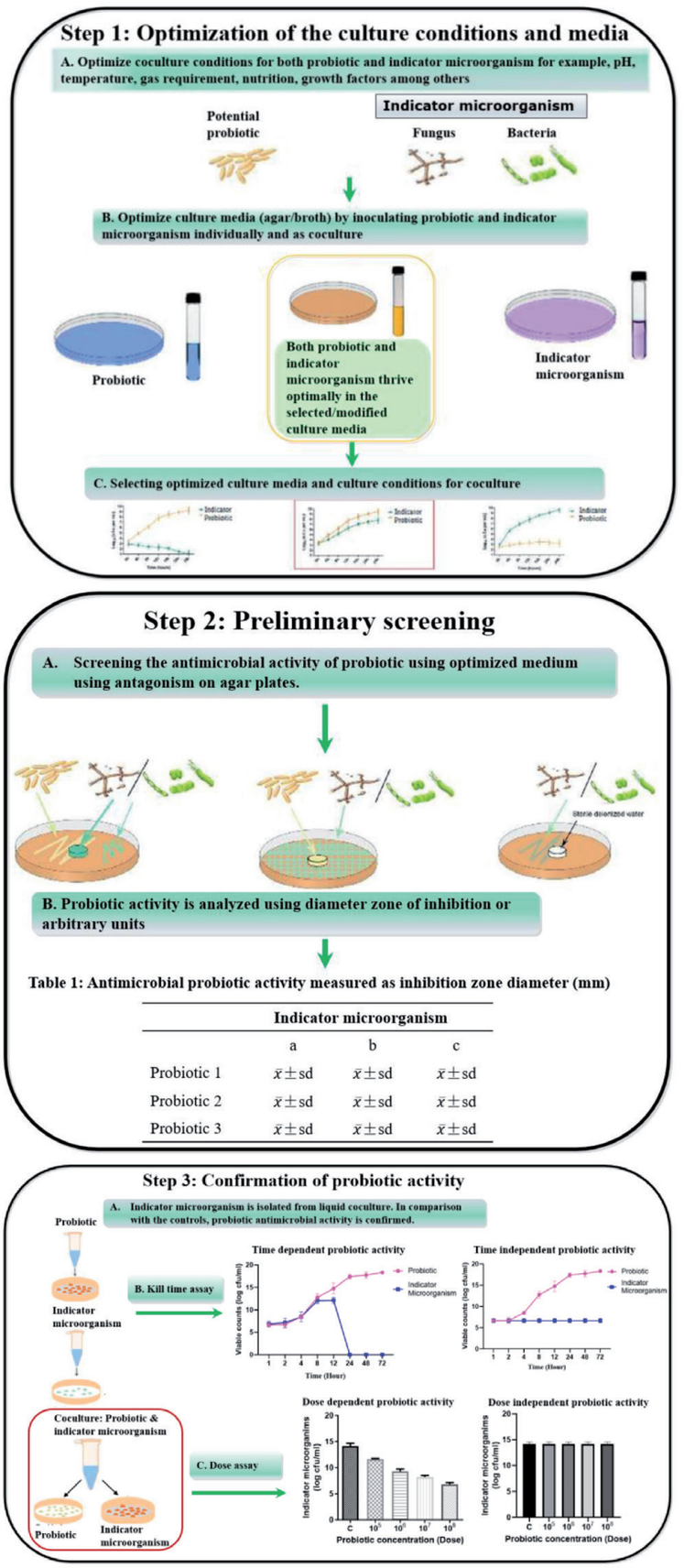

Figure 2.
Detailed proposed method for conclusively screening probiotic antimicrobial activity. Step 1 entails choice of optimal media and growth conditions, step 2 is the preliminary screening on agar and step 3 is the confirmation of probiotic antimicrobial activity in liquid cocultures.

3. Technology for investigation and confirmation of the antimicrobial activity of probiotics

3.1 Experiments for investigation of antimicrobial potential of probiotics *In vitro*

Included in this section are the *In vitro* antagonism methods on agar plates and liquid coculture for checking probiotic antimicrobial activity with their strengths and drawbacks. In antagonism on agar plate's methods, probiotic and indicator microorganism is introduced in the same plate. The difference is the sequence and manner of the inoculation of either indicator pathogen or probiotic microorganisms. After incubation, the diameter zone of inhibition which is the clear zones around the inoculated area, is then read in millimeters or reported or arbitrary units (AU) (**Figures 2** and **3**).

3.1.1 Antagonism on agar plate methods

3.1.1.1 Simple spot-on lawn assay

To screen postbiotics or probiotic microorganism using this method, the indicator pathogen is first inoculated, then probiotic microorganisms are spotted at specific points on solid media [37]. **Modification** to the method entails spotting probiotic microorganisms as parallel lines [33]. Its strength includes (a) media can be modified [33, 34], (b) it has an option of different incubation conditions, i.e., probiotic microorganism incubation conditions are first optimized, followed by optimizations for indicator pathogen.

3.1.1.2 Spot on agar assay

Probiotic microorganisms are first spotted on agar media and then incubated [39]. An indicator pathogen is added, and soft agar at around 45–50°C is poured to

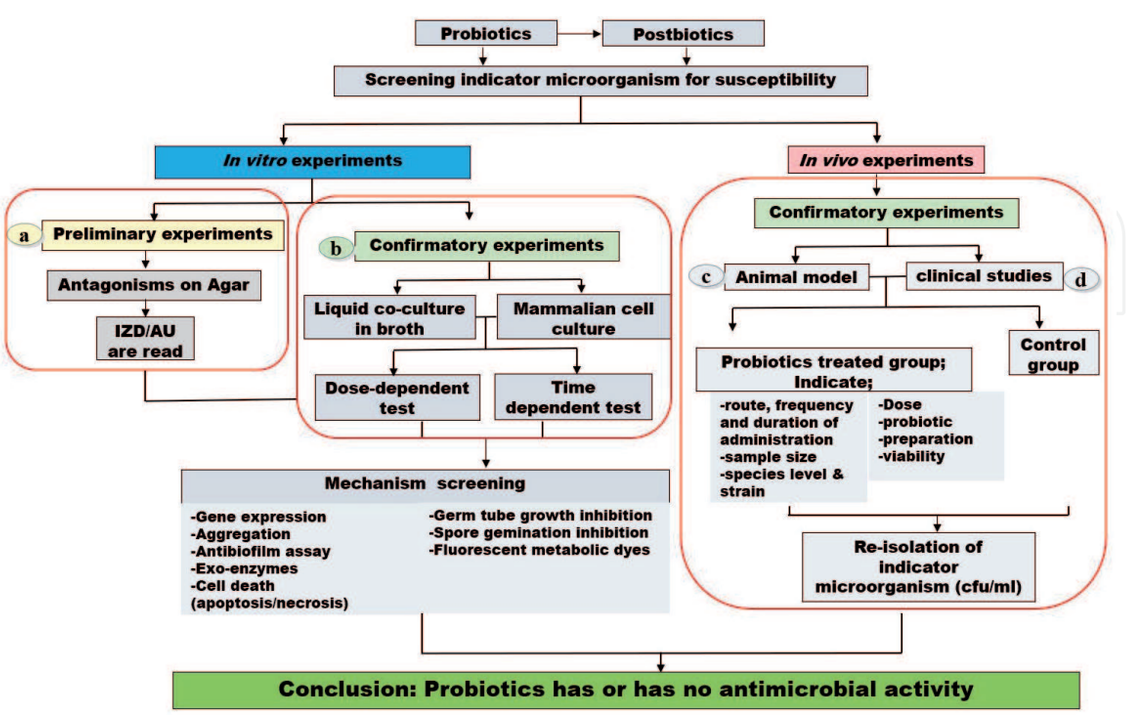

Figure 3.
Abridgement of methods involved in screening probiotic microorganisms both In vitro *and* In vivo. *(a)* In vitro *preliminary screening experiments on agar. (b)* In vitro *confirmatory experiments. (c)* In vivo *animal confirmatory experiment. (d)* In vivo *clinical confirmatory experiment.*

the previously prepared plate spotted with a probiotic microorganism [25, 35]. The advantage of the Agar spot test is that two different media can be used, one for spotting and the other as overlaid soft agar. Indicator and probiotic microorganisms can be grown at different times, meaning incubation conditions can be adjusted for each microorganism. The disadvantage is the high temperature of soft agar, i.e., between 45°C and 50°C, killing heat-labile indicator microorganisms. The strict aerobes may not grow well due to the pour plate method.

3.1.1.3 Spot on lawn assay with wells also referred to as Agar well diffusion assay or as conventional whole plate method

The wells are dug and indicator pathogen inoculated. Then postbiotic/Probiotic is dispensed [37, 40]. Unlike the simple spot-on lawn assay method, the probiotic microorganism can be allowed to grow first before introducing the indicator microorganisms or vice versa.

3.1.1.4 Paper disc assay

The postbiotic/probiotic is dispensed on the paper discs and placed on the inoculated media. The inoculation of both indicator pathogen and probiotic microorganism is simultaneous. The disadvantage of this method is that the results are not reproducible [41]. This is mainly attributed to the production of non-diffusible antimicrobials.

3.1.1.5 Cross streak on agar assay

Entails streaking the probiotic microorganism as parallel lines on media. A perpendicular line of indicator pathogen is then streaked. Growth inhibition is determined at the interception point [40].

3.1.1.6 The radial streak on agar assay

The probiotic microorganism is inoculated as a circle in the middle of the agar plate. The indicator pathogen is then streaked as radial lines from the edge of the petri dish to the center, and growth inhibition is examined [42]. Another method closely related to this method is cutting the media with the probiotic microorganisms and placing it on top of the indicator pathogen inoculated plate.

3.1.2 Liquid coculture method

The probiotic and indicator pathogens are both introduced to optimized broth culture media, then incubated. Samples are intermittently collected, and viability (cfu/ml) of indicator pathogen is established. It is used to determine if the probiotic effect is static or cidal [13, 24]. It may also be used to reveal the mechanism by which the probiotic bacteria exert their antimicrobial activity [35]. Microtitre assay is used to screen minimum inhibitory concentration (MIC) of postbiotics using microdilution method, macro serial dilution, or conventional kill time assay [35, 43]. Liquid coculture assay is recommended as a confirmatory test (**Figures 2** and **3**).

3.1.3 Summary of antagonism assay

Antagonism assay on agar plates has the advantage of being fast and straightforward. The disadvantage is that it does not directly interact with the probiotic

microorganism or postbiotics and indicator pathogen. Consequently, the probiotic microorganism should produce sufficient antimicrobial agent that should have the potential to diffuse through solid media in terms of size and spatial centrifugation [44]. Accordingly, it is not prudent to use these methods solely to ascertain antimicrobial activity of probiotic microorganisms. Hence, it is recommended to combine antagonism on agar plates and liquid coculture to establish the antimicrobial activity of probiotic microorganisms and postbiotics (**Figures 2** and **3**).

3.1.4 Cell culture and tissues

To closely mimic human infection, human cell cultures are infected with indicator pathogen, then treated with probiotic cultures or postbiotics [41].

3.2 Experiments for the discovery of antimicrobial mechanisms of probiotics

The methods used to ascertain probiotic microorganism mechanisms of antimicrobial activity include; the ability to inhibit virulence factors and cell death.

3.2.1 Ability to inhibit virulence factors

The virulence factors of pathogenic microorganisms vary from one microorganism to the other. For example, the virulence factor in bacteria includes adhesion, immunoevasion and immunosuppression, exo-enzymes, and exotoxin, among others [45]. The virulent factors in *Candida* include secretion of hydrolases, yeast to hypha transition, contact sensing, thigmotropism, biofilm formation, phenotypic switching, and range of fitness attributes [27, 37]. The following methods can be used to examine the ability of the probiotic microorganism to inhibit the virulence factors;

3.2.1.1 Gene expression levels

The expression levels of specific genes controlling one or more of these virulence factors can be ascertained when checking for probiotic activity [14, 25, 35, 36, 46–48]. The methods used include microarray analysis, RT-PCR techniques, and western blot [49].

3.2.1.2 Aggregation and coaggregation assay

Aggregation assay using spectrophotometric autoaggregation and coaggregation is used to ascertain the antimicrobial activity of probiotics [26, 38, 50]. The morphological transition of *C. albicans* that is, germ tube formation contributes to adherence and invasion to the host tissue and increases virulence [51, 52]. Lactobacilli build aggregates and co-aggregates with Candida cells, and this process neutralizes germ tube growth [53]. In addition, the coaggregation protects access of pathogens to a cell receptor and, as a result, inhibit pathogen adhesion which is a prerequisite step for colonization and the subsequent development of disease [26, 44, 50, 54].

3.2.1.3 Antibiofilm Assay

Biofilm produced by pathogens serves as a physical barrier and increases virulence. Antibiofilm assay includes (a) **static systems** like microtiter plate, Molony biofilm, Calgary biofilm device, biofilm ring test (b) **open systems** such as Kadouri

system, flow cell, perfused (membrane) biofilm fermenter, microfermentors, Modified Robbins Device, sorbarod devices (SBF), drip flow reactor, constant depth film fermenter, microfluidic biochips, rotating disc reactor, BioFlux device, annular reactors, CDC biofilm reactors (c) **microcosm** example airway epithelial cell model, reconstituted human epithelia (RHE), endothelial cells under flow model, Zürich oral biofilm-model, microfluidic coculture model, Zürich burn biofilm-model, multiple Sorbarod devices (MSD) (d) **ex-vivo which include;** candidiasis in the vaginal mucosa, RWV bioreactor, cardiac valve *ex vivo* model, root canal biofilms [55]. Viable colonies can also be used. While fluorescent labeling of biofilm coupled with mathematical labeling is used [41].

3.2.1.4 Exo-enzymes

The indicator microorganisms are treated with probiotics or CFS. The indicator microorganism is then examined for the ability to produce exo-enzymes on agar plate assays. The agar plate contains a suitable substrate specific to each enzyme activity [56].

3.2.1.5 Electron microscopy

Scanning electron microscopy and Transmission electron microscope are used to examine cell integrity which includes morphological adherence, distortion, biofilm, or apoptosis [27, 50, 57].

3.2.1.6 Germ tube and hyphal growth inhibition

The pelleted spores of dermatophytes and dimorphic pathogenic fungus are allowed to develop germ tubes and hyphae. Probiotic or CFS is then added and incubated. Growth is determined by examining germ tubes and hyphae [36, 58].

3.2.1.7 Spore germination inhibition assay

The pelleted mycelia and probiotic or CFS are added to media and incubated. Samples are withdrawn and microscopically examined. Percentage spore germination is calculated by the following formula [33, 36, 58]:

% spore germination = [Numbers of germinated spores /Numbers of total spores] × 100

3.2.1.8 Fluorescent metabolic dyes and Confocal laser scanning microscopy

The indicator microorganisms are treated with probiotic cultures or CFS then stained with fluorescent dyes according to the manufacturer's instructions. The live or dead cells are counted, and their metabolic activity is ascertained [26, 27]. Live/dead cells can also be confirmed by viable counts (cfu/ml).

3.2.2 Ability to induce cell death

A sequence of unique morphological changes outlines apoptosis. These include; visible cell shrinkage, extensive plasma membrane blebbing, chromatin condensation, nuclear fragmentation, formation of apoptotic bodies, which later undergoes decomposition within the phagosome and finally terminates with complete recycling of the components [59, 60]. Accumulation of reactive oxygen species (ROS) decreased membrane potential, biochemical and cytological

responses well known in programmed cell death (PCD), for instance, apoptosis [60]. Very high ROS concentrations induce necrosis [61]. These changes can be used to determine cell integrity. Of the biochemical and cytological methods used to check pathogen cell integrity after treatment with probiotics include but are not limited to; nuclear fragmentation using DAPI/Tunnel [62–67]; *in situ* ligation assay [65]; DNA laddering [65, 66]; externalization of Annexin V/PI by cell membrane [62, 64, 67–70]; mitochondrial and cytosolic calcium [66, 67, 69, 71]; depolarization of the mitochondria using mitochondria membrane potential detection kits for instance, JC fluorescent probes [62, 63, 66–71]; reactive oxygen species (ROS) accumulation [66, 67, 69–71]; detecting cytochrome c in cytoplasm using western blotting or color metric kits [63, 66–69, 71, 72]; cytosol / mitochondria intracellular glutathione [67, 69]lipid peroxidation [67, 69]; potassium release [67] and metacaspace activation detection using kits like CaspACE FITC-VAD-FMK *in situ* Marker [63, 67–69]. The antimicrobial activity of a probiotic microorganism can be assessed using a combination of a number of these methods, which can corroborate the integrity of the indicator pathogen. Careful choice of positive (example, antimicrobial drug) and negative (untreated) controls are important for interpreting the results.

3.3 Experiments that confirm the antimicrobial activity of probiotics *in vivo*

The *in vitro* studies offer required information about antimicrobial agents on susceptibility responses [73], exposure times, and optimal concentrations [74]. However, these studies have their limitations, for instance, the bulk of antimicrobial agents that are active *in vitro* lack significant antimicrobial activity *in vivo,* and vice versa sometimes occurs [73]. The strength of animal models in determining antimicrobial efficacy is that the study can be ascertained at specific body sites, for example, skin, thigh, lung, peritoneum, meninges, and endocardia [74]. Furthermore, antimicrobial agents are altered by host factors such as metabolism and the immune system in an animal model [74]. Consequently, animal models bridge the gap between in vitro and clinical trials [73] and are indispensable for authentication of probiotic antimicrobial activity. In brief, *in vivo* animal models and clinical studies are an absolute requirement to provide proof of beneficial activities of probiotic antimicrobial activity. To achieve this, appropriate infectious models for the two groups are critical. One infected with indicator pathogen and treated with probiotic cultures, and the other group infected with indicator pathogen only (negative control).

3.3.1 In vivo experiments on animal models

The infection route of dermatophytes is strictly dependent on the goal of the study, indicator fungus, and animal disease model of interest. Examples, to study geophilic and anthrophilic dermatophytes; *Microsporum gypseum* and *Trichophyton rubrum* that is difficult to establish infections in laboratory animals', zoophilic dermatophytes especially *Trichophyton metangrophytes* var. *mentagrophtes*, var. *quinckeanum* and var. *granulae*, *Trichophyton verrucosum,* and *Microsporum canis* are used instead. The most recommended animal model for dermatophytoses is hairless guinea pigs as the infection resembles infections in humans, and topical treatment is applicable. Mouse, rat, hamster, and dog are disadvantaged for dermatophytoses animal model since they defecate, lick, and bite itching or irritating lesions intensively [75].

C. albicans and *Candida tropicalis* have high virulence in systemically induced mice model [76–79]. Pregnant mice [75], zebrafish [80] and *Caenorhabditis elegans* [46] have also been utilized in disseminated systemic infection models. *Candida metapsilosis* is virulent in the vaginal mouse model [81]. Furthermore, oophectomised rats are used for chronic vaginitis [47, 75]. However, *C. parapsilosis, C. glabrata,* and *C. krusei* do not induce mice mortality [77]. Further, *C. albicans* [82], *Ctropicalis, C. parapsilosis* complex (*C. parapsilosis, C. orthopsilosis,* and *C. metapsilosis*), are virulent in the invertebrate *Galleria monella* model [77]. Induced immunosuppressed mice in murine oral candidiasis model of choice. To cause the immunosuppressed condition, administration of prednisolone 100 mg per kg [83] or ketamine: xylazine 90-100 mg/kg and 10 mg/kg respectively [84] of body weight administered by injected subcutaneously 24 h before inoculation with Candida orally is given. Additionally, avian and rats species can be used as oral candidiasis models [75, 84]; a summary of these *in vivo* models is given in **Table 1**.

Disease	Animal	Route of infection	Target organ	Reference
Dermatomycosis				
Dermatomycosis	Guinea pig	Skin abrasion	Skin localized infection	[75]
Dermatomycosis	Guinea pig	Intravenous	cutaneous disseminated infection	[75]
Candidosis				
Bacterial and fungal systemic infection	*Caenorhabditis elegans* (Round worm)	Skin	media	[46]
Bacterial and fungal systemic infection	Pregnant mice	Intravascular	placenta	[75]
Bacterial and fungal systemic infection	*Galleria mellonella* (Wax moth caterpillar)	Injection	systemic	[20, 85]
Bacterial and fungal systemic infection	Zebra fish (*Danio rerio)*	Microinjection	disseminated infection	[80]
Chronic vaginitis	Rats; oophectomised and kept permanently in pseudoestrous-weekly injection of estrogen	Intravaginal with blastospores	Vaginal swabs	[47, 75]
Localized oral candidosis (thrush)	Rats and several avian species	Peroral challenge with blastospores; favored by carbohydrate rich diet, antibiotic treatment and use of germ free or specific pathogen free animals	Mouth swabs	[75, 84]

Table 1.
Précis of in vivo *animal models for dermatomycosis, candidiasis and bacterial infections.*

3.3.2 Clinical trials

Clinical trials are conducted after promising *in vitro* and *in vivo* animal model experiments. The randomized placebo-controlled clinical trial is the most recommended method [10]. The number of clinical researches conducted on probiotics is about 1000, with *Lactobacillus rhamnosus* GG and *B. animalis* sp. *Lactis* being the most studied [41, 86]. The majority of these studies are on gastrointestinal diseases and the digestive system [86]. However, currently, there is a shift to metabolic disorders, communicable and infection [86]. The primary concerns in these clinical studies that need to be addressed for harmonization of probiotic clinical research include:

1. The probiotic dosage administered; only 42% of the clinical studies reported dosage correctly. It is recommended that the probiotic dosage is reported in colony-forming unit (CFU). Some clinical studies reported the number of drops, grams, or not indicated at all [86].

2. The amount of probiotic administered should be adequate [7, 87]; however, the amount used varied from 10^7 to 9×10^{11} per day [86].

3. The description of how the probiotic was prepared was incomplete in many studies [86].

4. Viability, which is the overall health of cells. It is crucial to check the viability of probiotics before administration and after a given duration since storage, transportation, and handling condition could kill some microorganisms.

5. It is essential to describe probiotic microorganisms not only to the species level but also to strain. This is because the diversity of probiotic microorganisms is enormous. Further, the probiotic activity is species and strain-specific [88–90]. This is incomplete in the majority of the clinical studies done. Only 49% of the studies conducted complete strain identification.

6. Route, frequency, and duration [91] of probiotic administration should always be reported. Many studies omit this vital aspect.

7. Sample size affects the power of the study to draw a conclusion and the precision of estimates. Therefore, the sample size should be big enough to reduce bias, especially when some patients discontinue the study.

It is important to note that, these details including probiotic dosage used in clinical studies, should be extrapolated from *in vitro* and *in vivo* models. Therefore, this emphasizes the importance of prior quality research.

Few clinical trials on confirmation of the antimicrobial effect of probiotics have been reported so far, yet they have been considered the final confirmative experiment. Probiotics are regarded as safe [13, 17]; thus, many researchers skip this critical step. This is the case in which many commercially marketed probiotics have pending clinical studies [92]. Probiotics clinical studies on the management of oral pathogens [9, 21, 93–95], urogenital infections [20, 96–99] and gastrointestinal systems [100] had promising results thus, supporting some probiotics as potential antimicrobial agents [10].

In conclusion, clinical studies are essential. Successful clinical studies require thorough *in vitro* and *in vivo* experiments, especially estimating the dosage,

duration, and frequency of probiotic administration. Areas that need urgent reporting and harmonization in clinical studies include probiotic viability, probiotic species and strain, dosage (CFU), duration, frequency of administration, and route of probiotics administration.

4. Summary and future prospects of probiotics as antimicrobial agents

The probiotics are offering a ray of hope to solve dwindling antibiotic efficacy. Further, the number of immunocompromised persons, number of microbial infections and drug resistance, and probiotics could come in handy to solve these problems. Therefore, there is a need for detailed conclusive research on *in vitro, in vivo,* and clinical trials of probiotic microorganisms, prebiotics, and postbiotics administration including, the benefits and side effects. The choice of probiotics, methods, and experimental designs need to be emphasized. Research has demonstrated that probiotics of a particular strain may have antimicrobial activity against one pathogen and not another [9, 10, 14]. This has been attributed to the great diversity of virulence factors expressed by these pathogens. Some pathogens can produce exoenzymes, encode resistance genes, form biofilms, and induce inflammatory responses, among others [37, 101, 102]. The probiotic dosage, duration, frequency, formulation, viability, species-level, and strain, among others, should always be reported for conclusive studies. Otherwise, it would be pretty challenging to compare these experiments and draw a definite conclusion. Some particular probiotics do not show any antimicrobial activity *in vitro* but present significant activity *in vivo* and vice versa. Hence, there is a need for meticulous screening of probiotic microorganisms before the antimicrobial activity is or is not confirmed.

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